BORON TREATMENT INDUCES METABOLIC REPROGRAMMING IN HEPATOCELLULAR CARCINOMA THROUGH SIRT3 ACTIVATION

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ABSTRACT

BORON TREATMENT INDUCES METABOLIC REPROGRAMMING IN HEPATOCELLULAR CARCINOMA THROUGH SIRT3 ACTIVATION

Sirtuins are members of NAD^+-dependent deacetylases and ADP ribosyltransferases, activated under stress conditions such as calorie restriction and starvation. Boron, mostly found in the form of boric acid (BA) or sodium borate (NaB), is known to bind NAD^+. In this study, particularly the effect of NaB on hepatoma cell line, HEP3B was investigated by analyzing the proteins harvested from NaB-treated and serum-starved HEP3B cells. It was discovered that treatment of cells with NaB (15 μg/ml) led to a decrease in the overall proteome acetylation specifically in mitochondria, in the synthesis rate of oxidative phosphorylation (OXPHOS) machinery subunits, in the amount of cellular reactive oxygen species (ROS), and in the proliferation rate of HEP3B cells. On the other hand, the cellular ratio of NAD^+/NADH and deacetylase activity of mitochondrial sirtuin, SIRT3 showed an increase. The results of this study suggest a link between boron treatment and activation of SIRT3 by means of affecting the cellular metabolism. In the scope of this study, it is expected to pave the way for new findings uncovering the metabolic changes in HEP3B cells related to SIRT3 activity upon NaB treatment.

ÖZET

BOR UYGULAMASI, HEPATOSELÜLER KARSİNOMDA SIRT3 AKTİVASYONU YOLUYLA METABOLİK YENİDEN PROGRAMLAMAYA NEDEN OLUYOR

Sirtuinler, kalori kısıtlaması ve açlık gibi stres durumlarında etkinleşen NAD^+-bağımlı deasetilaz ve ADP riboziltransferaz enzimleri üyeleridir. Çoğunlukla boric asit (BA) veya sodyum borat (NaB) formunda bulunan bor elementinin, NAD^+'ye bağlandığı bilinmektedir. Bu çalışmada, özellikle NaB'nin karaciğer kanseri hücre hattı, HEP3B üzerindeki etkileri NaB uygulanan ve serum açısından yoksun bırakılan HEP3B hücrelerinden elde edilen proteinlerin analizi ile araştırılmıştır. Bu hücrelerde 15 μg/ml konsantrasyonunda NaB uygulamasının mitokondrideki genel proteom asetilasyonunda, oksidatif fosforilasyon mekanizmasında görevli alt birimlerin sentezlenme hızında, hücresel reaktif oksijen türlerinin seviyelerinde ve HEP3B hücrelerinin çoğalma hızında azalmaya yol açtığı bulunmuştur. Bunların yanısıra, hücresel NAD^+/NADH oranında ve mitokondriyel sirtuin, SIRT3 deasetilaz aktivitesinde artış gözlemlenmiştir. Bu çalışmanın sonuçları, bor elementi uygulaması ve SIRT3 aktivasyonu arasında hücresel metabolizmayı etkilemek suretiyle bir ilişki ortaya koymaktadır. Bu çalışmanın, HEP3B hücrelerinde NaB uygulaması sonucu SIRT3 aktivitesine bağlı olarak metabolik değişimlerin ortaya çıkarılmasında yeni bulgulara zemin hazırlaması hedeflenmektedir.

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LIST OF SYMBOLS / ABBREVIATIONS

ANT	Adenine nucleotide translocator
AceCS	Acetyl-CoA synthetase
Akt	Protein kinase B
AMPK	Adenosine monophosphate-activated protein kinase
BA	Boric acid
CDK	Cyclin-dependent kinase
CPS	Carbamoyl phosphate synthetase
CypD	Cyclophilin-D
C-myc	A regulator gene that codes for a transcription factor
ETC	Electron transport chain
FOXO	Forkhead box protein
GDH	Glutamate dehydrogenase
Glut	Glucose transporter gene
G6P	Glucose-6-phosphate
GPX	Glutathione peroxidase
HAT	Histone acetyltransferase
HCC	Hepatocellular carcinoma
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HK	Hexokinase
HIF	Hypoxia inducing factor
IGF	Insulin-like growth factor
IMS	Inter-membrane space
IDH	Isocitrate dehydrogenase
KO	Knock-out
KAT	Lysine acetyltransferase
LDH	Lactate dehydrogenase
MPT	Mitochondrial permeability transition

