BOOLEAN NETWORK MODEL OF HYPOXIA STRESS RESPONSE PATHWAY

A Thesis

by

RAJANI RAJAN VARGHESE

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Chair of Committee,	Aniruddha Datta
Committee Members,	Shankar P. Bhattacharyya
	Raffaella Righetti
	V. S. Venkatraj
Head of Department,	Chanan Singh

May 2014

Major Subject: Electrical Engineering

ABSTRACT

Hypoxic stress is a consequence of the decrease in the oxygen reaching the tissues of the body. The coupling of energy with oxygen makes low tension oxygen sensing and adaptation very essential for survival. The intracellular partial pressure of oxygen is regularly measured by a family of hydroxylase enzymes named as prolyl hydroxylase domain containing proteins (PHD). Hypoxia Inducible Factor (HIF) is the transcription factor that controls the ability of the cell to balance between adaptation and cell death during hypoxia.

During normoxia, HIF1α undergoes non-reversible hydroxylation in the presence of PHD2. The hydroxylated HIF1α interacts with von Hippel-Lindua tumor suppressor protein (pVHL) and is degraded by ubiquitination. During hypoxia, PHD2 is inhibited which results in HIF-1α stabilization. Stabilized HIF-1α enters the nucleus and heterodimerizes with Hypoxia Inducible Factor-1β and the dimeric transcription factor HIF-1 is formed which binds to the response elements of the target genes. HIF-regulated target genes enable cells to induce an adaptive response by increasing glycolysis; angiogenesis etc. or undergo cell death by promoting apoptosis or necrosis.

In this work, a Boolean network is generated whose state transitions realize the hypoxic stress response pathway. The simulated behavior of the Boolean network obtained is consistent with the experimental results from the already published pathway literatures.

DEDICATION

I dedicate this thesis to my loving family.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Datta, and my committee members, Dr. Bhattacharya, Dr. Righetti and Dr. Venkatraj for their guidance and support throughout the course of this research. I thank Dr. Venkatraj (CVM, TAMU) for his proper guidance and encouragement which helped me to master the biological aspects of hypoxia. During the course, I was able to volunteer at Dr. Venkatraj's lab in College of Veterinary Medicine, TAMU, which helped me to understand various techniques used in the wet lab. Thanks to my friends, colleagues, the department faculty and staff for making my time at Texas A&M University a great experience. Finally, thanks to my mother, father and brother for their encouragement and to my husband for his love and support.

NOMENCLATURE

HIF	Hypoxia Inducible Factor
VHL	Von Hippel Lindau tumor suppressor protein
VEGF	Vascular Endothelial Growth Factor
ETC	Electron Transport Chain
HRE	Hypoxia Response Element
PHD	Prolyl Hydroxylase Domain containing proteins
ARNT	Aryl Hydrocarbon Nuclear Trans locator
mRNA	messenger Ribo Nucleic Acid
NADH	Nicotine Amide Dinucleotide (reduced form)
FAD	Flavin Adenine Dinucleotide
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
LDHA	Lactate Dehydrogenase A
PDH	Pyruvate Dehydrogenase
PDK	Pyruvate Dehydrogenase Kinase
ETC	Electron Transport Chain
ATP	Adenosine Tri Phosphate
ADP	Adenosine Di Phosphate
NAD^+	Nicotine Amide Dinucleotide

TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Page

1. INTRODUCTION

1.1 Introduction

Hypoxia is a condition in which there is an inadequate supply of oxygen to the tissues of the body. If the oxygen entering inside a cell is not matching the oxygen demand of the same cell, a hypoxic condition is created. [1] Hypoxic stress is caused when the amount of oxygen available in a cell is insufficient to meet the energy demands of that cell. In a living cell, this imbalance between oxygen supply and energy demand arises due to physiological and pathophysiological processes. [2]. A cell faces hypoxic stress due to normal physiological variations during fetal development, wound healing, adapting to a high altitude, inflammation etc. [2] In a developing embryo, hypoxic stress is due to reduced oxygen supply, whereas while doing vigorous exercise, a hypoxic condition is created in the exercising muscles due to an increased demand of energy. [3] The optimum oxygen tension for tubulogenesis, vasculogenesis and angiogenesis in an embryo of a mammal is about 23-38 mm Hg. [2]

1.2 Partial pressure of oxygen and hypoxia

Different cells in the body have different partial pressures of oxygen that are considered to be normal and dropping of the partial pressure of oxygen these normal levels, create a hypoxic condition. The difference of the partial pressures of oxygen between the blood and the mitochondria of the cells is responsible for the transfer of oxygen between blood and all the cells in the body. [2] Hypoxia detection in human beings is carried out by the most vascular tissue in the human body, the carotid body,

which is located near the bifurcation of the carotid artery. [4] The partial pressures of inhaled air, arterial blood and venous blood are 150 mm Hg, 100 mm Hg and 40 mm Hg respectively. [2] The carotid body detects the gradient of the partial pressure of oxygen of the arterial blood that flows through the carotid body. [5] The intracellular PO_2 is regularly measured by three hydroxylases. These hydroxylases are named as prolyl hydroxylase domain containing proteins (PHD), namely PHD1, PHD2, and PHD3. [2]

1.3 Metabolic reactions and hypoxia

Almost 95% of the oxygen that we breathe in is used up by the reactions of the Electron Transport Chain (ETC), catalyzed by cytochrome oxidase, to produce energy. [2] A gradient of protons is created across the innermost membrane of mitochondria as a result of some redox reactions taking place in the mitochondrial ETC, due to the transfer of electrons from an electron donor to the terminal electron acceptor, oxygen. This proton gradient is used to phosphorylate ADP to form ATP in the presence of ATP synthase. The inhaled air, which has a partial pressure of 150 mm Hg, passes into the alveoli which are the small air sacs located inside the lungs. Oxygen mixes with the water vapor and carbon dioxide (external respiration) and then enters into the arterial blood with a partial pressure of 13.3 KPa. Arterial blood carries oxygen to the mitochondria in each and every cell of the body, where it extracts hydrogen from the food to react with oxygen to produce water vapor. Carbon dioxide produced in the mitochondria enters into the venous blood and is expelled from the lungs. The normal partial pressure of oxygen in the arterial blood is 100 mm Hg, and when it falls to 40 mm Hg, it is very dangerous. [6] Hence, low oxygen tension should be identified and proper

adaptation measures should be taken by the cell for survival, because oxygen consumption is coupled to release of energy in the form of ATP, which is essential for the proper functioning of the cell.

1.4 Hypoxia and cancer

Hypoxia can also occur due to pathophysiological processes, especially cancer. Hypoxia is a prominent condition observed in solid tumors, stroke, atherosclerosis, asthma, chronic bronchitis, emphysema, neuronal cell death and disability. Myocardial hypoxia, cerebral hypoxia, alveolar hypoxia etc. are very detrimental to the human body. [2] Hypoxia is a very prominent character displayed by almost all solid tumors, since rapid cell growth demands a lot of energy, and hence oxygen becomes a limiting factor. As the tumor grows to about 2-3mm in diameter, the normal oxygen supply falls short to satisfy the increase in energy requirement of cancer tissues. The cells adapt to this scarcity by creating new blood vessels from the already existing blood vessels (angiogenesis) and also by increasing the glycolytic rate for easy and faster energy production. [7]

1.5 Adaptation to hypoxic stress

To maintain the proper tissue function, an adaptive response must be incorporated to overcome the scarcity of oxygen in order to harvest energy from food. The transcription factor HIF (Hypoxia Inducible Factor) has a prominent role in the gene expression regulation by oxygen. HIF achieves this response by regulating the transcription of thousands of hypoxia responsive genes. The interaction of HIF was

identified for the first time in erythropoietin (EPO) gene which is involved in angiogenesis. [2] EPO gene was up regulated almost 100 fold by hypoxia. [8]

1.6 Hypoxia Inducible Factor (HIF) and its regulation

Hypoxia Inducible Factor (HIF) is a heterodimer comprising of α and β subunits. HIFα consists of 3 subunits, HIF1α, HIF2α and HIF3α.The HIFβ subunit consists of ARNT which is the aryl hydrocarbon nuclear translocator and ARNT2. HIF1 transcription factor consists of HIF1α and HIF1β (ARNT). [9] The concentration of oxygen in the cells regulate the HIF1α subunit, but the HIF1β subunit is unaffected by the concentration of oxygen. HIF1α quickly stabilizes and is accumulated on hypoxic conditions by the inhibition of PHD2, but on re oxygenation, it is suddenly destroyed and its half-life is less than 5 minutes. [10] This short half –life shows that HIF1α accumulation is not good for the body. In fact, prolonged hypoxia is observed in most of the tumors.

HIF1 α undergoes hydroxylation of the amino acid proline located in its oxygen dependent degradation (ODD) domain by PHD2. [2] HIF1 α undergoes hydroxylation of the asparagine 803 residues located in the C terminal activation domain (CTAD) by FIH (factor inhibiting HIF). [11] The hydroxylation of HIF1α by PHD2 increases its interaction with the tumor suppressor protein, pVHL, the von-Hippel Lindau tumor suppressor protein which marks HIF1α for degradation with the help of E3 ubiquitin – ligase. [2] PHD2 hydroxylates HIF1α and that create a binding site for pVHL [12] Again, the hydroxylation of HIF1α by FIH reduces the transcriptional activity of HIF1α by preventing the binding of coactivators p300/CBP to HIF1α. [13] The hydroxylation

of HIF1α by FIH results in p300/CBP independent transcription of HIF1α. PHD and FIH require oxygen, iron, α Keto Glutarate (αKG) and ascorbate as substrates to function properly. Oxygen and αKG are the co-substrates required for PHD2 functioning whereas, ascorbate and Fe^{2+} (iron) are the cofactors required for PHD2 functioning. PHD2 hydroxylates HIF1α and simultaneously PHD2 decarboxylates αKG to succinate. [2] αKG is an anion of α keto- glutaric acid which is produced by the deamination of glutamate, an intermediate of the citric acid cycle. [14] During hypoxia, the substrate oxygen required for PHD functioning is not available and hence PHD is inhibited. This results in the activation and stabilization of HIFα subunit which enters into the nucleus and binds with HIFβ subunit to form HIF heterodimer. [12], [13] HIF transcription factor binds to the Hypoxia Responsive Elements (HRE) of the target genes and this interaction drives the transcription in a hypoxia responsive manner up regulating the genes involved in angiogenesis (EPO), vasculogenesis (VEGF), glycolysis (GLUT1, PDK1, LDHA) etc. Electron Transport Chain (ETC) directs the electron transport between the electron donors NADH (Nicotinamide Adenine Dinucleotide) and FADH2 (Flavin Adenine Dinucleotide) to the terminal electron acceptor oxygen. ETC takes place inside the mitochondria where O_2 is reduced to H_2O, NADH is oxidized to NAD+ and succinate in the presence of Succinate Dehydrogenase (SDH) enzyme is converted to fumarate. [15] The gradient of proton generated across the membrane of mitochondria, pumps protons inside the mitochondrial space which converts Adenosine Di Phosphate to Adenosine Tri Phosphate with the enzyme ATP synthase. This whole process is what is termed as oxidative phosphorylation. [16] Some of the electrons will not be transferred from the

electron donor through the complexes I-IV to the terminal electron acceptor, but there may be premature electron leakage through complexes I and III forming reactive oxygen species (ROS) such as superoxide that creates oxidative stress. [17] During hypoxia ETC is inhibited since, there is not enough oxygen available, which generates more ROS creating an oxidative stress. [18] This oxidative stress does not allow iron to cycle between the oxidation states which results in PHD inhibition since, iron is one of the cofactors required for PHD to function properly. PHD inhibition results in the accumulation and stabilization of HIF1α which results in the induction of anti-apoptotic as well as apoptotic genes depending on the extent of hypoxia. [19]

1.7 Switch from aerobic metabolism to anaerobic metabolism

Activation of the transcription factor HIF1 results in a shift from aerobic metabolism to anaerobic metabolism. Pyruvate kinase is an enzyme that converts Phospho Enol Pyruvate (PEP) to pyruvate. Pyruvate Kinase Muscle Isozyme 2(PK-M2) is one of the isozymes of the glycolytic enzyme Pyruvate Kinase [20]. PHD3 hydrolyzes PK-M2 on proline-403/408, and the hydrolyzed PK-M2 interacts directly with HIF1α subunit which enhances the binding of HIF1 to the HRE of the target genes. But, PKM2 itself is a hypoxia target gene since, a hypoxia responsive element (HRE) was observed in the intron 1 of PK-M2. [21] PK-M2 promotes the Warburg effect. [22], [23] PK-M2 is identified in almost all the cancer cells and is essential for rapidly dividing cells since quicker energy harvesting is possible by shifting from aerobic to anaerobic metabolism. [24] Pyruvate Dehydrogenase Kinase 1(PDK1) is a HIF1 target gene. PDK1, which is a hypoxia target gene, inhibits the action of the enzyme PDH, (Pyruvate Dehydrogenase)

which converts pyruvate to acetyl-co enzyme A. Hence, pyruvate is unable to enter into the citric acid cycle and is trapped in the cytosol and the rate of glycolysis is increased. This explains the Warburg effect and the relation between prolonged glycolysis and HIF1 activation.

So, in effect PK-M2 catalyzes the conversion of PEP to pyruvate and PHD3 hydrolyzes PK-M2 and the hydrolyzed PK-M2 enters the nucleus and interacts with HIF1α subunit and enhances the binding of HIF1 to the hypoxia responsive elements of the target genes. But, PK-M2 and PHD3 are hypoxia target genes. Moreover, PDK does not allow pyruvate to enter into citric acid cycle and traps pyruvate in the cytosol. Pyruvate inhibits PHD which stabilizes HIF1α, but PDK1 itself is a HIF target gene. Most of the glycolytic enzymes like GLUT1, LDHA, ALDA etc. are the hypoxia target genes and this explains the feed forward mechanism for HIF 1α activation and increased rate of glycolysis.

1.8 Feedback inhibition

PHD2 hydrolyzes HIF1α and the hydrolyzed HIF1α is degraded by the VHL tumor suppressor protein. But, PHD2 itself is a hypoxia target gene. [25], [26], [27] This feed-back inhibition acts a regulative control for hypoxic response when normoxia is re-established. [2] Thus, HIF1:PHD2 linked transcription sets up the appropriate HIF1 signaling to face the hypoxic stress effectively. [2]

1.9 Hypoxic response based on the extent of hypoxia

Hypoxic response can be either adaptive or apoptotic depending on the level of hypoxia. During normoxia, both catalytic enzymes PHD and FIH are active, which

results in the hydroxylation of proline and asparagine residues of HIF1α, resulting in the complete degradation of HIF1α by interacting with VHL. During moderate hypoxia, PHD is inactive, whereas FIH is active since FIH requires only less oxygen concentration than PHD to function properly. [28] Here the transcription of HIF1 is limited, it occurs in a p300 independent manner. P300 is a coactivator which helps in rapid transcription. In this condition, the cell tries to adapt to the hypoxic stress by up regulating the anti-apoptotic genes involved in angiogenesis (EPO), vasculogenesis (VEGF) and glycolysis (GLUT1, LDHA, ALDA etc.). During, severe hypoxia, both the catalytic enzymes PHD and FIH are inactive and thus, there is a strong HIF1 transcription resulting in a full- blown apoptotic response by up regulating the apoptotic genes p53, BNIP3, NIX etc. [25], [29], [30] There is an increased level of pro-apoptotic and apoptotic factors and p53 facilitated cellular injury in such a situation. Inhibiting the adaptive response could decrease the chance of survival of a cell when faced by hypoxic stress. [2]

2. METABOLIC SWITCH AND HYPOXIA

2.1 Introduction

Activation of the transcription factor HIF1 results in a switch from oxidative metabolism to glycolytic metabolism. Hypoxia prevents the switch from glycolysis to citric acid cycle and glycolysis continues indefinitely so that the increasing energy demand during hypoxia can be met rapidly. The analysis of the mRNA of the HIF-1α deficient cells indicated that most of the enzymes involved in glycolysis is regulated by HIF-1 [2].

2.2 Glycolysis

Glycolysis is the central pathway of the glucose catabolism. It is a process by which glucose, which is a 6 carbon compound, is degraded to yield 2 molecules of pyruvate, which is a 3 carbon compound, in a sequence of ten enzyme catalyzed reactions. [15] The end product of glycolysis stores a lot of free energy and releases only a small fraction of the overall energy available inside the glucose molecule. The free energy released during glycolysis is preserved as ATP (Adenosine Tri Phosphate). The remaining free energy can be released only when the glycolytic products are completely oxidized into carbon dioxide and water with oxygen being the terminal electron acceptor. Glycolysis takes place in the cytoplasm and ten intermediate compounds are formed as a result of ten intermediate reactions catalyzed by glycolytic enzymes.

There are two phases of glycolysis, the preparatory phase during which the ATP is consumed and the payoff phase during which ATP is generated. There are total ten

reactions in glycolysis, out of which the first five are preparatory phase and the last five are payoff phase. In the preparatory phase, phosphorylation of glucose by ATP occurs in the presence of enzymes and finally 2 molecules of glyceraldehyde 3-phosphate is formed. During the pay-off phase of glycolysis, NAD^+ oxidizes glyceraldehyde 3-phosphate with the help of glyceraldehyde phosphate dehydrogenase enzyme forming 1, 3-diphosphoglycerate and simultaneously NAD^+ is reduced to NADH. [15] The 1, 3-diphosphoglycerate releases its phosphate group rich in energy to ADP to generate ATP and forms 3-phosphoglycerate. [15] This is the first ATP forming reaction. It then isomerizes into 2-phosphoglycerate in the presence of phosphor glycerate mutase enzyme and the latter undergoes dehydration with the help of enolase and forms Phospho Enol Pyruvate (PEP). [15] Then PEP releases its phosphate group rich in energy to ADP to form ATP in the presence of Pyruvate Kinase and pyruvate is formed simultaneously. [15] The reaction involving Pyruvate Kinase enzyme is the second point of regulation for glycolysis.

Thus, two molecules are used in the preparatory phase whereas; four molecules of ATP are formed in the pay-off phase, resulting in a net yield of two ATP molecules for every glucose molecule undergoing glycolysis. During conversion of glyceraldehyde- 3-phosphate to 1, 3-diphosphoglycerate in the presence of the enzyme glyceraldehyde phosphate dehydrogenase (GAPDH), NAD^+ is reduced to NADH. This NADH is transferred from cytosol to mitochondria by GAPDH which is activated by calcium ions (Ca^{2+}). NAD is the product of cytosolic GAPDH activity. The pictorial representation of glycolysis is shown in figure 1.

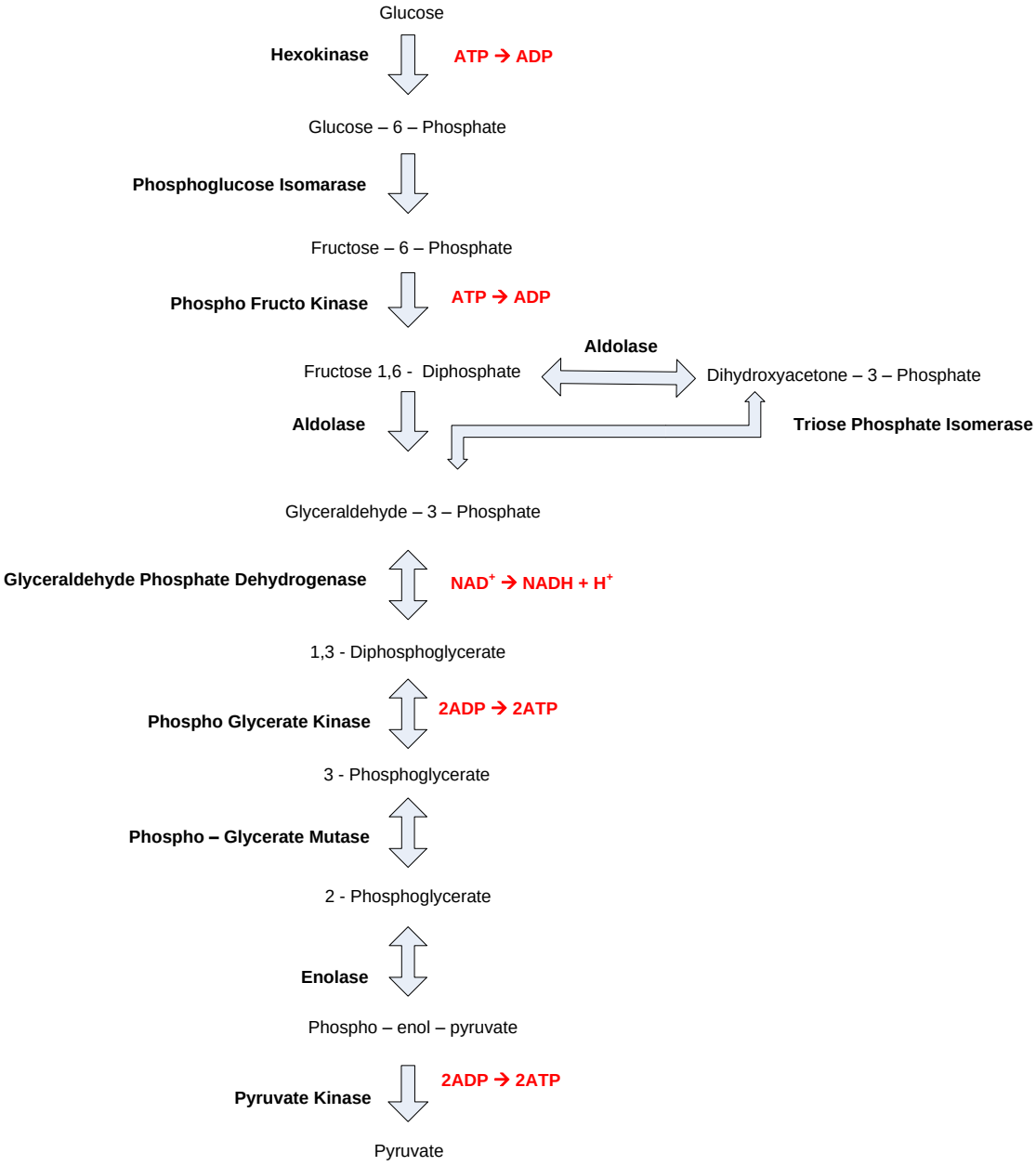

Figure 1: Anaerobic glycolysis

2.3 Hypoxia and glycolysis

Hypoxia and glycolysis is very much related since almost all the glycolytic enzymes are up regulated during hypoxia. The analysis of m-RNA expression of HIF-1α deficient cells showed that the enzymes hexokinase (HK), glucose phosphate isomerase (GPI), phospho fructo kinase (PFK), aldolase, (TPI) triose phosphate isomerase, (GAPDH) glyceraldehyde phosphate dehydrogenase, (PGK) phospho glycerate kinase, phospho gluco mutase (PGM), enolase, (PK) pyruvate kinase and lactate dehydrogenase A (LDHA) are regulated by HIF-1. [31] Thus, the rate of glycolysis increases when cells undergo hypoxic stress. The end product of glycolysis, pyruvate inhibits PHD2 which results in the activation of HIF-1α and it enters into the nucleus and binds with HIF-1β to form the transcription factor HIF-1. Hypoxia Inducible Factor-1 which binds to the HRE of the hypoxia target genes which include almost all the glycolytic enzymes, thus increasing the rate of glycolysis. Moreover, Pyruvate dehydrogenase Kinase (PDK) is a hypoxia target gene which inhibits pyruvate dehydrogenase (PDH). PDH is the enzyme that converts pyruvate to Acetyl- coenzyme A. Hence, hypoxia inhibits the formation of acetyl coenzyme A and thereby prevents pyruvate from entering into the citric acid cycle and glycolysis continues indefinitely.

Pyruvate kinase is the glycolytic enzyme that converts Phospho Enol Pyruvate (PEP) to pyruvate. Pyruvate Kinase Muscle Isozyme 2(PK-M2) is one of the isozymes of the glycolytic enzyme Pyruvate Kinase. [21] PHD3 hydrolyzes PK-M2 on proline-403/408, and the hydrolyzed PK-M2 interacts directly with HIF1α subunit which enhances the binding HIF1 to the HRE of the target genes. But, PKM2 itself is a hypoxia

target gene since, a hypoxia responsive element (HRE) was observed in the intron 1 of PK-M2. PK-M2 promotes Warburg effect. Warburg effect postulates that cancer cells produces a lot of energy rapidly by an increased rate of glycolysis which is followed by lactic acid fermentation rather than the decreased rate of glycolysis subsequently followed by oxidizing pyruvate inside the mitochondria by citric acid cycle and oxidative phosphorylation. [32] PK-M2 is identified in almost all the cancer cells and is essential for rapidly dividing cells since quicker energy harvesting is possible by switching from oxidative to glycolytic metabolism.

If glycolysis continues indeterminately, all NAD^+ will be used up and glycolysis would come to a stop. So, NADH must be oxidized back to NAD^+ for the glycolysis to continue. There are three different mechanisms by which NADH can be oxidized back to NAD^+. They are listed below.

2.4 Lactic acid fermentation

Pyruvate is reduced to lactate by Lactate Dehydrogenase A (LDHA) enzyme anaerobically. During this reaction, NADH is oxidized back to NAD^+. This process is an important source of ATP during hypoxic conditions, for instance in infarcted heart muscle cells and while doing strenuous exercise. In many tissues, anaerobic lactic acid fermentation is a cellular last option for energy. The lactic acid fermentation by lactic acid bacteria causes milk to curdle (souring of milk) while making yogurt. [33]

2.5 Alcoholic fermentation

Fermentation is defined as the anaerobic degradation of glucose into various products which are different for different organisms, solely for obtaining energy in the form of ATP [15]. The pyruvate formed from glucose is converted to ethanol and carbon dioxide, simultaneously oxidizing NADH to NAD^+.

2.6 Aerobic respiration

In aerobic organisms, glucose is completely oxidized to CO_2 as well as water and glycolysis is only the first stage of this aerobic oxidation. Then, pyruvate is decarboxylated in the presence of Pyruvate Dehydrogenase (PDH) enzyme to form the acetyl group of Acetyl-coenzyme A. This acetyl-coenzyme A then enters into the citric acid cycle where it is oxidized into CO_2 and water and reduces NAD^+ to NADH.

This reduced NADH is oxidized back to NAD^+ by passing through the Electron Transport Chain (ETC). The electron is transferred through the Electron Transport Chain to oxygen and is finally reduced to water.

This generates a proton gradient which is used to produce 2.5 moles of ATP for every NADH oxidized which is termed as Oxidative Phosphorylation. The three different mechanisms by which NADH is oxidized back to NAD^+ is illustrated in figure 2.

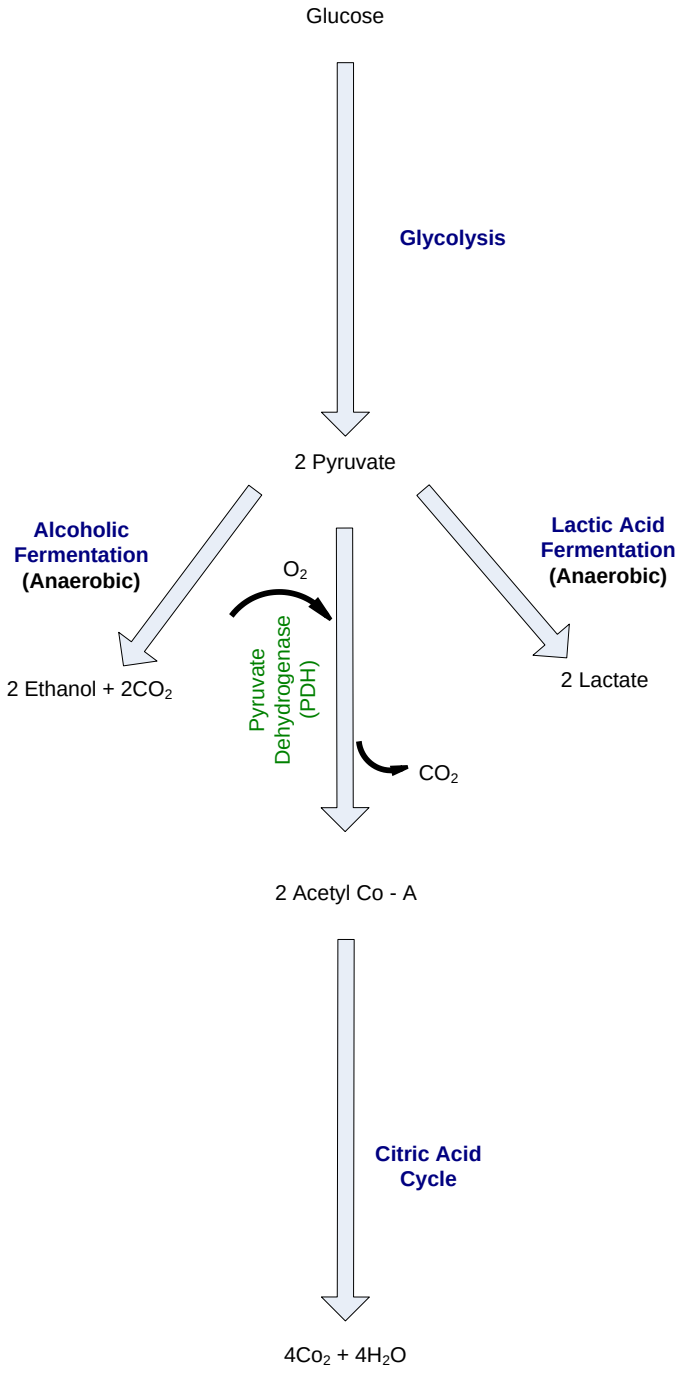

Figure 2: Mechanisms by which NADH is oxidized to NAD^+

2.7 Citric acid cycle

Citric Acid Cycle or Tri Carboxylic Acid cycle (TCA cycle) or Krebs cycle is the final common pathway by which almost all the energy producing nutrients are finally oxidized to a CO_2 and water. [15] Majority of living cells is normally aerobic and the organic fuels are completely oxidized to a CO2 and water with the help of oxygen." Citric Acid Cycle" name is derived from the tricarboxylic acid, citric acid which is first used up at the beginning of the cycle and later regenerated to complete the cycle. In eukaryotes, citric acid cycle takes place inside the mitochondria, whereas in prokaryotes, TCA cycle takes place inside the cytoplasm. The product of glycolysis, pyruvate is decarboxylated to acetyl coenzyme A in the presence of Pyruvate Dehydrogenase (PDH) and enters into the citric acid cycle. Pyruvate is directly converted into Oxalo Acetic Acid (OAA) in the presence of the enzyme, pyruvate carboxylase and ATP is consumed during this process. This cycle consumes acetate obtained from fuels (carbohydrates, fats and proteins) and water and oxidizes it to carbon dioxide. During this process, NAD^+ is reduced to NADH. The energy released by the oxidation of glucose to CO_2 and water is more than energy released by the anaerobic oxidation of glucose to pyruvate.

The end product of glycolysis, pyruvate undergoes oxidative decarboxylation with the help of PDH, pyruvate dehydrogenase to form acetyl-coenzyme A. [15] Acetyl-coenzyme A is converted to citrate by donating its acetyl group to oxaloacetate. Then isocitrate is formed from citrate and isocitrate undergoes dehydrogenation with the loss of carbon dioxide to form α-Keto Glutarate (α-KG). [15] α-KG is decarboxylated to form succinate. Succinate is converted to fumarate by Succinate Dehydrogenase (SDH)

and malonate is a competitive inhibitor of SDH and results in the accumulation of citrate, α-KG and succinate in the mitochondria. Fumarate is converted to malate by Fumarate Hydratase (FH). Malate to oxaloacetate (OAA) dehydrogenation is done in the presence of Malate Dehydrogenase and oxalo acetate intakes the acetyl group of acetyl co-A to form citrate again and the cycle continues. Pyruvate is directly converted to oxaloacetate (OAA) in the presence of the enzyme Pyruvate Carboxylase. After each and every turn of the citric acid cycle, oxaloacetate (OAA) is regenerated and it reacts with Acetyl-co A to start the next turn of the cycle. During this cycle, one molecule of Acetyl-co A is taken in and two molecules of carbon dioxide are produced and at the end of the cycle, one molecule of oxaloacetate was regenerated. One pair of hydrogen atom were removed from isocitrate, α-KG, succinate and malate respectively and out of this four pairs of hydrogen atoms, three pairs reduced three molecules of NAD^+ to three molecules of NADH and remaining one pair of hydrogen atom reduced Flavin Adenine Dinucleotide (FAD) of succinate dehydrogenase to $FADH_2$. Citric acid cycle yields one molecule of ATP during each turn of the cycle. The citric acid cycle figure is shown in figure 3.