MAC	Mitochondrial apoptosis-induced channel
MS	Mass spectroscopy
mTOR	Mammalian target of rapamycin
mPTP	Mitochondrial permeability transition pore
NAD	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NaB	Sodium borate
NAM	Nicotinamide
NF-κB	Nuclear factor kappa B
NOR	Nuclear organizer region
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
OXPHOS	Oxidative phosphorylation
OMM	Outer mitochondrial membrane
PPP	Pentose phosphate pathway
PCNA	Proliferating cell nuclear antigen
PTM	Post-translational modification
PGC	Proliferator-activated receptor gamma co-activator
PDK	Pyruvate dehydrogenase kinase
PDH	Pyruvate dehydrogenase
ROS	Reactive oxygen species
Ras	A protein superfamily of small GTPases
SIRT	Sirtuin protein
Sir2	Silent information regulator gene
SOD	Superoxide dismutase
COXII	Cytochrome *c* oxidase subunit 2
TCA	Tricarboxylic acid
TLR	Toll-like receptor
TIGAR	TP53-induced glycolysis regulator
UCP	Uncoupling protein

1. INTRODUCTION

1. 1. Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is a type of liver cancer, which is primarily caused by viral hepatitis B or C infections and alcohol-induced cirrhosis. Aflatoxin is the second most common cause of HCC, with hepatitis B virus (HBV) and hepatitis C virus (HCV) accounting for 70% of HCC cases worldwide [1-3]. Apart from these, heavy alcohol drinking, obesity, and diabetes increase the risk of HCC [4, 5]. This increase is considered to be related to metabolic alterations caused by enhanced hepatic activity [6]. Inherited disorders associated with deficiencies in iron absorption have also been shown to be linked with HCC [7].

HCC cases are especially high in Eastern and South-Eastern Asia and Middle and Western Africa [3]. Following an increase in the early 1990s, HCC related deaths decreased in areas with traditionally high mortality rates of southern Europe countries while an increase was observed in low mortality areas of Central and Northern Europe since 1994 [8]. According to microarray studies, molecular mechanisms leading to HCC are significantly different between HBV- and HCV-infected livers [9-12]. HBV uses a double-stranded DNA genome to integrate into the host genome. On the other hand, replication of an RNA virus in the cell cytoplasm is responsible for HCV infection [13]. Pathways related to immunity, cell cycle and RNA metabolism are especially enriched in HBV-induced HCC. Common alterations in several pathways also exist in HBV- and HCV-induced HCC such as those associated with protein metabolism, translation, glycolysis, OXPHOS, amino acid metabolism, and nucleotide metabolism [14].

A multistep progression is mostly observed in HCC, starting with hepatitis infection or cirrhosis, followed by the occurrence of precancerous lesions which eventually leads to the early HCC to the progressed HCC [2]. Early HCCs and progressed HCCs can be differentiated based on histological examinations. Small, nodular, and well-differentiated formations are observed in early HCCs, while poorly differentiated and distinct gross tumour formations are observed in progressed HCC [15, 16].

Liver transplantation, surgical resection, and radiofrequency ablation at an early tumor stage can provide 5-year survival rate in 70% of HCC patients [17]. However, given the best treatment outcomes considering survival, hepatic resection and liver transplantation mostly result with recurrence in 30% to 50% of cases [9, 18, 19].

1. 2. Cancer Cells and The Warburg Effect

Cancer cells mostly maintain higher rates of glycolysis by down-regulating their oxidative phosphorylation (OXPHOS) machinery regardless of environmental oxygen concentrations. This situation is known as the Warburg effect. The decreased OXPHOS in cells displaying the Warburg effect is mostly associated with malfunctioning mitochondria [20], which is in turn related to the cellular energy metabolism, maintained by the NAD^+ / NADH ratio (Figure 1.1.) [21]. The increased glucose uptake related to Warburg effect not only serves as a source for ATP generation through tricarboxylic acid (TCA) cycle, but also as source for carbon and metabolites harnessed in the synthesis of macromolecules supporting cellular proliferation [22]. This is predominantly seen in the fast growing tumor cells [23] providing growth advantage over healthy cells as they become less exposed to the oxidative stress by avoiding high levels of ROS [24, 25].