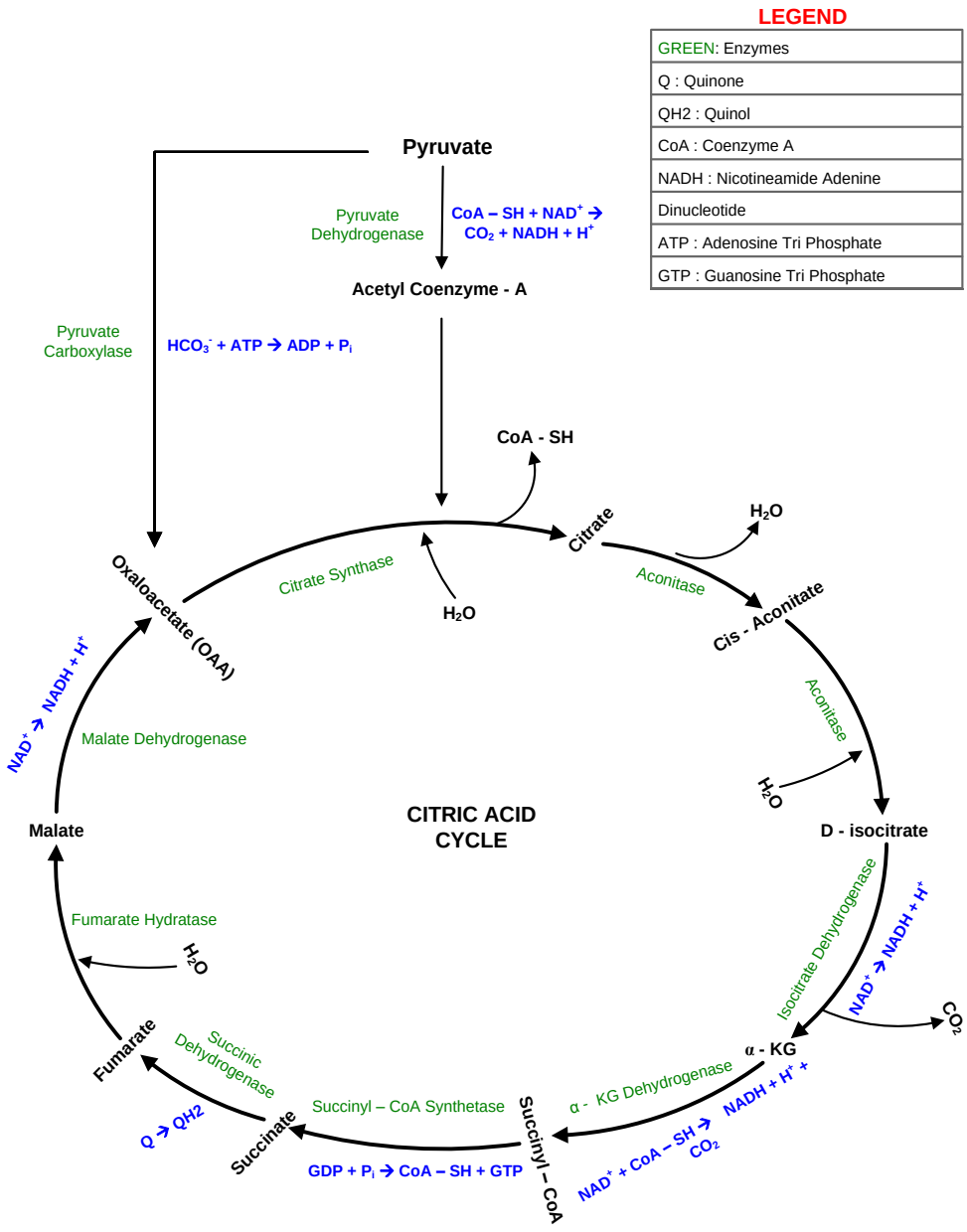

Figure 3: Citric acid cycle

2.8 Citric acid cycle and hypoxia

Hypoxia prevents acetyl co-A from entering into the citric acid cycle and hence inhibits citric acid cycle and promotes glycolysis. PHD2 is the enzyme which hydroxylates HIF1α at the proline residue, but simultaneously PHD2 decarboxylates αKG to succinate. One of the intermediates of citric acid cycle, α-KG acts as a co-substrate for PHD2 to function properly. Succinate Dehydrogenase (SDH) is the enzyme that converts succinate to fumarate. SDH mutation increases the concentration of succinate in the mitochondria, and diffuses through the inner and outer mitochondrial membrane into the cytoplasm. Once inside the cytoplasm, succinate inhibits α-KG decarboxylation to succinate by product inhibition. This decarboxylation step is mandatory for PHD2 catalytic functioning and the inhibition of the same leads to PHD2 dysfunction. This causes stabilization of HIF-1α and up regulation of hypoxia target genes which further inhibits citric acid cycle. People with mutations in SDH gene has been identified with pseudo hypoxic response nature. Similarly, Fumarate Hydratase (FH) mutation results in the accumulation of fumarate inside the mitochondria, and this acts as a competitive inhibitor to α-KG binding site in PHD2, thereby inhibiting PHD2.

2.9 Electron transport chain

The four pair of electrons from the citric acid cycle is transferred through the ETC to the terminal electron acceptor oxygen located in the complex IV of ETC, reducing two molecules oxygen to four molecules of water. ETC couples the transfer of electrons from the electron donors NADH and $FADH_2$ released by the citric acid cycle and to the terminal electron acceptor oxygen. ATP is generated by creating a

transmembrane proton gradient across the inner membrane of mitochondria by pumping protons. [15] The electron acceptor is more electronegative than the electron donor and the electrons passes from the donor to the acceptor until it reaches oxygen.

There are four complexes in the ETC, namely complex I, complex II, complex III and complex IV. [15] Complexes I, II and IV are proton pumps and these complexes are connected by lipid-soluble and water -soluble electron carriers. Complex-I accepts two electrons from NADH released by the citric acid cycle and passes these electrons to a lipid-soluble carrier ubiquinone (coenzyme Q). FAD is reduced to $FADH_2$, during the conversion of succinate to fumarate. [15] Complex II accepts electrons from $FADH_2$ and transfers it to ubiquinone. SDH catalyzes the oxidation of succinate to fumarate and during this process, ubiquinone (Q) is converted into ubiquinol (QH_2).

SDHA subunit functions as a part of citric acid cycle and converts succinate to fumarate and FAD to $FADH_2$. SDHB subunit functions as a part of respiratory chain. The iron clusters [2Fe-2S], [4Fe-4S], [3Fe-4S] receives the electrons from $FADH_2$. [15] SDHB protein is encoded by SDHB gene, which is a tumor suppressor gene. SDHC cytochrome b560 subunit and SDHD cytochrome b small subunit are located at the inner mitochondrial membrane and functions as a part of respiratory chain. Q gets reduced to QH_2 as a result of electron transfer from SDHB to Q (ubiquinone).

Complex III of ETC is also known as the Q cycle. Q cycle lists a series of reactions that explains how the redox reactions of coenzyme Q can pump protons across membrane of mitochondria to produce ATP. There are two different sites in complex III, the Q_0 site and the Q_i site. At the Q_0 site, quinol is oxidized and two electrons from

quinone are transferred to cytochrome c. At the Q_i site, quinone is reduced to quinol. Hence, there is a total transfer of six protons across the membrane; two protons reduce quinone to quinol and two quinol molecules releases two protons each.

Complex IV is also known as cytochrome AB or cytochrome c oxidase. The electrons from complex III are transferred to oxygen to produce two molecules of water. Oxygen is the terminal electron acceptor. Simultaneously, four protons are removed from the mitochondria resulting in a proton gradient across the membrane.

2.10 Electron transport chain and hypoxia

Hypoxia inhibits ETC and results in the formation of Reactive Oxygen Species (ROS) from complexes I and ubisemiquinone sites of complex III. ROS accumulates in the cytoplasm and inhibits PHD2 by inhibiting the ability of iron (a co-factor for PHD2 catalytic activity) to cycle between the oxidation states. This again leads to the HIF-1α stabilization. HIF-1α combines with HIF-1β and HIF -1 heterodimer is formed which up regulates a lot of anti-apoptotic genes. During hypoxia, cells lacking mitochondria cannot induce HIF-1α stabilization. [17] Usually electrons from NADH and $FADH_2$ transfer through complexes I to IV of ETC into oxygen and reduce oxygen to water. But, during hypoxia, there is not much energy to transfer electrons and instead of moving from SDHA subunit to SDHB subunit, some electrons may directly leak into oxygen and generate ROS such as superoxides or peroxide anions which are dangerously reactive, resulting in oxidative stress. ROS oxidizes proteins and cause DNA a mutation which is the main reason for premature aging and causes a lot of diseases. High mitochondrial membrane potential is one of the main reasons for ROS production. So, mitochondria

always try to maintain the membrane potential at a narrow range to reduce ROS production. To lessen the aging effects of ROS cells contain many antioxidants like vitamin C and vitamin E.

2.11 Oxidative phosphorylation

The transfer of electrons between electron donor and electron acceptor through ETC releases a lot of energy which pumps protons (H^+) from the matrix of the mitochondria into the inner mitochondrial membrane (IMM) space. This creates a transmembrane electro chemical proton gradient called ψ. This electrochemical proton gradient allows ATP- synthase enzyme to phosphorylate ADP to ATP and this process is termed as oxidative phosphorylation.

2.12 Metabolic switch

Figure 4 shows how hypoxia causes switch from aerobic metabolism to anaerobic metabolism. Glycolysis takes place inside the cytoplasm where glucose is converted to pyruvate. Pyruvate is then converted to acetyl-coenzyme A with the help of PDH which the get into the TCA cycle inside the mitochondria. [15] Citric acid cycle converts acetyl-coA finally into CO_2 and water and releases reduced from of Nicotine Amide Dinucleotide (NADH) and Flavin Adenine Dinucleotide ($FADH_2$) which are electron donors. The electrons from these electron donors pass through the ETC to O_2 and water is formed as a result of the reduction of oxygen. The transmembrane gradient of proton produced as a result of proton pumping from the mitochondrial matrix to the inner mitochondrial membrane results in the phosphorylation of ATP to ADP.

PHD2 is the enzyme that catalyzes the non-reversible hydroxylation of the proline residues of HIF-1α and marks it for ubiquitination and degradation by pVHL. Once the PHD2 enzyme is inhibited, HIF-1α gets stabilized and enter into the nucleus where it heterodimerizes with Hypoxia Inducible Factor -1β and the dimeric transcription factor HIF-1 is formed. [2] PDH converts pyruvate to acetyl-coA, but one of the hypoxia target genes Pyruvate Dehydrogenase Kinase (PDK) inhibits PDH. So, acetyl-co A is unable to enter into the citric acid cycle and glycolysis continues indefinitely. Almost all the glycolytic enzymes are hypoxia target genes. This, hypoxia promotes glycolysis for a rapid and easy energy harvesting. The intermediate metabolites of citric acid cycle, fumarate and succinate inhibit PHD2 thereby stabilizing HIF-1α. Hypoxia inhibits ETC and generates ROS which in turn inhibits PHD2.

PHD3 hydrolyzes the proline-403/408 residues of PKM2 and this increases the coactivator function of PKM2. The hydrolyzed PKM2 enters into the nucleus and enhances the rate of HIF-1 binding to the responsive element of the target genes of hypoxia. PKM2 also stimulates the recruitment of p300 and CBP to the HRE of the target genes thereby increasing hypoxic response. Moreover, a new HRE was identified in the intron1 of PKM2 which shows that PKM2 itself is a hypoxia target gene. Also, PHD3 is also a hypoxia target gene. Thus, PKM2 increases the transcriptional activity of HIF-1α. The relationship between different metabolic reactions and hypoxia is shown in figure 4.

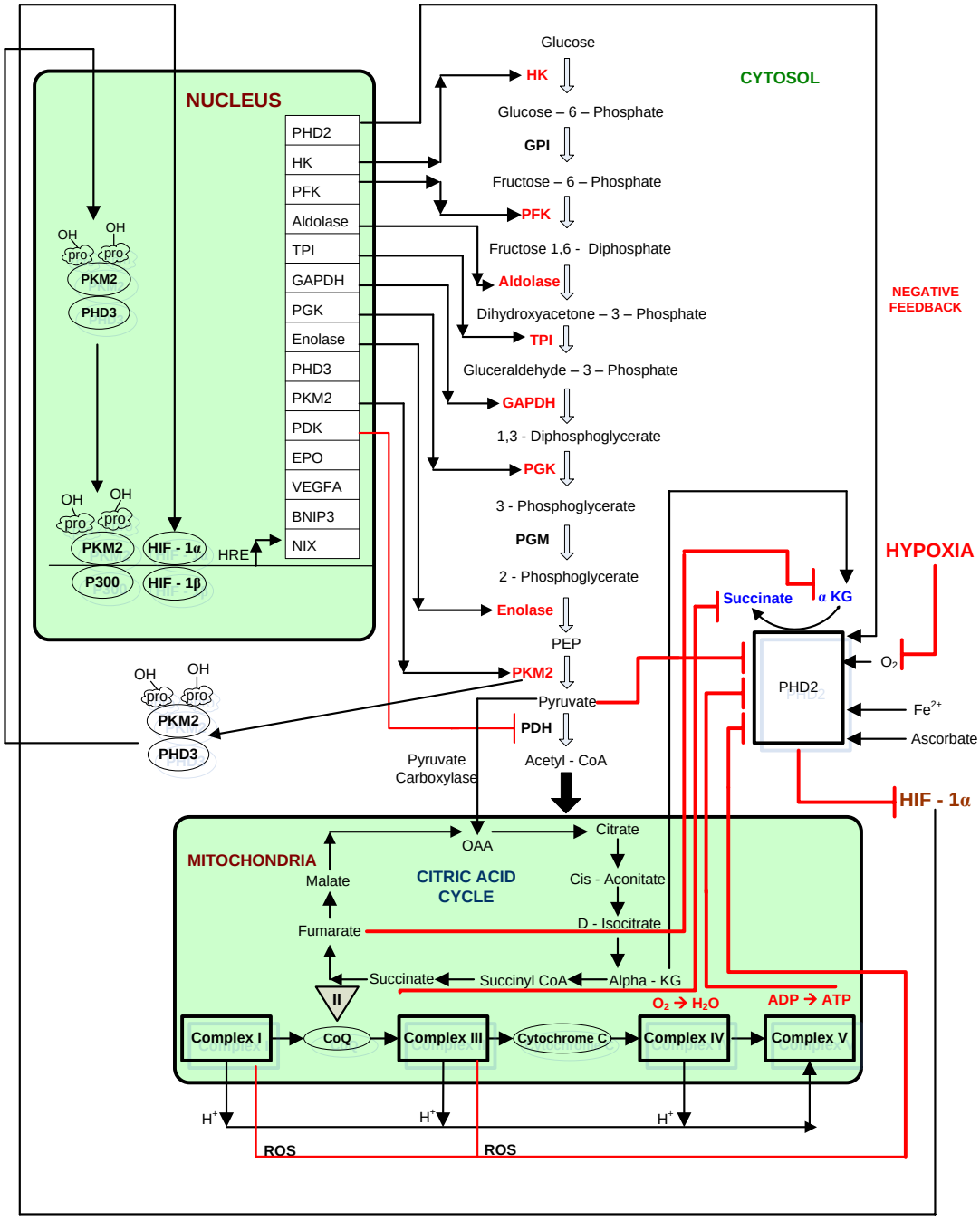

Figure 4: Metabolic switch and hypoxia

3. P300 AND CYCLIC AMP RESPONSIVE ELEMENT BINDING PROTEIN-BINDING PROTEIN (CBP) TRANSCRIPTIONAL CO-ACTIVATORS AND HYPOXIA

3.1 Introduction

HIF-1 heterodimer is formed once the stabilized HIF-1α gets into the nucleus and binds with the HIF-1β. [2] The so formed HIF-1 transcription factor binds to the response elements of the target genes and then recruits the transcriptional co-activating proteins p300 (E1A (Adenovirus early region A) binding protein p300 or EP300) and CBP (cyclic AMP responsive element-binding protein-binding protein). [34] P300 is a protein that regulates cell growth, cell division and cell differentiation and carries out these functions by activating transcription. P300/CBP is considered as a single entity because these two proteins are structurally and functionally similar and are considered as structural homologs and functional homologs. [35] In contrast, there are some functional differences between CBP and p300. For instance, the kinase cyclin E/cyclin Cdk2 negatively regulates p300 whereas; cyclin E/cyclin Cdk2 increases the Histone Acetyl Transferase (HAT) activity of CBP. [36] P300 and CBP connect with transcription factors and these interactions are managed by their 5 protein interaction domains. [37] CH1, CH2 and CH3 are the 3 zinc finger domains and also has histone acetyltransferase (HAT) domain and a bromo domain which binds to the lysine amino acid of the histone that is acetylated. The organization of p300/CBP is shown in figure 5.

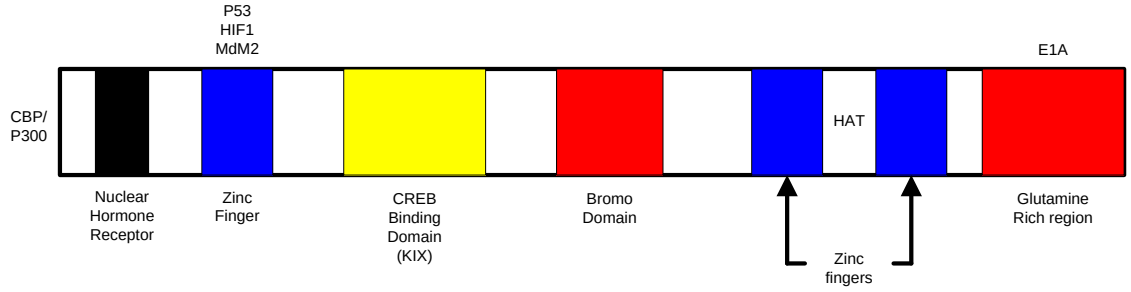

Figure 5: Organization of p300/CBP

3.2 Histone acetyl transferase

Histone acetyl transferases (HAT) are the enzymes which can acetylate lysine amino acids on the histones that surround the DNA. [35] Acetylation of histone enhances the gene expression because, DNA is wrapped around the histone proteins and hence by the acetylation of histone, more binding sites are created for specific protein-protein interaction domains such as the bromo domain which binds to the acetylated lysine. Both CBP and p300 function as HAT and plays a major role in cellular differentiation and cellular proliferation. HATs can even acetylate the transcription factors. CBP/p300 acts as the transcriptional coactivators of the transcription factor HIF1α and enhance the gene expression of hypoxia target genes. Both CBP and p300 are type A HATs which are found inside the nucleus and they directly regulate gene expression by acetylating the lysine amino acids of the histones surrounding the DNA which is located within the chromatin of the nucleus. [35] One of the protein-protein interaction domains, the bromo domain binds to the acetylated lysine on the histones. [36]

3.3 P300/CBP as transcriptional coactivators

P300 and CBP are the transcriptional coactivators that can increase the rate of gene transcription. For instance, CREB (cyclic AMP response element- binding protein) is one of the transcription factors. [38] It binds to a sequence of DNA within the target genes called CRE (cAMP response elements) and can increase or decrease the transcription rate of the target genes. CREB binds to CRE and CREB is phosphorylated. This increases the interaction of CBP and CREB and enhances the transcription of the target genes of CREB. [38]

3.4 Hyper phosphorylation of P300/CBP during hypoxia

When a cell is under hypoxic stress, the transcriptional coactivators p300 and CBP is hyper phosphorylated. PC12 cell lines are obtained from the pheochromocytoma of the medulla of the adrenal gland of rats. Pheochromocytoma is a disease caused by the mutation of pVHL and the loss of VHL activity results in a drastic increase in the amount of HIF-1α which results in tumor. Pheochromocytoma results in the tumor of the medulla of the adrenal gland. [39] The reason for hyper phosphorylation of p300 is due to the calcium release from IP_3 (inositol 1, 4, 5-triphosphate) sensitive stores and this hyper phosphorylation can be inhibited by inhibiting the glucose metabolic pathway. [40] During glycolysis, NADH is released into the cytoplasm by the formation of 1,3-biphosphoglycerate from glyceraldehyde 3- phosphate with the help of glyceraldehyde 3- phosphate dehydrogenase (G3PDH/GAPDH). [15] This NADH is transferred from the cytoplasm to the mitochondria by GAPDH. This transfer is activated by $Ca2^{+}$. Glycerate phosphate shuttle transfers NADH from cytoplasm to ubiquinone (Q) and

complex III of the electron transport chain (ETC). [15] This NADH transfer releases a pool of ATP that enhances the activity of the kinases responsible for the phosphorylation of p300 and p300 is hyper phosphorylated. [40]

3.5 P300/CBP and tumor suppression

P300 and CBP are involved in several tumor suppressor pathways and is demonstrated by its interaction with the tumor suppressor gene p53. [41] P53 interacts with the carboxyl terminal region of CBP/p300 and thus the rate of transcription of the p53 response genes MDM-2, p21 were increased. [42] Adenovirus E1-A interacts with p300/CBP at the zinc finger region and block its activity. P300/CBP can regulate p53 degradation. During DNA damage, p53 activates its target gene MDM-2, but MDM-2 inhibits p53 and this is a negative feedback. E1-A does not allow p53 to induce MDM-2 and hence p53 is stabilized by suppressing the feedback loop. MDM2 and p53 binds to the first zinc finger domain of p300. MDM-2 can inhibit p53 by competing for p300/CBP binding site. For instance, MDM-2 competes with p73, which is a homolog of p53 for binding with the first zinc finger domain of p300 and prevent its interaction with p300. [43]

The knowledge that p300/CBP can act as tumor suppressors are further strengthened by the interaction of p300/CBP with the tumor suppressor gene BRCA1 (breast cancer type 1), by acting as a transcriptional regulator of BRCA1. [44] Moreover, CBP negatively regulates Wnt pathway in drosophila. If the Wnt pathway is up regulated or activated, then the gene cyclin D1 and the proto oncogene c-myc will be induced and

this can cause tumor. [45] The negative regulation of wnt pathway by CBP is shown in figure 6.

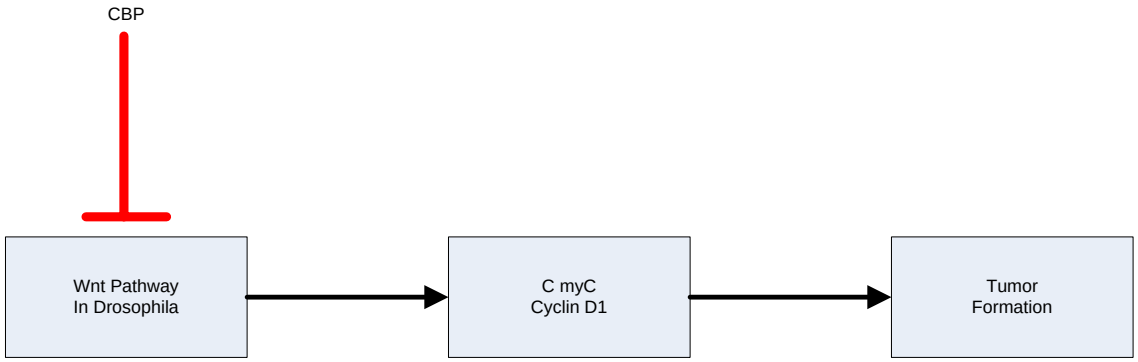

Figure 6: Negative regulation of wnt pathway by CBP

3.6 P300/CBP and E1A

CBP/p300 interacts with a lot of viral onco- proteins such as v-myb. C-myb is a proto oncogene and v-myb is an oncogene which is carried by the avian myeloblastosis virus (AMV). C-myb binds to the CREB binding domain (KIX domain) of CBP and thus enhances its transcription. But, at the same time, there are other viral onco-proteins such as adenovirus E1-A, human papilloma virus E6 etc. which can block the complete functioning of CBP/p300. These viral onco-proteins do not bind to the DNA but, the amino terminus of E1A bind to CH3 of CBP/p300. So, E1A functions by blocking the interaction of p300/CBP with these positive effectors of these coactivators. But, the carboxyl terminus (C-terminus) of E1-A inhibits the histone acetyl transferase activity of CBP/p300 directly, even without having to displace the positive effectors. But, a very

high level of E1-A is required to inhibit the HAT activity of CBP/p300 directly. [44] The interaction of E1-A with CBP/p300 is shown in figure 7.

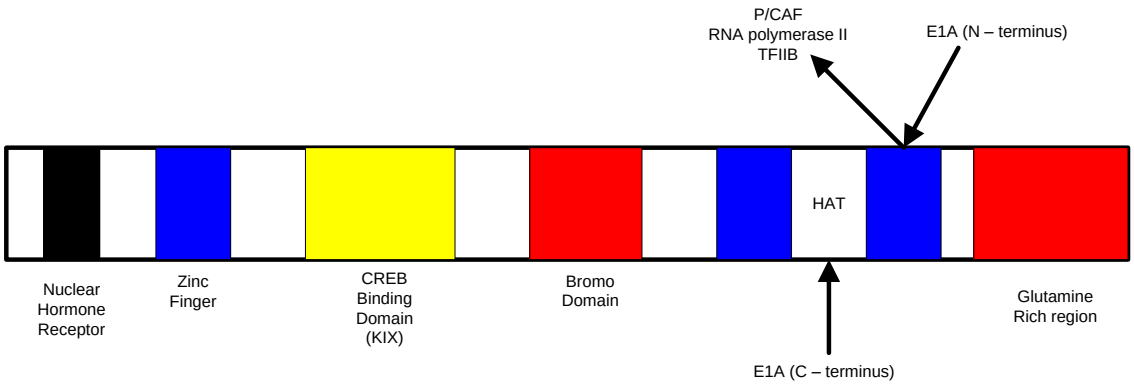

Figure 7: E1A and CBP/p300

The E6 protein of human papilloma virus (HPV) degrades the tumor suppressor gene p53, by recruiting p53 into a complex that contains the ubiquitin ligase E6AP. E6 proteins from the invasive cervical carcinoma bind to the third zinc finger domain, displacing p53 from its transcriptional coactivator because p53 also binds to the third zinc finger domain (CH3). This results in p53 degradation by ubiquitination. [46]

Both CBP and p300 are regulated by phosphorylation. Cyclin Cdc2/Cyclin Cdk2 are the kinases that phosphorylate CBP/p300 and enhances their transcriptional coactivator functioning. But, adenovirus E1A has the ability to block the phosphorylation of CBP/p300 by these kinases. These coactivators are also negatively regulated by the kinase cyclin E/cyclin Cdk2. But, the cyclin-dependent kinase inhibitor,

p21 blocks this negative regulation of p300 by cyclin E/cyclin Cdk2. P21 enhances the efficiency of p300 as a transcriptional coactivator. Thus, p21 participates in a positive feedback loop as follows: p53 which depends upon CBP/p300 induces p21 which removes the block on CBP/p300 by the kinase cyclin E/cyclin Cdk2 and enhances the transcriptional coactivator function of CBP/p300. [47]

3.7 P300/CBP mutations

The mutations in CBP/p300 results in a variety of developmental disorders like RTS (Rubinstein-Taybi Syndrome). People with mutations in CBP/p300 possess an increased risk of developing cancerous and non-cancerous tumors, leukemia and lymphoma. Most of these tumors develop during childhood. RTS occurs because of the mutation that happens in only one copy of CBP which reduces the production of CREB binding protein by half. This protein is very much essential for growth and development before and after birth. RTS is a developmental disorder and is characterized by mental retardation, broad thumb, unusual facial appearance and broad and big toes. One out of every 1, 00,000 newborns are affected by RTS due to deletions, point mutations, or translocations in the CBP gene. [48] The thyroid hormone receptors depend on the transcriptional coactivators CBP/p300 for their function. Even though RTS patients have mutant CBP, the thyroid functions of these patients appear to be normal. This is probably because, the affinity of CBP towards the activated thyroid hormone receptors are so high that a reduction in the amount of CBP cannot disrupt the interaction between CBP and thyroid hormone receptors or may be some other hormone receptor coactivators are masking the consequences of CBP deficiency. [44]

3.8 P300/CBP associated factor

P/CAF or PCAF (CBP/p300 associated factor) is a transcriptional coactivator associated with the tumor suppressor gene p53. [49] The protein encoded by PCAF and adenovirus E1A binds to the third zinc finger domain (CH3) of CBP/p300 and hence both compete for the same binding site within CBP/p300. PCAF inhibits the cell cycle progression and adenovirus E1A functions by blocking the interaction between CBP/p300 and PCAF. [35]

3.9 Interaction with transcription factors

Both CBP and p300 play a major role in regulating transcription by its interaction with hundreds of transcription factors. CBP/p300 is called transcription coactivators because they bind to the transcription factor and enhances the rate of transcription by positioning histone acetyl transferase (HATs) near precise nucleosomes. [35] There are three zinc finger domains for CBP/p300, CH1, CH2 and CH3 and they are the binding site for numerous transcription factors which indeed makes CBP/p300 an important regulator of transcription mechanism. [40] P300 contains a Protein Kinase A (PKA) site near to the third zinc finger domain (CH3) and new studies suggest that phosphorylation by PKA regulates p300/CBP. [36]

HAT activity links chromatin remodeling and transcriptional activity. [40] HAT activity is not required for the transcription of naked DNA templates, but histone acetyl transferase activity is required for releasing the chromatin structure of the histones surrounding the DNA. This weakens the DNA –histone bond and hence protein translation increases because more binding sites are available for the transcription factors

to associate with the DNA. [36] P300/CBP contributes to transcriptional synergy by interacting with several transcription factors simultaneously. Even though they bind to hundreds of transcription factors, the concentration of CBP/p300 in the cells is very limited and hence a small decrease in the amount of these coactivators is very much damaging to the cell.

Both p300 and CBP are targeted by the adenovirus E1A. The transcription factor HIF-1α binds to the first zinc finger domain (CH1) of p300. [50] P300 upregulates the EPO gene in response to hypoxia. When a cell was induced with hypoxic conditions, a DNA binding complex was formed which contained the transcription factor HIF1α as well as the transcriptional co-activators p300 and CBP. [50] When enough energy is not available, cAMP (the energy sensor), is upregulated and then the transcription factor CREB bound to the CRE recruits CBP which functions as a transcriptional coactivator and binds to the transcription factor CREB and upregulates the cAMP responsive genes. The adenovirus ze1A can transform normal cells to malignant cells only when it is associated with p300/CBP. So, p300/CBP plays a major role in suppressing neoplastic transformation. [50]

E1A targets CBP/p300 and inhibits hypoxic induction of the target genes EPO and VEGF. P300/CBP is involved in the upregulation of a variety of hypoxia target genes. [50] E1A has the ability to bind to a number of transcription factors and thus act as scaffolds binding to different transcription factors directly and integrating genetic information from various transcription factors. Thus, they contribute to transcriptional synergy. [51] So, even though p300/CBP is known for its tumor suppressor

characteristics, it can also support tumor progression by associating with oncoproteins such as E1A. Hence, p300/CBP –HIF complex can be a target for cancer therapy. [50]

3.10 P300/CBP and p35srj protein

p35srj protein, a 35KDa cellular protein was identified, cloned and named as p35srj for its serine – glycine rich junction by Bhattacharya in the year 1999 and is a novel protein. The p35srj protein which binds to CH1 (the same binding site for HIF1α), inhibits the HIF1α-CBP/p300 association.

But, p35srj protein itself is a hypoxia inducible protein. So, this is a negative feedback regulation for hypoxia. [52] The p35srj protein is very unstable and is completely bound to p300/CBP through the carboxy terminus. P35srj blocks the association of HIF1α –CTAD (C terminal activation domain) with p300/CBP and that is how it inhibits HIF1 activity. [40] Thus, p35srj regulated HIF1 transcription in a negative feedback mechanism which is shown in the figure 8.