1. 3. Reactive Oxygen Species and Mitochondria

ROS are a group of oxygen containing compounds resulting from the incomplete reduction of molecular oxygen in oxidation-reduction reactions, forming superoxide (O_2^-) [26]. The primary source of ROS generation is the cellular respiration in which Complex I and Complex III are the mostly involved respiratory complexes in this mechanism [27]. Low ratio of NAD^+ / NADH also increases production of ROS. On the other hand, mitochondrial ROS is required for the proper functioning of biological processes such as signaling, adipocyte differentiation, insulin release, and hypoxic signaling [27-30]. However, the effects of ROS on cellular mechanisms depend on their concentrations produced. Enhanced ROS generation and accumulation are lethal for cells. This results in oxidative damage to the cellular components, nicks in DNA, malfunction in the DNA repair mechanism, elevated cell membrane permeability, induction of cell death, and lipid peroxidation [31-33]. Moreover, proteins are the most affected groups of compounds from

ROS accumulation. Proteins, which are labile to functional impairments, get altered due to the increased production of ROS, which leads to detrimental changes in their three-dimentional structure upon folding.

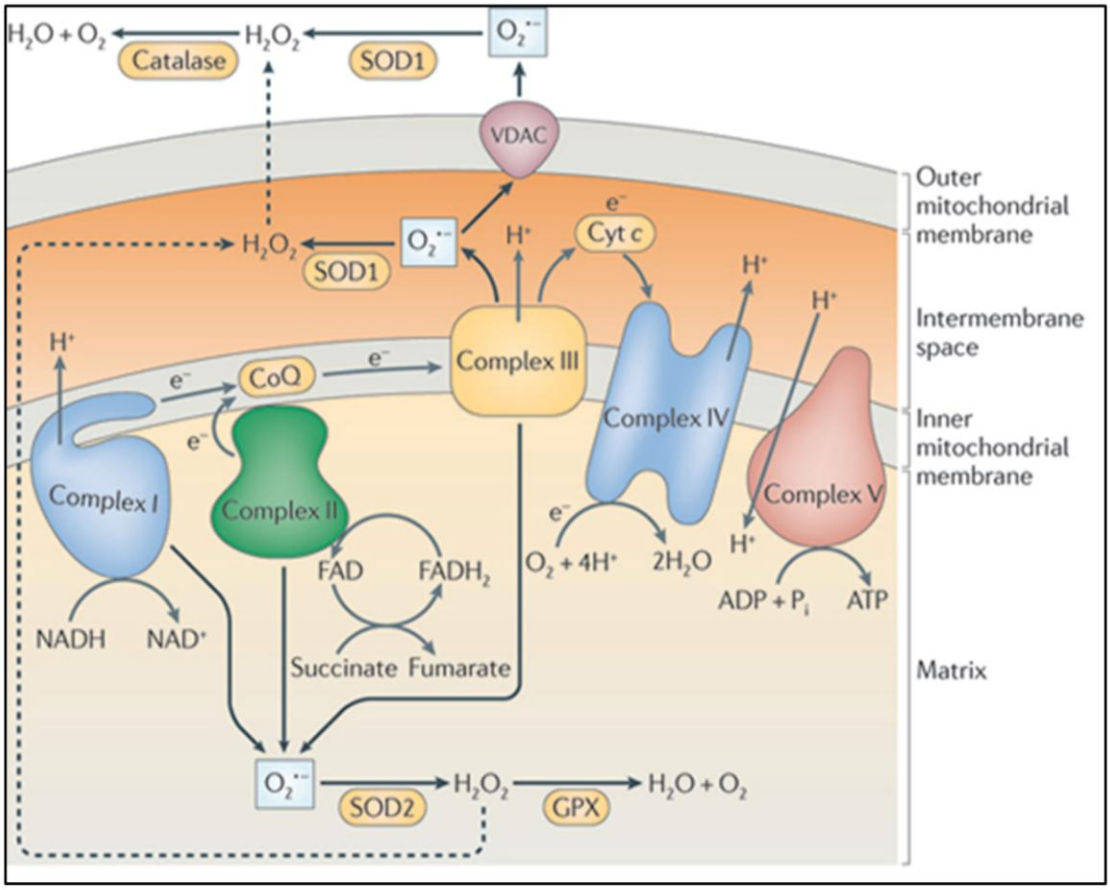

Figure 1.1. Reactive oxygen species and mitochondria. Reactions taking place through oxidative phosphorylation complexes (CI: NADH-ubiquinone oxidoreductase, CII: Succinate dehydrogenase, CIII: Cytochrome *b-c1* complex, CIV: Cytochrome *c* oxidase, CV: ATP synthase, SOD: superoxide dismutase, GPX: glutathione peroxidase) [21].

Altered protein structures, in turn, can have severe impact on human health. Many cancer states are related to ROS accumulation. Enhanced generation of ROS significantly promotes tumor development and progression by altering protein functions that result in cellular proliferation, evasion of apoptosis, tissue invasion, and metastasis [25]. Superoxide (O_2^-) can directly react with the iron-sulfur clusters of several enzymes

involved in TCA cycle, leading to their deactivation [34, 35]. The proper functioning of antioxidant enzymes is essential for eliminating the harmful effects caused by the elevated levels of ROS. When ROS generation exceeds the capacity of antioxidant defense, mitochondria are the primary sites to be affected. Mitochondrial function is hampered, further leading to cancerous diseases [36]. It is important to point out that high levels of ROS are less deleterious in cancer cells than normal cells. Tumorigenic cells can induce new redox balances within their metabolisms. This permits cancer cells to increase their metabolic rate and proliferation while escaping free radical damage [25]. Given OXPHOS being the major ROS generating pathway, mitochondria in cancer cells are of great importance. Having a repair capacity much lower than that of nuclear DNA and lack of histones, mitochondrial DNA is much more prone to free radical damage [37-39]. Any damage in key cellular components might lead to severe consequences such as apoptosis [25]. The maintenance of mitochondrial potential and oxidative equilibrium controls apoptotic mechanisms and cell viability. Loss of structural integrity, disruption of OXPHOS machinery leads to the impairment of membrane potential, which is crucial for a proper mitochondrial function [40].

1. 4. Mitochondria and Cellular Energy Metabolisms

Mitochondria pose a great impact on tumor cell survival by mediating apoptotic pathways [36]. For instance, decreased expression of p53 protects tumor cells from apoptosis through alterations in mitochondrial respiration [41]. An important apoptotic trigger is the induction of outer mitochondrial membrane (OMM) permeabilization. This can happen as a result of different mechanisms such as voltage-dependent anion channel (VDAC) function or the formation of mitochondrial permeability transition (MPT) pore or mitochondrial apoptosis-induced channels (MACs) [42-44]. Glycolytic enzymes and hexokinases (HK), are upregulated and implicated in preventing the OMM permeabilization in tumor cells [36, 45]. HKI and HKII are especially important as they can directly bind to mitochondria and occupy the binding sites on OMM, that are specific for pro-apoptotic proteins, hence providing an apoptotic resistance [46, 47]. Stress-induced senescence is associated with an enhanced ROS generation, thus with oxidative damage [48, 49]. The activity of ROS scavengers are coupled to the balance between NADP and NADPH, which are generated in Pentose Phosphate Pathway (PPP). PPP branches from

glycolysis, and in order to synthesize macromolecules, cancer cells follow the PPP upon enhanced glycolysis. This metabolic shift is vital in terms of their apoptotic resistance [50-52]. Accordingly, by targeting metabolic mechanisms, it becomes possible to overcome this apoptotic inhibition observed in most cancer cells. When OXPHOS mechanism is restored, metabolic shifting can be achieved. This way, the balance between NADP and NADPH will be reconstituted, further recovering the activities of ROS scavengers. The proper functioning of OXPHOS will most importantly restore the ratio between NAD^+ and NADH, indicating the elimination of harmful ROS generation. In light of the importance in reducing ROS levels to improve cellular antioxidant capacity, developing a treatment strategy involving compounds with healing properties seems plausible.