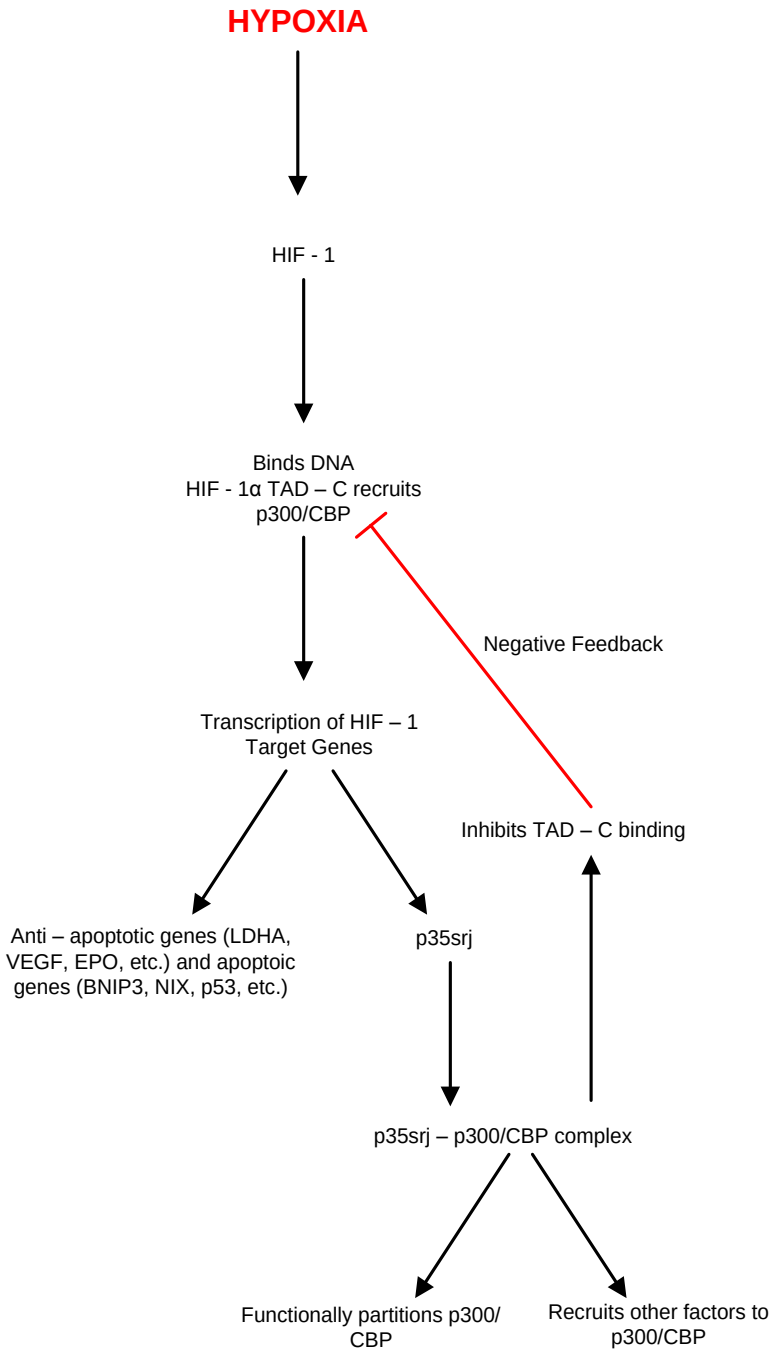

Figure 8: Negative feedback regulation of HIF1 by the protein p35srj

3.11 Hypoxia induces hyper phosphorylation of P300/CBP

P300 and CBP both being phospho proteins, hyper phosphorylation of p300/CBP is caused due to the release of Ca^{2+} ions from the IP_3 sensitive stores. [40] In the paper Adriana et al. the inhibitors of IP_3 receptors 2APB and Xestospongin C were used in the cells exposed to 1% hypoxia for 3 hours and that inhibited the hyper phosphorylation of p300/CBP. [40] Specific glucose metabolic pathways need to be activated for the hyper phosphorylation of p300. Calcium ions (Ca^{2+}) activate the enzyme G3PDH. [40] NADH passes through the mitochondrial membrane with the help of glycerol phosphate shuttle. [40] G3PDH reduces dihydroxyacetone to glycerol- 3- phosphate inside the cytoplasm with the help of NADH and oxidizes glycerol-3-phosphate back to dihydroxyacetone inside the mitochondria with the help of FAD and during this process, FADH2 is generated which is produce reducing equivalents to ubiquinone (Q) and to complex III of the mitochondria. [40] Glycolysis is inhibited by 2-deoxyglucose, which prevents the hyper phosphorylation of p300 and iodoacetate an inhibitor of GAPDH prevented the accumulation of HIF1α during hypoxia. [40] Moreover, the cells treated with substrates hydroxybutarate, pyruvate and α-keto Glutarate (α-KG) which activates the Krebs's cycle and generate NADH at complex I was not able to induce hyperphosphorylation of p300 in hypoxia induced cells which indicates that, for the hyperphosphorylation of p300 to occur, glycolysis is very much essential. [40] Palmitoyl coenzyme A, an inhibitor of G3PDH and DPI (diphenyliodonium) which is an inhibitor of $FADH_2$ decreased the p300 hyperphosphorylation. [40] The pathways responsible for the hyper phosphorylation of p300 during hypoxia is shown in the figure 9.

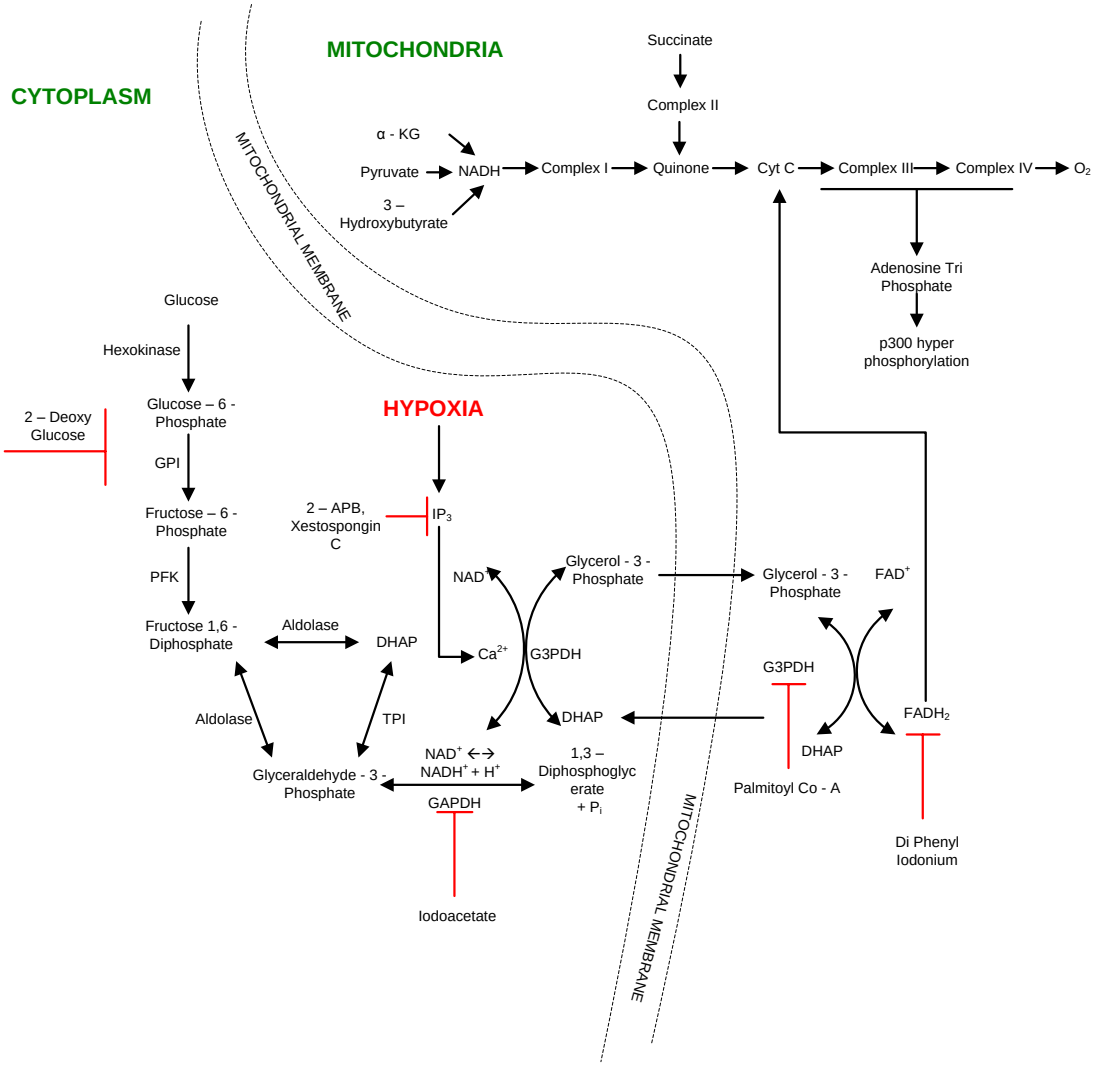

Figure 9: Pathways responsible for the hyper phosphorylation of p300 during hypoxia

4. TRANSLATIONAL CONTROL OF GENE EXPRESION DURING HYPOXIA

4.1 Introduction

Translation is the process by which messenger RNA (mRNA) is decoded by the ribosome complex to produce amino acid chain which folds to produce proteins. [53] During hypoxia, the gene expression of the target genes is controlled by regulating the process of translation and this is done by controlling the initiation steps of mRNA translation. [54] This is also the strategy adapted by solid tumors for growth and development, as it faces severe oxygen crisis since, the normal supply of oxygen falls short to satisfy the increasing demand of oxygen. [2] The phenomenon of protein synthesis requires a lot of energy. In order to save energy, protein synthesis is suppressed during hypoxia by regulating the initiation steps of mRNA translation by two different pathways, either by the phosphorylating eIF2 which is the eukaryotic initiation factor 2α or by inhibiting eIF4F, which is another eukaryotic initiation factor. [54] During hypoxia, even though there is a decrease in translation, there is not much translation inhibition at the individual gene level because of the presence of 3' and 5' untranslated regions of messenger RNA. [54]

4.2 PI3K/AKT/mTOR pathway

The growth factors like IGF1 (Insulin Growth Factor 1), IGF2 (Insulin Growth Factor 2) etc. sustains oxygen homeostasis in growing cells by activating the signaling pathways that includes phosphoinositide 3-kinase (PI3K), Protein Kinase B (PKB aka AKT) and mTOR (mammalian Target of Rapamycin). [2] EIF-4E (eukaryotic translation

initiation factor -4E) is increased due to this activity, which increases the translation of HIF-1α messenger RNA. [2] MTOR (Mammalian Target of Rapamycin) is a protein encoded by FRAP1 (FK506 binding protein 12-Rapamycin associated Protein 1) gene in humans. [55] Rapamycin is a product of bacteria which can inhibit mTOR activity. MTOR has FK506 binding protein 12 – Rapamycin (FBR) binding domain. [56] Once this rapamycin binds to mTOR, it impedes the mTOR activity. The main function of mTOR is to collect the inputs from the growth factor pathways. The activities of mTOR include regulation of synthesis of proteins, transcription of various genes, cell movement, growth, proliferation and survival of cells. [56] PI3K (phosphoinositide -3 kinase) are a family of intracellular enzymes which can phosphorylate phosphatidylinositol at its 3 position hydroxyl group of inositol ring. The main functions of PI3K are cell differentiation, cell motility, cellular growth, survival, proliferation and trafficking. [57] AKT is a protein kinase which is threonine/serine specific protein kinase that can activate mTOR. [58] AKT is involved in several important cellular processes such as metabolism of glucose, migration and proliferation of cells etc. and is able to induce protein synthesis pathways necessary for growth and development. [58] AKT binds to PIP_3 (Phosphatidylinositol (3, 4, 5)-triphosphate) or PIP_2 (Phosphatidylinositol (4, 5)-biphosphate) with high affinity. Once PI3K is activated by the signals from the upstream growth factors like IGF1, IGF2 etc. PI3K phosphorylates PIP_2 to PIP_3. Then, AKT binds to PIP_3 and AKT is phosphorylated by its kinases mTORC2 (Mammalian Target of Rapamycin Complex 2) and PDPK1 (phosphoinositide dependent kinase 1) Phosphorylation of mTORC2 results in the

phosphorylation of AKT by PDPK1. Once AKT is phosphorylated, it results in mTOR activation by its kinase activity. [59] PI3K/AKT/mTOR pathway is very important in the context of cancer because, they integrate signals from the upstream growth factors like IGF1, IGF2 etc. and reduces apoptosis and increases proliferation which contributes to cancer. PI3K activation leads to AKT activation which finally leads to mTOR activation. [60] PI3K/AKT/mTOR pathway is over reactive in cancer cells due to the faults or deficiencies in the tumor suppressor gene, Phosphatase and Tensin homolog (PTEN). The action of the phosphatase protein product of PTEN is responsible for its tumor suppression characteristic. It inhibits PI3K/AKT/mTOR pathway by converting PIP_3 to PIP_2. This tumor suppressor gene is involved in cell cycle regulation, and prevention of rapid cell division. [59] PTEN is one of the most mutated or lost tumor suppressor gene in almost all types of human cancers and approximately 70% of men with prostate cancer have already lost one copy of tumor suppressor PTEN gene by the time this disease is diagnosed. [61] Defect in the tumor suppressor genes PTEN and p53 results in a decrease in the neuronal energy production which leads to abnormal energy level production in hippocampus as well as cerebellum of the brain leading to autism. [62] To sum up, PI3K/AKT/mTOR pathway is inhibited by PTEN that works antagonistically to PI3K. The drastic reduction in AKT activation is because of the membrane localization factor from AKT. [61]

4.3 Unfolded protein response

Hypoxia results in suppression of protein synthesis by regulating the initial steps of mRNA translation and is linked to the activation of Unfolded Protein (UPR) due to Endoplasmic Reticulum stress. In this case, the process of translation is inhibited because the Eukaryotic Initiation Factor (eIF2α) is phosphorylated in a PERK dependent manner. [54] The stress in the organelle endoplasmic reticulum results in a cellular state response called Unfolded Protein Response (UPR). Due to this stress many proteins that are not folded properly accumulates inside the endoplasmic reticulum lumen. In this scenario, the UPR has two aims. First, it will stop the protein synthesis for some time and activate those signaling pathways that could increase the synthesis of molecular chaperones that are involved in proper folding of the proteins. This has to be done within a certain time limit. If they are unable to correct the misfolding with a certain time frame, unfolded protein response results in apoptosis of the cells facing ER stress. [63] EIF2α is the α subunit of eIF2 dimer and is involved in the initiation of translation. Ribosomes are the basic building blocks of proteins and they build proteins based on the messenger RNA information. EIF2 is a eukaryotic initiation factor that facilitates the binding of transfer RNA (tRNA) to ribosomes. A ternary complex (TC) is formed by the combination of eIF2, GTP (Guanosine Tri Phosphate) and the initiator methionine-tRNA (met-tRNA).Methionine is an amino acid that is coded by the initiation factor AUG codon which is the most common eukaryotic start codon.

The GDP has to be exchanged with another GTP and only the GTP-bound eIF2 can participate in another translation initiation. [64] After the formation of the ternary

complex, 43S Pre Initiation Complex (PIC) is formed once it binds with the small ribosomal subunit. Then, eIF4S unwinds the mRNA, to which the 43SPIC will later bind. Then the initiation factor eIF4S and 43S PIC forms the complex 48S on the mRNA and runs along the mRNA in search of the start codon AUG. [64] Now, once the start codon AUG is located, then the met-tRNA base pairs with the AUG codon and a protein that activates the GTP-ase, eIF5 recruits to the 48S complex and GTP is hydrolyzed to GDP by eIF2. [65] This results in the release of eIF2-GDP from the 48S complex and recruits the large ribosomal subunit, and forms the 80S initiation complex and finally the process of translation begins. [64] The Guanine Nucleotide Exchange Factor, eIf2B helps eIF2 to exchange its GDP for a GTP and a new round of translation initiation begins. [65]

The translation inhibition during the initial phase of mRNA translation is achieved by the phosphorylation of eIF2α. [54] The kinases act as a result of Endoplasmic Reticulum stress (PERK). The affinity of eIf2α for eIF2B increases due to the phosphorylation of eIF2α. But, eIF2B exchanges GDP (Guanosine Di Phosphate) to GTP (Guanosine Tri Phosphate) only with the unphosphorylated eIF2. EIF2B is inhibited by phosphorylated eIF2. The cellular concentration of eIF2B is very low when compared to the cellular concentration of eIF2. [66]

PERK or Eukaryotic Translation Initiation Factor2- Alpha Kinase -3 (EIF2AK3) is related to the Unfolded Protein Response (UPR). If there is an increase in the amount of proteins not folded properly in the endoplasmic reticulum lumen, BiP/GRP78 chaperones bind to these proteins and so BiP/GRP78 dissociates from their from their

corresponding receptors to bind to these proteins. [67] PERK oligomerizes with BiP in the ER stressed cells. [67] The activated PERK attenuates transcription by phosphorylating the α subunit of translation machinery, eIF2. PERK also can induce apoptosis by activating the pro apoptotic protein, CHOP (CCAA1/ enhancer binding protein homologous protein). [67] CHOP down regulates anti apoptotic protein BCL2. [68]

4.4 Regulation of translation during hypoxia through eIF2

Both severe and moderate hypoxia can cause the phosphorylation of eIF2α. [54] The difference is that during severe hypoxia, the phosphorylation of eIF2 occurs very rapidly within just 30 minutes when compared to the eIF2α phosphorylation by moderate hypoxia. [69] Moreover, this raid phosphorylation process is totally reversible due to ATP depletion during hypoxia. Due to the negative feedback loop in the hypoxic stress response pathway, even though the rapid phosphorylation occurs within half an hour, within a time span of about 4-8 hours, it partially recovers. By analyzing the levels of ribosomal association with mRNA, it is validated experimentally in ref. 54 that hypoxia reduces the number of ribosomes required per translation and shift to lower ribosomal density. [54] Thus, the translation inhibition by the phosphorylation of eIF2α impairs the ternary complex (TC) and decreases the ribosomal recruitment to transcripts of messenger RNA. [54] The pictorial representation of the regulation of the inhibition of translation is shown in the figure 10.