1. 5. Boron

Boron is a highly beneficial trace element found in human body as well as in other animals and plants. It is widely distributed in nature and released by natural weathering processes; however, it is found only in compounds and in combinations with sodium and oxygen [53]. Following the discovery of boron deposits; sodium borate (NaB), also known as borax, initially became useful as an antiseptic and as an essential constituent of household products [54]. As a naturally occurring compound, NaB is refined through recrystallization [54]. With its refined forms such as borax decahydrate, borax pentahydrate, and anhydrous borax, Turkey is the major producer of boron products in the world [55]. The importance and essentiality of this element was perceived initially through plant studies back in 1920s [56]. As its role in maintaining the structural integrity in plants, beneficial roles of boron are also well established in animal studies. Playing a major role in bone health, boron is also involved in the modulation of synthesis and the release of steroid hormones [57], embryogenesis and most importantly immune defense. Boron is an active inducer of the immune system in humans and animals. Though not an antioxidant itself, it is able to strengthen tissue antioxidant defenses in animal systems, which are highly impaired in cancer [58, 59]. The immune induction mechanism of boron is not fully uncovered yet; however, recent studies on cancer cells have shown that boron treatment in fact helps to improve the biological and physiological state of cancer cells and increase the activities of antioxidant enzymes, such as glutathione, in cancerous diseases [60-62]. Boron is also shown to play a role in decreasing the serum biological markers related to diseases [63].

Boron treatment on rat models with fulminant hepatic failure (FHF) significantly decreases ROS production and the activity of FHF serum marker enzymes, which are severely altered in hepatic injury [60]. Another study on rat models with hepatocellular carcinoma (HCC) confirms the beneficial effects of boron treatment in liver pathology [63].

Boron treatment, in this study, was shown to decrease hepatic nodules that form focal lesions and inhibit tumor growth in rat hepatocytes. By down-regulating proliferating cell nuclear antigen (PCNA) which is only expressed in proliferating cells for mechanisms such as DNA synthesis, repair, cell cycle regulation, apoptosis, boron inhibited the tumor cell proliferation [64].

Boron binds to NAD^+ and $NADP^+$ through reactions involving the N group or hydroxyl groups on these pyridine nucleotides [59]. The binding between NaB and NAD^+ might significantly alter the intracellular NAD^+ / NADH ratio, which in turn, would affect the cellular ROS levels and SIRT3 activity in a stress-dependent manner.

1. 6. Acetylation of Histones as a Post Translational Modification

Genomic DNA is packaged into chromatin by both histone and non-histone proteins, which can either be acetylated or deacetylated. Acetylated histones are unbound to DNA and allow for the transcription processes while deacetylated histones bind tightly to DNA, restricting these processes [65]. Acetylation is the covalent addition of an acetyl group to a ε-lysine residue (Figure 1.2). This reaction alters the three-dimentional structure of a given protein by neutralizing its positive charge [66, 67]. Regions with ordered secondary structure are mostly the ideal sites for acetylation [68]. α-helical regions and depleted coils are abundant in calorie restriction-related acetyl sites [69]. Deacetylation of histones specifically at lysine residues results in the compaction of chromatin and repression of gene transcription. Peculiar lysine acetylation or the deregulation of this process is being related to cancer [70, 71]. It is especially implicated in the modulation of protein subcellular localization, protein stability, enzymatic activity, protein-protein interactions and gene expression [72, 73]. Proteomics studies demonstrate that metabolic reprogramming, which confers an advantage to cancer cells over normal cells by providing

more building blocks for their increased proliferation, is mediated mostly through PTMs [22, 74].

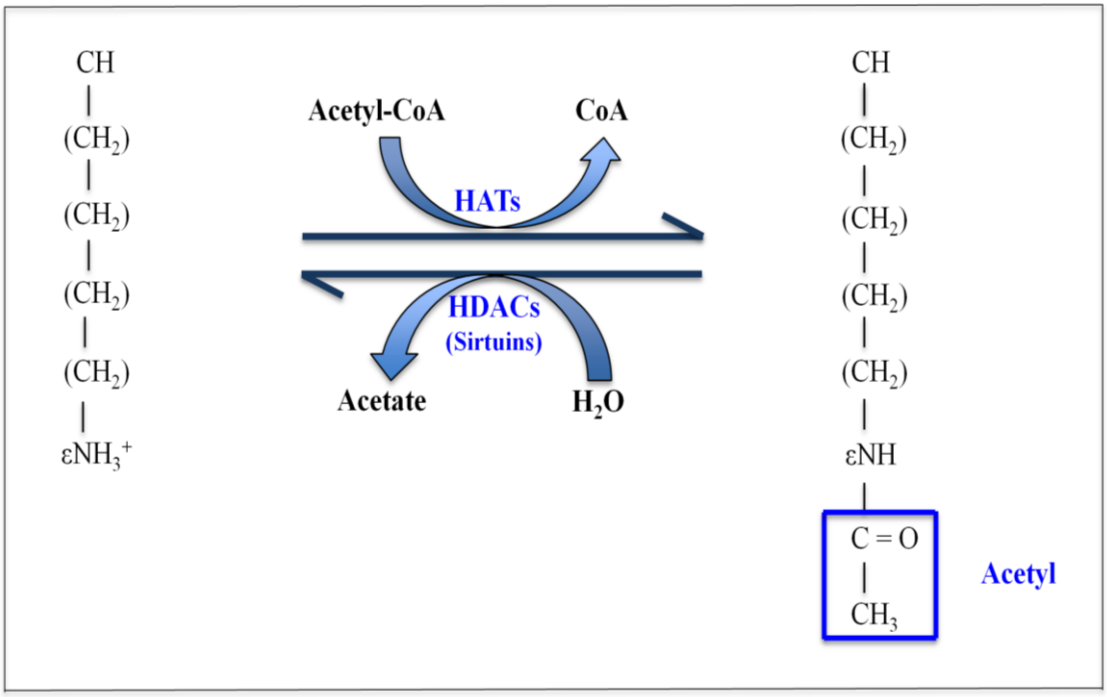

Figure 1.2. Acetylation of lysine as a post-translational modification. Mechanisms of acetylation and deacetylation reactions are depicted in diagram.

1. 7. Mitochondrial Lysine Acetylation

Mitochondria are central in cellular structure for active regulation of cell signaling pathways, which is crucial for cellular homeostasis. Mitochondrial function is tightly controlled by Post-Translational Modifications (PTMs) [72, 75, 76]. Acetylation is a common PTM taking place in mitochondria, especially on proteins involved in metabolism [68, 77-79]. It occurs on the 'tail' domains of histones extending from the surface of chromatin polymer, allowing interactions with other proteins [80]. These interactions lead to the neutralization of the positive charges of proteins [81]. Caloric restriction and fasting regulate the acetylation levels of mitochondrial proteins, which are generally acetylated at multiple sites [68, 77]. A proteomics study conducted on fed and fasted mice liver

mitochondria revealed 277 acetylation sites on 133 proteins, some of them being unique to fed mice, from central metabolic pathways such as OXPHOS, TCA cycle, urea cycle and β-oxidation [77]. Mass Spectrometry (MS) based studies relate the acetylation of metabolic proteins such as enzymes of TCA cycle, fatty acid oxidation and OXPHOS pathway to metabolic stress [77, 78, 82]. The differences in amino acids favored in specific positions for protein acetylation suggests the presence of a unique mechanism taking place in mitochondria.

The most common and crucial type occurring within mitochondria is lysine-acetylation [68, 77]. The amount of lysine-acetylated proteins in mitochondria is more than that of phosphorylated proteins by three folds [83]. The majority of these proteins are acetylated at multiple sites. MS studies based on a comprehensive atlas recently revealed 65% of mitochondrial proteins to have at least one acetylation site that is identifiable [69]. Furthermore, higher number of unique acetylation sites were observed in SIRT3 KO mice fasted for twenty-four hours before tissue harvesting to maximize SIRT3 expression [73]. Among the mitochondrial proteins acetylated, most of them were found to be multiacetylated, with 61% baring at least two acetylated lysine sites. While 65% of the proteins involved in citric acid cycle were acetylated, specific mitochondrial pathways involved in acetyl-CoA production and utilization were also mostly acetylated [73]. Regulating the activities of major metabolic enzymes; lysine-acetylation, which is conserved among species, involves the competing activities of protein lysine acetyltransferases (KATs) and deacetylases [22, 78, 79].

Proteomic studies show that among 133 lysine-acetylated mitochondrial proteins, 91 of them were found to be involved in the metabolic processes and to be reversibly acetylated in response to calorie restriction [77]. Other acetylated proteins play important roles in chromatin remodelling, cell cycle, nuclear transport, actin nucleation and splicing [68]. Highly metabolic tissues such as liver bare the most of the acetylated mitochondrial proteins [83]. Proteins of mitochondrial oxidative metabolism are hyperacetylated in liver in response to high-fat diet, which in turn, decreases the activities of their respiratory chain complexes [84]. Liver from mice undergone a calorie-restricted diet revealed that most of the lysine-acetylated proteins were involved in metabolic or cellular processes and that 87% of these proteins were conserved in humans [73].