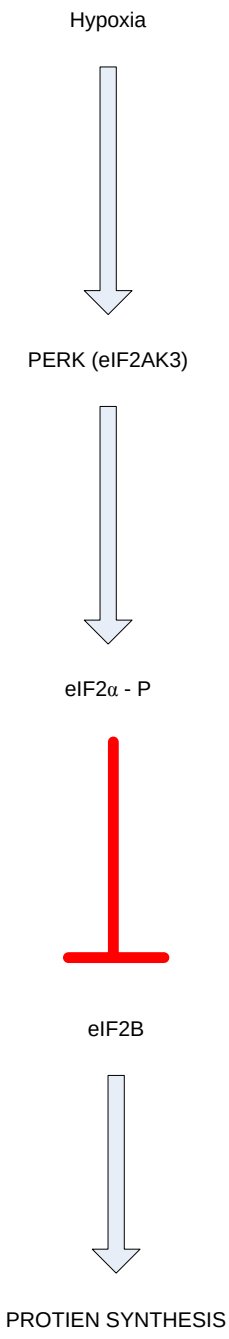

Figure 10: Translation inhibition by phosphorylation of eIF2α

5. RESULTS AND CONCLUSIONS

5.1 Introduction

In Biology, almost all the knowledge and information is available as signaling pathways. They are unable to represent the multivariate interaction between the genes, even though they can give a pictorial representation of the univariate interactions. [70] There are also possibilities that two or more pathways can share the same genes or the same node. Thus, a biological pathway gives a clear cut idea of the relationship between different genes in that pathway, but fails to provide the information about how these genes interact globally when they are present in different pathways. [71] The main aim of this work is to generate a Boolean network whose state transitions realize the hypoxic stress response pathway. The resulting Boolean network obtained shows dynamic behavior which is consistent with the experimental results and observations from the already published literatures.

By incorporating the expert knowledge obtained from the biological pathways, the cardinality of the search space of the network can be reduced. The main drawback of the pathway knowledge is that it provides only partial information and that too restricted to a specific context [71]. By mathematically modeling the multivariate interaction between the genes in a pathway, these networks can be used to differentiate between normal cell behavior and diseased cell behavior [70]. By working with the genetic regulatory networks instead of the biological pathways, the holistic behavior of the genes can be captured and the appropriate therapeutic interventions can be developed. The

main aim of the biological pathways is to demonstrate the complex interactions taking place inside a cell when a stimulus or stress is applied. [71]

5.2 Stress response pathways

The main aim of adaptive stress response pathways is to activate the transcription of cytoprotective genes [34]. Metazoans respond to the stress via growth factors to promote the growth of the organisms. [34] But, if a stress is applied to a cell exogenously such as xenobiotic, radiation, heat etc. these cells respond via several highly conserved adaptive stress response pathways that will try to attenuate the consequences of these stress and re-establish the homeostasis. [34]

The rapid response of these stress response pathways can be attributed to the architecture of the stress response pathways. [70]. Basic components of this architecture include a transcription factor, a sensor and a transducer. A schematic diagram of the architecture of the stress response pathways is shown in figure 11.

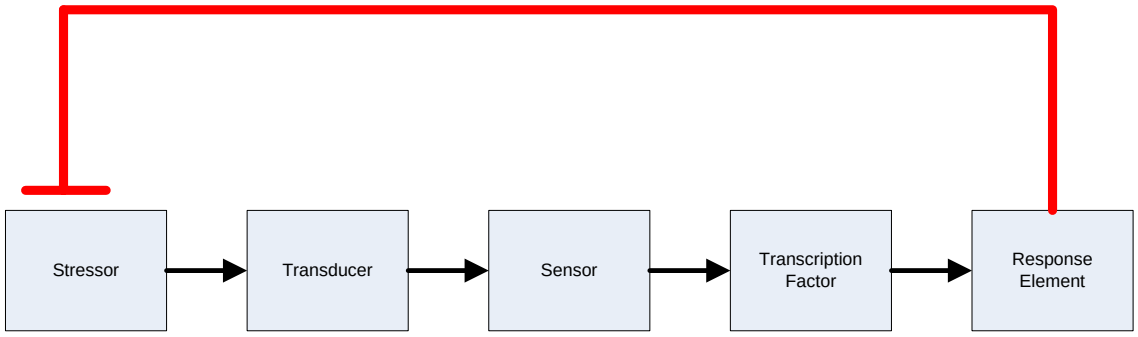

Figure 11: Architecture of the stress response pathways

A transcription factor is a protein which binds to the DNA through the response elements within its promoter region and thus activates or upregulates the expression of the target genes. [34] During the absence of stress, the transcription factor is prevented from entering into the nucleus with the help of a protein known as a sensor. It interacts with the transcription factor in such a way that the transcription factor is sequestered in the cytoplasm. [34] When a cell is faced by a stress, an enzymatic protein, transducer, transfers a cue from a pathway which lies upstream of the sensor/transcription factor complex. [34] The transducer either modifies the transcription factor or modifies the sensor which destabilizes the sensor/transcription factor complex. The activated transcription factor then enters to the nucleus and upregulates the target genes. [72]

5.3 Network modeling

Given two genes/proteins A & B and their binary values a, b ϵ {0, 1}, a pathway segment, $A \xrightarrow{t:a,b} B$ means that, if gene A assumes the value a then, gene B transitions to the value b in no more than t subsequent time steps. [71] Similarly, a

pathway is defined as a sequence of pathway segments of the form, $A \xrightarrow{t1:a,b} B \xrightarrow{t2:b,c} C$. [71] A Boolean network (BN), $\gamma = (V, F)$, on n genes is defined by a set of nodes/genes $V=\{x_1, \ldots, x_n\}$, $x_i \in \{0,1\}$, i =1,…., n, and a list $F= (f_1, \ldots, f_n)$, of Boolean functions, $f_i : \{0, 1\}^n \rightarrow \{0, 1\}$, i=1, …., n . [73] The gene expression of each gene is quantized to two levels, 0 and 1. The node x_i represents the gene expression of the gene *i*, where $x_i= 0$ means that, gene *i* is off and $x_i = 1$, means that gene *i* is ON. [70] The predictor function f_i updates the states of all the genes in γ at every time step. The network state also called the Gene activity Profile (GAP) of the network at time t is given by, $x (t) = (x_1(t), x_2(t), \ldots, x_n (t))$. [71] The prior biological knowledge of the hypoxia stress response pathway is used to model the Boolean networks of hypoxia stress response pathway. In order to get an update equation, for each and every gene in the Boolean network obtained, the Karnaugh map reduction technique is applied to it. [71] The hypoxia stress response pathway is shown in figure 12.

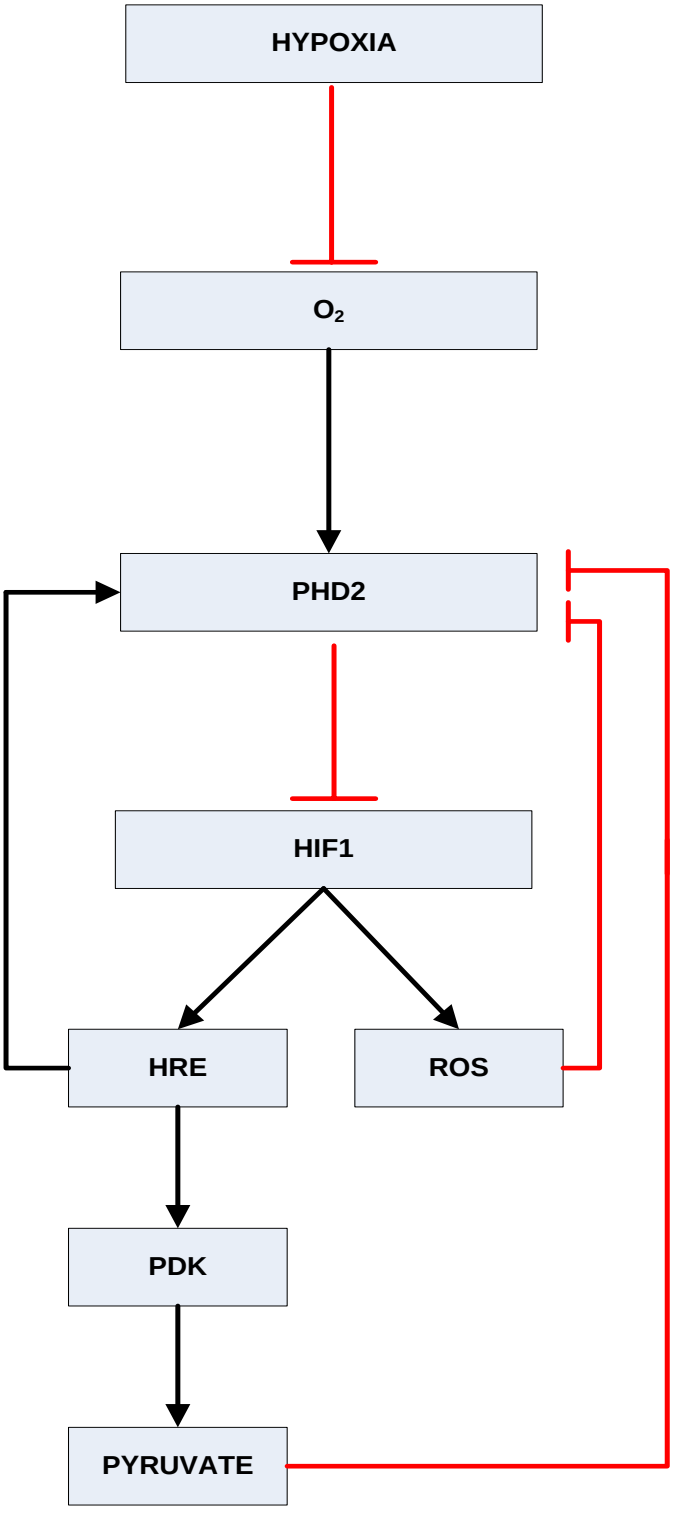

Figure 12: Hypoxia stress response pathway

The pathway segments from the pathways in the above figure are given below:

$$Hypoxia \xrightarrow{1:1,0} O2 \quad (5.1)$$

$$O2 \xrightarrow{1:1,1} PHD2 \quad (5.2)$$

$$PHD2 \xrightarrow{1:1,0} HIF1 \quad (5.3)$$

$$HIF1 \xrightarrow{1:1,1} HRE \quad (5.4)$$

$$HRE \xrightarrow{1:1,1} PDK \quad (5.5)$$

$$HRE \xrightarrow{1:1,1} PHD2 \quad (5.6)$$

$$PDK \xrightarrow{1:1,1} PYRUVATE \quad (5.7)$$

$$PYRUVATE \xrightarrow{1:1,0} PHD2 \quad (5.8)$$

$$HIF1 \xrightarrow{1:1,1} ROS \quad (5.9)$$

$$ROS \xrightarrow{1:1,0} PHD2 \quad (5.10)$$

From the above described pathway segments and applying the techniques of K map reduction explained in [71], the update equation of each and every gene is obtained. The state space is defined as [PHD2 HIF HRE ROS PDK PYRUVATE]. A set of possible Boolean networks were obtained, out of which the one that matched most to the prior literature knowledge were chosen. The update equations are given below:

$$O2_{next} = \overline{Hypoxia} \quad (5.11)$$

$$PHD2 = \overline{(PYRUVATE + ROS)*(O_2 + HRE)} \quad (5.12)$$

$$HIF_{next} = \overline{PHD2} \quad (5.13)$$

$$HRE_{next} = HIF \quad (5.14)$$

$ROS_{next} = HRE$ (5.15)

$PDK_{next} = HRE$ (5.16)

$PYRUVATE_{next} = PDK$ (5.17)

This Boolean network will have two different contexts based on the value of the hypoxic stress, i.e., when Hypoxia =0 and when Hypoxia =1. Figure 13 shows the diagram of state transition when hypoxia=0. In this diagrams showing the state transition the order of the genes in the binary state representation are as such: [PHD2 HIF HRE ROS PDK PYRUVATE]. For the ease of demonstration, the decimal equivalents are used to represent their binary states. For example, the decimal equivalent 31 is used to represent its binary state (011111). The attractors give rise to the steady state properties of the network obtained.

There are two singleton attractors 32(100000) and 31(011111) in the diagram showing the state transition during normoxia. This is shown in figure 3. Actually, the singleton attractor, 31(011111) is present in both cases of Hypoxia=0 and Hypoxia =1, which is not biologically possible. When hypoxic stress is not applied, that is during normoxia, PHD2 is active and HIF1α is hydroxylated by PHD2, which results in the degradation of HIF1 and the genes/proteins ROS, PDK and Pyruvate are OFF. Thus, when hypoxia is 0, the presence of the singleton attractor 31(011111) does not make any sense because PHD2 will be ON and the rest of the genes will be OFF in this case. From the previous biological knowledge from ref.2, it is clear that during the absence of hypoxic stress all other genes except PHD2 is inactive. Thus, the presence of singleton

attractor 32(100000) when Hypoxia=0 is consistent with the biological literature. The state transition diagram when hypoxia = 0 is shown in figure 13.

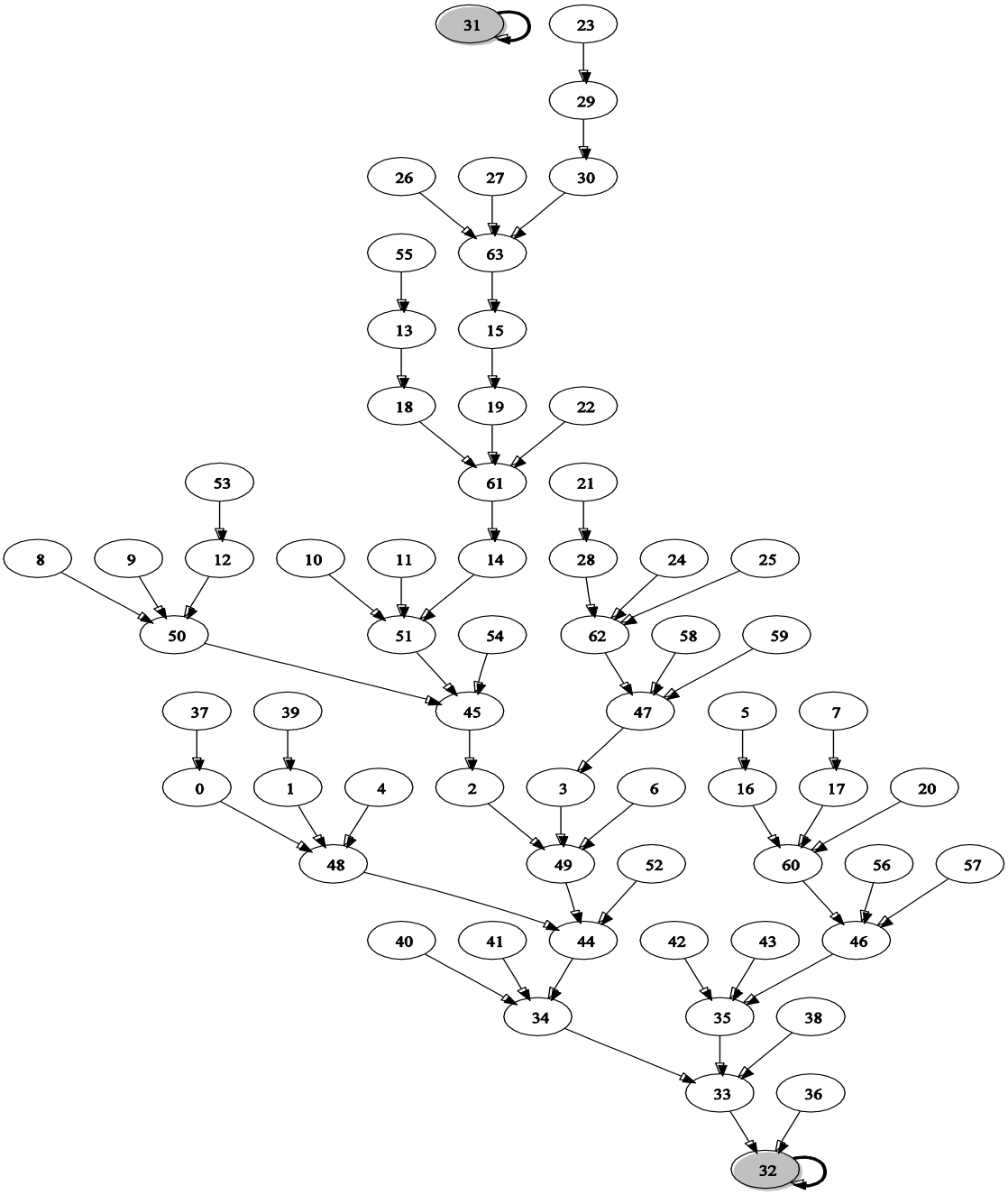

Figure 13: State transition diagram when hypoxia = 0

Next, when Hypoxia=1, the state transition diagram in figure 14 is obtained and in this case, the state space is partitioned. There are two steady state cyclic attractors involving cyclic variation in the experimental patterns of all the 6 genes/proteins. The final attractor cycle will depend on the starting state, one of them cycling through the states 47 (101111)→ 3 (000011)→17 (010001)→28 (011100)→62 (111110)→ 47(101111) and the other attractor cycle cycling through the states, 63 (111111)→15 (001111)→19 (010011)→29 (011101)→30 (011110)→63 (111111). So, the time domain response during hypoxia exhibit cyclic oscillations. Hence, during normoxia, the system rests in one state only, whereas during hypoxia, the genes or nodes oscillate in a cyclic manner. The state transition diagram when hypoxia =1 is shown in figure 14.