1. 8. Sirtuins and Lifespan Regulation

There are mainly three mechanisms in mammalian systems that mediate lifespan, by regulating the cell stress responses. These major pathways are the mammalian target of Rapamycin (mTOR), insulin/ insulin-like growth factor 1 (IGF-1) and sirtuins [85].

Mammals have seven homologues of the silent information regulator gene, Sir2, which promotes longevity and exerts beneficial effects on disease conditions including cancer, neurological disorders and insulin sensitivity through activation upon calorie restriction and fasting [65, 85-87]. The redox-sensitive family of sirtuins regulates the expression of genes involved in stress response, energy metabolism and longevity by controlling the acetylation state of lysine residues on histones, transcription factors and transcriptional co-activators [88]. They catalyze the removal of acetyl groups from the side chain amino groups of lysine residues, in a reaction consuming NAD^+ and generating nicotinamide (NAM) and 2'-*O*-acetyl-ADP-ribose [89] (Figure 1.3.)[90]. Sir2 catalytic core domain is conserved among seven mammalian sirtuins. The changeable amino- and carboxyl-terminal extensions, which mediate their catalytic activity, also designate their subcellular localization [91]. The conserved sirtuin core domain comprises amino acid residues essential for NAD^+ binding [40].

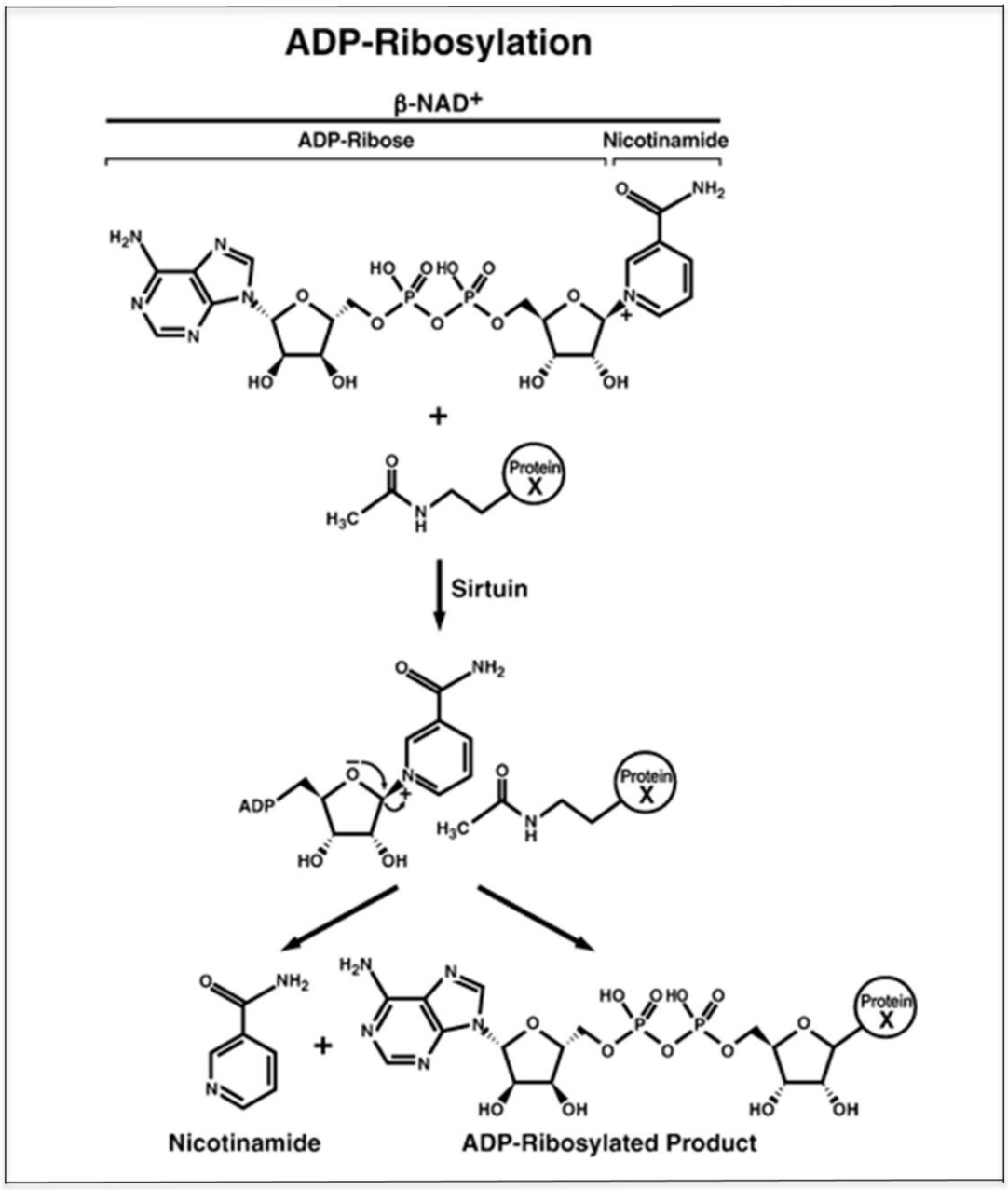

Figure 1.3. Mechanism of ADP-ribosylation reaction. Sirtuins ADP-ribosylate the target protein by using NAD^+ as a cofactor and release nicotinamide [90].

Initially discovered in yeast genome as silent information regulator, Sir2; silencing telomeres and rDNA, regulating aging processes and genomic integrity [92], Sir2 reduces the negative outcomes of ROS production by decreasing the hydrogen peroxide (H_2O_2) toxicity and by mediating the asymmetric segregation of oxidatively damaged proteins in the course of cell division in yeast [35]. Sirtuins are central in the direction and regulation

of diverse metabolic pathways such as energy homeostasis, energy sensing in mammals and tumor biological processes [93]. As cellular energy sensors, they are activated in response to stress conditions such as calorie-restriction and fasting. Through deacetylation mechanisms, sirtuins are shown to prevent carcinogenesis in some mammalian models such as mouse embryonic fibroblasts (MEFs), mouse embryonic stem cells (MES) and HepG2 cells [88, 94-96]. Among the seven sirtuin homologues; SIRT1, SIRT2 and SIRT3 show the closest homology to yeast deacetylase, SIR2 [97]. SIRT1, SIRT6 and SIRT7 are localized to the nuclear compartment while SIRT2 functions in the cytoplasmic compartment and SIRT3, SIRT4, SIRT5 are localized to mitochondria [93, 97, 98].