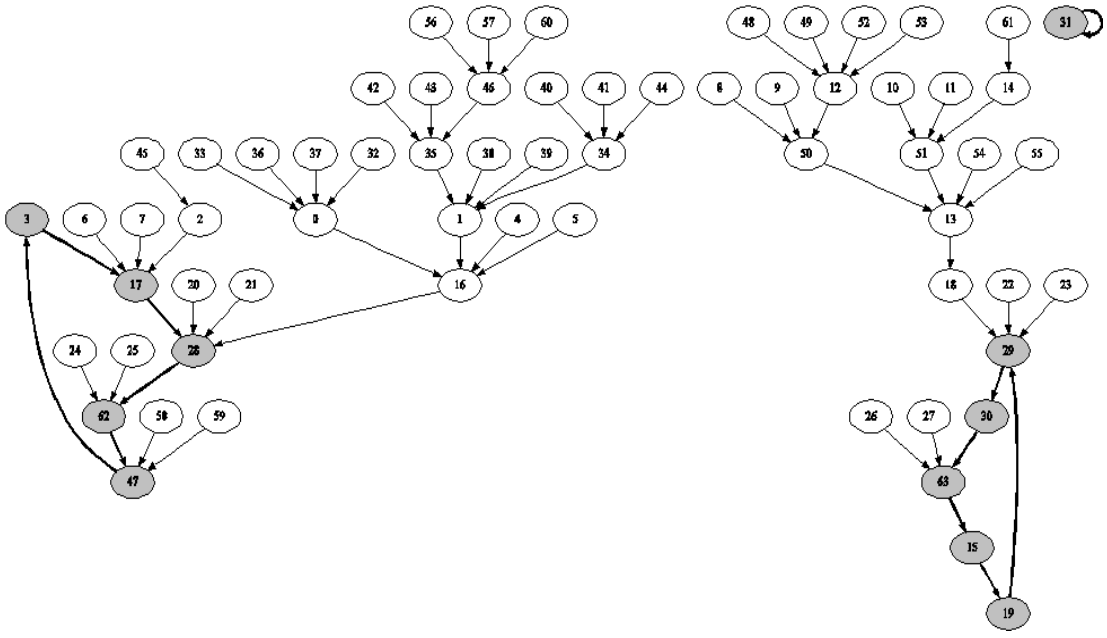

Figure 14: State transition diagram when hypoxia = 1

5.4 Time domain simulation results

To understand the functionality of the Boolean network described by equations from (5.11) to (5.17), an external stress input signal was given for a duration of 100 time steps to simulate the Boolean network obtained using MATLAB. The simulated time course behavior of the expression pattern of the nodes, PHD2, HIF, HRE, ROS, PDK, and Pyruvate are shown in figure 15 below for the case when hypoxia = 0.

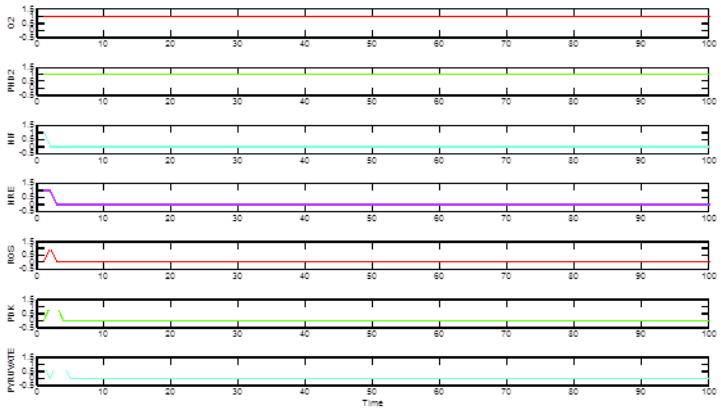

Figure 15: Time response behavior when hypoxia = 0

When $O_2 = 1$, it implies that hypoxic stress = 0 and when $O_2 = 0$, it implies that hypoxic stress = 1. From the simulations obtained in figure 15, it is clear that PHD2 is activated and the rest of the genes are deactivated when the cell is not subjected to hypoxic stress. From the time response behavior in figure 15 it is clear that there is an out of phase relationship between PHD2 and the rest of the genes. The time response behavior of the system when hypoxia =1 is shown in figure 16.

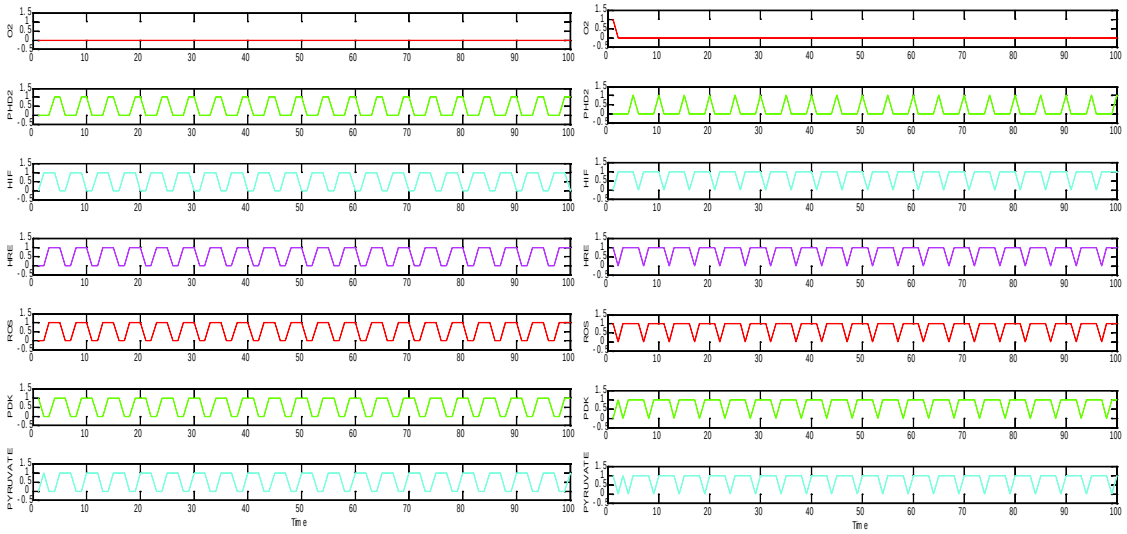

Figure 16: Time response behavior of the system when hypoxia = 1

5.5 Literature validation

Reference 74 shows that the optimal HIF1α transcriptional activity requires sequential inhibition of PHD2. [74] Here, they used the pharmacological inhibitor JNJ-42041935which inhibits the hydroxylation of HIF1α by PHD2, via displacing the endogenous 2-oxalogluatarte co-substrate required for the proper functioning of PHD2. [75] The figure 2 of that paper shows the densitometric analysis of the western blot which demonstrates the stabilization of HIF1α at 3% of oxygen and more amount of HIF1α stabilization at 1% of oxygen in Human Embryonic Kidney cells transfected with pGluc-HRE At 21% of oxygen concentration that is during normoxia, there was no HIF1α stabilization (figure 2(C)), which shows that HIF1α stabilizes during low oxygen tension. Figures 3(B) to 3(D) and 3(G) and 3(H) experimentally validate the HIF1α stabilization when PHD/FIH is inhibited.

Reference 76 presents an experimental based computational method that shows the in phase relationship between ROS and HIF1α. Therapeutic agents target five compounds that is involved in HIF1α hydroxylation, i.e. iron (Fe), ascorbate (Asc), hydrogen peroxide (H_2O_2) 2-oxaloglutarate (2-OG) and succinate. H_2O_2 oxidizes ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) and prevents the binding of ferrous iron to PHD2. [76] Thus, the PHD2 action is inhibited and HIF1α is stabilized. Moreover, H_2O_2 recruits ascorbate as a free radical scavenger not allowing to reduce ferric iron to ferrous iron and also prevents ascorbate from binding to PHD2 which inhibits the PHD2 action. [76] Free radicals and mitochondrial dysfunction alter the concentration of 2-OG and succinate which is involved in HIF1α hydroxylation. ROS affects HIF hydroxylation by changing H_2O_2, Fe^{2+}, ascorbate, 2-OG and succinate. [76] The figure 2(A) in that paper shows the effect of ROS on HIF1α by changing the ascorbate and Fe^{2+} levels. In the normal conditions, when Fe^{2+} and ascorbate were upregulated, HIF1α reached its peak value within an hour. Next, when ROS production was increased by 10 fold by decreasing Fe^{2+} and ascorbate concentrations, it led to 5 fold increase in the maximum HIF1α expression. This clearly explains that ROS and HIF1α are in phase. Figure 2(B) in this paper shows the experimental result that a 10 fold decrease in Fe^{2+} and 2-OG concentration results in increased ROS and HIF1 activity, whereas a 10 fold increase in Fe^{2+} and 2-OG concentration results in decreased HIF1 concentration. [76] Thus, this paper experimentally validates the in phase relationship between HIF1α and ROS.

Reference 17 experimentally validates that hypoxia activates the transcription of several hypoxia target genes by increasing ROS concentration. [17] In this paper, they

selected ρ_0 cells by exposing Hep3B cell culture to mitochondrial inhibitors rotenone and antimycin A. [17] The measurement of ROS was done using 2', 7'-dichloroflurescin (DCFH) diacetate. ROS oxidizes DCFH to 2', 7'-dichloroflurescin DCF). Experimental results in figure 1(A) of that paper shows that the cellular oxygen uptake by ρ_0 cells was approximately only 10% of the total cellular uptake of oxygen by wild type Hep3B cells which indicates that almost all the oxygen is used up in the mitochondria. [17] Figure 1(C) of that paper shows that there is no EPO secretion in ρ_0 cells during hypoxia (1.5% of oxygen), which means that the organelle mitochondria is very much essential for the induction of hypoxia target genes, whereas in wild type Hep3B cells, there is a high amount of EPO secretion during hypoxia (1.5% of oxygen).

When ρ_0-Hep3B cells were exposed to hypoxia, it did not display any HIF-1 DNA binding activity [17]. This paper experimentally validates that mitochondria is very much essential for transcriptional activity of hypoxia. DCFH diacetate was used to assess the ROS production in the Hep3B wild type cells. H_2O_2 oxidizes DCFH to DCF. [17] Figure 2(A) of that paper shows the fluorescence of DCF at various levels of oxygen. During normoxia, (8% of oxygen), there was no DCF fluorescence (ROS generation) at all. To strengthen the fact that the complex III of mitochondria is the major site of ROS production, inhibitor of complex I of mitochondria, rotenone and inhibitor of complex III of mitochondria, myxothiazol were administered during hypoxia. [17] Both these inhibitors resulted in a decrease in DCF fluorescence (ROS generation) during hypoxia indicating that complex III is the major site of ROS

production during hypoxia. Figures 3(B) to 3(C) and 3(D) of that paper shows the same results.

Reference 77 experimentally validated that the glycolytic enzymes which are the hypoxia target genes are very much important for the metabolic adaptation to hypoxia by increasing the conversion of glucose to pyruvate and finally the accumulation of lactate. [77] They observed that, HIF1α null mouse embryo fibroblasts (MEFs) failed to upregulate PDK1. This shows that PDK1 is a direct hypoxia target gene i.e. an in phase relationship between HRE and PDK1. The immunobolt assay in figure 1 in that paper shows the increased expression of PDK1 in hypoxic cells by HIF1. [77] Moreover, HIF1α null MEFs did not express PDK1, whereas the wild type MEFs showed a drastic increase in PDK1 gene expression clearly depicted in figure 1(C) of that paper.

5.6 Conclusion and future work

Thus, the Boolean network was developed that could generate trajectories consistent with the hypoxia stress response pathway. The resulting network exhibited dynamic behavior consistent with the published literature where ever applicable.

The literature validations considered only 2 genes/proteins at a time. They did not experimentally validate the simultaneous expression of these 6 genes i.e, PHD2, HIF, HRE, ROS, PDK and Pyruvate during hypoxia. There is need for more experiments validating the multivariate relationship between these genes rather than focusing on the univariate relationships.

Since hypoxia is related to almost all the pathological conditions, especially cancer, stroke, atherosclerosis and asthma, proper drug interventions can be hopefully developed by targeting to control the simultaneous oscillations of these six genes during hypoxia.

REFERENCES

[1] West, John B. (1977). "Pulmonary Pathophysiology- The Essentials". Baltimore. Williams and Wilkins.

[2] KangAe Lee, Robert, A. Roth, John, J. Lapres. (2007). "Hypoxia, Drug Therapy and Toxicity". Pharmacology and Therapeutics. **113**(2): 229-246.

[3] Semenza, G. L. (2006). "HIF1 and Human Disease: One Highly Involved Factor". Genes Dev. **2000**(14): 1983-1991.

[4] Gonzalez, C., Almaraz, L., Obeso, A., Rigual, R. (1994). "Carotid Body Chemoreceptors: From Natural Stimuli to Sensory Discharges". Physiol. Rev. **74** (4): 829–98.

[5] Ward, J.P. (2008). "Oxygen Sensors in Context". Biochim Biophys Acta. **1777** (1): 1–14.

[6] Lowe, C.H., Vance, V.J. (1955). "Acclimation of the Critical Thermal Maximum of the Reptile Urosaurus Ornatus". Science. **122** (3158): 73-74.

[7] Hockel, M., Vaupel, P. (2001). "Tumor Hypoxia: Definitions and Current Clinical, Biologic and Molecular Aspects". J Natl Cancer Inst . **93** (4): 266-276.

[8] Beck, I., Ramirez, S., Weinmann, R., Caro, J. (1991). "Enhancer Element at the 3′-Flanking Region Controls Transcriptional Response to Hypoxia in the Human Erythropoietin Gene". J Biol Chem. **266** (24):15563−15566.

[9] Wang, G. L., Jiang, B. H., Rue, E. A., Semenza, G. L. (1995). "Hypoxia Inducible Factor 1 is a Basic-Helix-Loop-Helix-PAS Heterodimer Regulated by Cellular O_2 Tension". Proc. Natl. Acad. Sci. U.S.A**. 92** (12); 5510−5514.

[10] Yu, A. Y., Frid, M. G., Shimoda, L. A., Wiener, C. M., Stenmark, K., Semenza, G. L. (1998). "Temporal, Spatial and Oxygen-Regulated Expression of Hypoxia-Inducible Factor 1 in the Lung". Am J Physiol. **275**(4 Pt 1): L818−L826.

[11] Mahon, P. C., Hirota, K., Semenza, G. L. (2001). "FIH-1: A Novel Protein that Interact with HIF-1 α and VHL to Mediate Repression of HIF-1 Transcriptional Activity". Genes Dev. **15** (20): 2675−2686.

[12] Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., et al. (2001). "C. Elegans EGL-9 and Mammalian Homologs Define a Family of Dioxygenases that Regulate HIF by Prolyl Hydroxylation". Cell. **107**(1):43-54.

[13] Lando, D. (2002). "Asparagine Hydroxylation of the HIF Transactivation Domain- A Hypoxia Switch". Science. **295**(8): 856−861.

[14] Guzy, R.D., Hoyos, B., Robin, E., Chen, H., Liu, L., Mansfield, K.D., et al. (2005b) "Mitochondrial Complex III is Required for Hypoxia Induced ROS Production and Oxygen Sensing". Cell Metab. **1**(6): 401-408.

[15] Albert, L. Lehinger. (1982). "Principles of Biochemistry". New York. Worth Publishers Inc.

[16] Karp, Gerald (2008). "Cell and Molecular Biology (5th ed.)". Hoboken, New Jersey. John Wiley and sons.

[17] Chandel, N. S., McClintock, D. S., Feliciano, C. E., Wood, T.M., Melendez, J. A., Rodriguez, A. M., et al. (2000). "Reactive Oxygen Species Generated at Mitochondrial Complex III Stabilize Hypoxia-Inducible Factor-1 Alpha During Hypoxia: A Mechanism of O_2 Sensing". J Biol Chem. **275** (33):25130-25138.

[18] Qun Chen, Shadi Moghaddas, Charles, L. Hoppel and Edward, J. Lesnefsky. (2008) "Ischemic Defects in the Electron Transport Chain Increase the Production of Reactive Oxygen Species from Isolated Rat Heart Mitochondria". Am. J. Physiol. Cell. Physiol. **294** (2):C460-C466.

[19] Gong, Y., Agani, F. H. (2005). "Oligomycin Inhibits HIF1α Expression in Hypoxic Tumor Cells". Am. J. Physiol. Cell. Physiol. **288** (5): 1023−1029.

[20] Tani Kenzaburo, Michihiro, C. Yoshida, Satoh Hitishi, Mitamura Keiji, Noguchi Tamio, Tanaka Takeihiko, Fuiji Hisaichi, Miwa Shiro. (1988). "Human M2-Type Pyruvate Kinase: CDNA Cloning, Chromosomal Assignment and Expression in Hepatoma". Gene. **73**(2): 509–516.

[21] Weibo Luo, Hongxia Hu, Ryan Cahng, Jun Zhong, Matthew Knabel, Robert O' Meally, Robert, N. Cole, Akhilesh Pandey, Gregg, L. Semenza. (2011). "Pyruvate Kinase M2 is a PHD3 –Stimulated Coactivator for Hypoxia –Inducible Factor 1". Cell. **145**(5):732-744.

[22] Christofk, H.R., Vander Heiden, M.G., Harris, M.H., Ramanathan, A., Gerszten, R.E., Wei, R., Fleming, M.D., Screiber, S.L., and Cantley, L.C. (2008). "The M2

Splice Isoform is Important for Cancer Metabolism and Tumor Growth". Nature. **452**(7184): 230-233.

[23] Hitosugi, T., Kang, S., Vander Heiden, M.G., Chung, T.W., Elf, S., Lythgoe, K., Dong, S., Lonial, S., Wang, X., Chen, G.Z., et al. (2009). "Tyrosine Phosphorylation Inhibits PKM2 to Promote Warburg Effect and Tumor Growth". Sci. Signal. **2**(97): ra73.

[24] Vengellur, A., Phillips, J. M., Hogenesch, J. B., Lapres, J. J. (2005). "Gene Expression Profiling of Hypoxia Signaling in Human Hepatocellular Carcinoma Cells". Phys Genom. **22**(3): 308−318.

[25] Vengellur, A., Woods, B. G., Ryan, H. E., Johnson, R. S., Lapres, J. J. (2003). "Gene Expression Profiling of the Hypoxia Signaling Pathway in Hypoxia Inducible Factor 1α Null Mouse Embryonic Fibroblasts". Gene Exp. **11**(3-4): 181−197.

[26] Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D., Pouyssegur, J. (2003). "HIF Prolyl-Hydroxylase 2 is the Key Oxygen Sensor Setting Low Steady-State Levels of HIF-1 Alpha in Normoxia". EMBO J. **22**(16): 4082−4090.

[27] Cioffi, C. L., QinLiu, X., Kosinski, P. A., Garay, M., Bowen, B. R. (2003). "Differential Regulation of HIF-1 Alpha Prolyl-4-Hydroxylase Genes by Hypoxia in Human Cardiovascular Cells". Biochem. Biophys. Res. Commun. **303**(3): 947−953.

[28] Stolze, I. P., Tian,Y. M., Appelhoff, R. J., Turley, H., Wykoff, C. C., Gleadle, J.M., et al. (2004). "Genetic Analysis of the Role of the Asparaginyl Hydroxylase Factor Inhibiting Hypoxia-Inducible Factor (HIF) in Regulating HIF Transcriptional Target Genes". J. Biol. Chem. **279**(41): 42719−42725.

[29] Piret, J. P., Motter, D., Raes, M., & Michiels, C. (2002). "Is HIF-1 Alpha a Pro- or an Anti-Apoptotic Protein?". Biochem. Pharmacol. **64**(5-6): 889−892.

[30] Denko, N. C., Fontana, L. A., Hudson, K. M., Sutphin, P. D., Raychaudhuri, S., Altman, R., et al. (2003). "Investigating Hypoxia Tumor Physiology through Gene Expression Pattern". Oncogen. **22**(37): 5907−5914.

[31] Gregg, L. Semenza. (1998). "Hypoxia-Inducible Factor 1: Master Regulator of Oxygen Homeostasis". Curr. Opin. Genet. Dev. **8**(5): 588-594.

[32] Gatenby, R.A., Gillies, R.J. (2004). "Why Do Cancers Have High Aerobic Glycolysis?" Nature Reviews Cancer. **4** (11): 891-899.

[33] Seagroves, T.N., Ryan, H.E., Lu, H., Wouters, B.G., Knapp, M., Thibault, P., Laderoute ,K., and Johnson , R.S. (2001) . "Transcription Factor HIF1 is a Necessary Mediator of the Pasteur Effect in Mammalian Cells". Mol. Cell. Biol.**21** (10): 3436-3444.

[34] Simmons, S.O., Fan, C.Y., Ramabhadran, R. (2009) "Cellular Stress Response Pathway System as a Sentinel Ensemble in Toxicological Screening". Toxicol. Sci. **111**(2): 202-225.

[35] Sterner, D. E., Berger, S. L. (2000). "Acetylation of Histones and Transcription-Related Factors". Microbiol. Mol. Biol. Rev. **64**(2): 435–459.

[36] Lee, K.K., Workman, J.L. (2007). “Histone Acetyltransferase Complexes: One Size Doesn't Fit All”. Nat. Rev. Mol. Cell Biol. **8** (4): 284–95.

[37] Richard Eckner, Mark, E. Ewen, David Newsome, Mike Gerdes, James, A. DeCaprio, Jeanne Bentley Lawrence, David, M. Livingston. (1994). “Molecular Cloning and Functional Analysis of the Adenovirus E1A-Assoclated 300-kD Protein (p300) Reveals a Protein with Properties of a Transcriptional Adaptor”. Genes Dev. **8**(8): 869-884.

[38] Mayr, B., Montminy, M. (2001). “Transcriptional Regulation by the Phosphorylation-Dependent Factor CREB”. Nat. Rev. Mol. Cell Biol. **2** (8): 599–609.

[39] Boulpaep, Emile, L., Boron, Walter F. (2003). “Medical Physiology: A Cellular and Molecular Approach”. Philadelphia. Saunders.

[40] Adriana Zakrzewska, Phillip, O. Schnell, Justin, B. Striet, Anna Hui, Jennifer, R.Robbins, Milan Petrovic, Laura Conforti, David Gozal, Marc, G. Wathelet, Maria, F. Czyzyk-Krzeska. (2005). “Hypoxia Activated Metabolic Pathway Stimulates Phosphorylation of P300 and CBP in Oxygen Sensitive Cells”. J. Neurochem. **94**(5): 1288-1296.

[41] Kung, A.L., Rebel, V.I., Bronson, R.T., Ch’ng, L.E., Sieff, C.A., Livingston, D.M., Yao, T.P. (2000). “Gene Dose-Dependent Control of Hematopoiesis and Hematologic Tumor Suppression by CBP”. Genes & Dev. **14**(3): 272–277.

[42] Gu, W., Roeder, R.G. (1997). “Activation of P53 Sequence- Specific DNA Binding by Acetylation of the P53 C-Terminal Domain”. Cell **90**(4): 595–606.

[43] Zeng, X., Chen, L., Jost, C.A., Maya, R., Keller, D., Wang, X., Kaelin, W.G., Jr., Oren, M., Chen, J., Lu, H. (1999). "MDM2 Suppresses P73 Function Without Promoting P73 Degradation". Mol. Cell. Biol. **19**(5): 3257–3266.

[44] Richard, H. Goodman, Sarah Smolik. (2000). "CBP/P300 in Cell Growth, Transformation, and Development". Genes Dev. **14**(13): 1553-1577.

[45] Waltzer, L., Bienz, M. (1998). "Drosphila CBP Represses the Transcription Factor TCF to Antagonize Wingless Signaling". Nature. **395**(6701): 521–525.

[46] Zimmermann, H., Degenkolbe, R., Bernard, H.U., O'Connor, M.J. (1999). "The Human Papilloma Virus Type 16 E6 Oncoprotein Can Down-Regulate P53 Activity by Targeting the Transcriptional Coactivator CBP/P300". J. Virol. **73**(8): 6209– 6219.

[47] Perkins, N.D., Felzien, L.K., Betts, J.C., Leung, K., Beach, D.H., Nabel, G.J. (1997). "Regulation of NF-B by Cyclin-Dependent Kinases Associated with the P300 Coactivator". Science. **275**(5299): 523–527.

[48] Rubinstein, J.H., Taybi, H. (1963). "Broad Thumbs and Toes and Facial Abnormalities". Am. J. Dis. Child. **105**: 588–608.

[49] Linares, L.K., Kiernan, R., Triboulet, R. (2007). "Intrinsic Ubiquitination Activity of PCAF Controls the Stability of the Oncoprotein Hdm2". Nat. Cell Biol. **9** (3): 331–338.

[50] Arany, Z., Huang, L. E., Eckner, R., Bhattacharya, S., Jiang, C., Goldberg, M. A., Bunn, H. F., Livingstone, D. M. (1996) "An Essential Role for P300/CBP in

Cellular Response to Hypoxia". Proc. Natl. Acad. Sci. USA **93**(23): 12969–12973.

[51] Arany, Z., Newsome, D., Oldread, E., Livingston, D. M., Eckner, R. (1995). "A Family of Transcriptional Adaptor Proteins Targeted by the E1A Oncoproteins". Nature **374**(6517): 81–84.

[52] Bhattacharya, S., Michels, C. L., Leung, M. K., Arany, Z. P., Kuang, A. L., Livingstone, D. M. (1999). "Functional Role of P35srj, A Novel P300/CBP Binding Protein During Transactivation by HIF-1". Genes Dev. **13**(1): 64–75.

[53] Joseph, F. Ross, Micheal Orlowski. (1981). "Growth-Rate-Dependent Adjustment of Ribosome Function in Chemostat-Grown Cells of the Fungus Mucor Racemosus" Journal of Bacteriology. **149**(2): 650-653.

[54] Twan van den Beucken, Marianne Koritzinsky, Bradly, G. Wouters. (2006). "Translational Control of Gene Expression During Hypoxia". Cancer Biology & Therapy. **5**(7): 749-755.

[55] Kim, J. W., Tchernyshyov, I., Semenza, G. L., Dang, C. V. (2006). "HIF-1-Mediated Expression of Pyruvate Dehydrogenase Kinase: A Metabolic Switch Required for Cellular Adaptation to Hypoxia". Cell Metab. **3**(3): 177−185.

[56] Eric, J. Brown, Mark, W. Albers, Tae Bum Shin, Kazuo Ichikawa, Curtis, T. Keith, William, S. Lane, Stuart, L. Schreiber. (1994). "A Mammalian Protein Targeted by G1-Arresting Rapamycin-Receptor Complex". Nature. **369**(6483):756-758.

[57] Zachary, A. Knight, Beatriz Gonzalez, Morri, E. Feldman, Eli, R. Zunder,

David, D. Goldenberg, Olusegun Williams, Robbie Loewith, David Stokoe, Andras Balla, Balazs Toth, Tamas Balla, William, A. Weiss, Roger, L. Williams, Kevan, M. Shokat. (2006). "A Pharmacological Map of the PI3-K Family Defines a Role for p110a in Insulin Signaling". Cell. **125**(4): 733-747.

[58] Timothy, A. Yap, Michelle, D. Garrett, Mike, I. Walton, Florence Raynaud, Johann, S. de Bono, Paul Workman. (2008). "Targeting the PI3K–AKT–mTOR Pathway: Progress, Pitfalls, and Promises". Curr. Opin. Pharmacol. **8**(4): 393-412.

[59] Thomas, F. Franke, David, R.Kaplan, Lewis, C. Cantley, Alex Toker. (1997). "Direct Regulation of the Akt Proto-Oncogene Product by Phosphatidylinositol-3, 4-Bisphosphate". Science.**275** (5300):665-668.

[60] Robert, S. Garofalo, Stephen, J. Orena, Kristina Rafidi, Anthony, J. Torchia, Jeffrey, L. Stock, Audrey, L. Hildebrandt, Timothy Coskran, Shawn, C. Black, Dominique, J. Brees, Joan, R. Wicks, John, D. McNeish, Kevin, G. Coleman. (2003). "Severe Diabetes, Age Dependent Loss of Adipose Tissue, and Mild Growth Deficiency in Mice Lacking AKT2/PKBβ". J Clin. Invest. **112**(2): 197-208.

[61] Jie-Oh Lee, Haijuan Yang, Maria-Magdalena Georgescu, Antonio Di Cristofano, Tomohiko Maehama, Yigong Shi, Jack, E. Dixon, Pier Pandolfi, and Nikola, P. Pavletich. (1999). "Crystal Structure of the PTEN Tumor Suppressor: Implications for its Phosphoinositide Phosphatase Activity and Membrane Association Cell". Cell. **99**(3): 323–334.

[62] Chen, Z., Trotman, L. C., Shaffer, D., Lin, H.K., Dotan, Z. D., Niki, M., Koutcher, J.A., Scher, H.I., Ludwig, T., Gerald, W., Cordon Cardo, C., Pandolfi, P.P. (2005). "Crucial Role of p53-Dependent Cellular Senescence in Suppression of PTEN Deficient Tumorogenesis". Nature. **436** (7051): 725-730.

[63] Julie, A. Moreno, Mark Halliday, Colin Molloy, Helios Radford, Nicholas Verity et al. (2013). "Oral Treatment Targeting the Unfolded Protein Response Prevents Neurodegeneration and Clinical Disease in Prion-Infected Mice". Sci Transl Med. **5**(206): 206ra138.

[64] Scot, R. Kimball. (1999). "Molecules in Focus-Eukaryotic Initiation Factor EIF2". The International Journal of Biochemistry & Cell Biology. **31** (1): 25-29.

[65] John, W. B. Hershey. (1989). "Protein Phosphorylation Controls Translation Rates". The Journal of Biological Chemistry. **264**(35): 20823-20826.

[66] Alan, G. Hinnebusch. (2005). "Translational Regulation of GCN4 and the General Amino Acid Control of Yeast". Annu. Rev.Microbiol. **59**(10): 407-450.

[67] Sebasti´an Bernales, Feroz, R. Papa, Peter Walter. (2006). "Intracellular Signaling by the Unfolded Protein Response". Annu. Rev. Cell Dev. Biol. **22**:487–508.

[68] Ruggero, D., Montanaro, L., Ma, L., Xu, W., Londei, P., Cordon Cardo, C., Pandolfi, P.P. (2004). "The Translation Factor EIF-4E Promotes Tumor Formation and Cooperates with C-Myc in Lymphomagenesis". Nat Med. **10**(5):484-486.

[69] Pause, A., Belsham, G.J., Gingras, A.C., Donze, O., Lin, T.A., Lawrence, J.C. Jr., Sonenberg, N. (1994). "Insulin-Dependent Stimulation of Protein Synthesis by Phosphorylation of a Regulator of 5'-Cap Function". Nature. **371** (6500): 762–767.

[70] Datta, A., Dougherty, E. R. (2007). "Introduction to Genomic Signal Processing with Control". Boca Raton, Florida. CRC Press.

[71] Layek, R., Datta, A., Dougherty, E.R. (2011) "From Biological Pathways to Regulatory Networks". Mol. BioSyst. **7**(3):843-851.

[72] Sridharan, S., Layek, R., Datta, A., Venkatraj, J. (2011). "Modelling Oxidative Stress Response Pathways". BMC Genomics. **13**(6): S4.

[73] Viswanathan, G.A., Seto, J., Patil, S., Nudelman, G., Sealfon, S.C. (2008). "Getting Started in Biological Pathway Construction and Analysis". PLoS Comput. Biol. **4**(2): e16.

[74] Nguyen, L. K., Cavadas, M.A.S., Scholz, C.C., Fitzpatrick, S.F., Bruning, U., Cummins, E.P., Tambuwala, M.M., Manresa, M.C., Kholodenko, B.N., Taylor, C.T., Cheong, A. (2013) "A Dynamic Model of the Hypoxia-Inducible Factor 1 (HIF-1) Network." J Cell Sci. **126**(Pt.6): 1261454–1463.

[75] Terrance, D. Barrett, Heather, L. Palomino, Theresa, I. Brondstetter, Kimon, C. Kanelakis, Xiaodong Wu, Peter, V. Haug, Wen Yan, Andrew Young, Hong Hua, Juliet, C. Hart, Da-Thao Tran, Hariharan Venkatesan, Mark, D. Rosen, Hillary, M. Peltier, Kia Sepassi, Michele, C. Rizzolio, Scott, D. Bembenek, Tara Mirzadegan, Michael, H. Rabinowitz, Nigel, P.

Shankley. (2011) “Pharmacological Characterization of 1-(5-Chloro-6-(trifluoromethoxy)-1H Benzoimidazol-2-yl)-1H-Pyrazole-4-Carboxylic Acid (JNJ-42041935), a Potent and Selective Hypoxia-Inducible Factor Prolyl Hydroxylase Inhibitor”. Mol. Pharmacol. **79**(6): 910–920.

[76] Qutub, A.A., Popel, A.S. (2008). “Reactive Oxygen Species Regulate Hypoxia-Inducible Factor 1 Differentially in Cancer and Ischemia,” Mol. and Cellular Biol. **28**(16): 5106–5119.

[77] Kim, J.W., Tchernyshyov, I., Semenza, G.L., Dang, C.V. (2006). “HIF- 1-Mediated Expression of Pyruvate Dehydrogenase Kinase: A Metabolic Switch Required for Cellular Adaptation to Hypoxia”. Cell Metab. **3**(3): 177–185.