1. 8. 1. Nuclear Sirtuins

SIRT1 exerts deacetylase activity on key histone residues involved in the mediation of transcription as well as on non-histone protein targets such as tumor antigen p53, Forkhead box proteins 1 and 3 (FOXO1, FOXO3), Peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1α) and Nuclear factor kappa B (NF-κB) [85]. Other vital roles of SIRT1 are DNA repair, muscle/fat differentiation, neurogenesis, mitochondrial biogenesis, glucose and insulin homeostasis, hormone secretion, all of which contribute to the longevity effects of calorie restriction [65, 85]. SIRT1 was shown to be effective in increasing lifespan in response to caloric restriction [35].

SIRT6 exhibits both NAD+-dependent deacetylation and mono-ADP ribosylation activity. Its expression was implicated with conditions related to aging. When overexpressed, SIRT6 promoted lifespan extension in male mice [85, 99]. Metabolic defects were observed in mice lacking SIRT6 and these mice developed age-related abnormalities when they reached to 2-3 weeks of age [100]. Being tightly bound to chromatin, SIRT6 is crucial in maintaining metabolic homeostasis through the regulation of DNA repair mechanism, glucose and lipid metabolism, resistance to oxidative stress, suppression of genomic instability and maintenance of telomeres, which relate its activity with conditions such as cancer and diabetes [101, 102]. SIRT6 in coordination with SIRT1, was known to promote a switch from glycolysis to fatty acid oxidation during acute inflammatory response. Toll-like Receptor 4 (TLR4) stimulation in human derived acute monocytic leukemia cells,

THP-1, showed that SIRT6 acts to down-regulated glycolysis by enabling a switch, while SIRT1 supported the activation of fatty acid oxidation pathway [103].

The other sirtuin homolog found in the nuclear compartment of mitochondria is SIRT7. SIRT7 is the only sirtuin localized in the nucleoli and it is known to participate in the regulation of ribosomal gene (rDNA) and rRNA transcription processes [100, 104]. Nuclear compartment of mitochondria is crucial in being the center of ribosomal biogenesis involving processes such as the rDNA transcription and the assembly of rRNAs with ribosomal proteins [105]. These ribosomal processes are of great importance for mammalian cells, since they are directly affected by the expression of rRNA synthesis, which is altered by aging, tumor formation, cellular proliferation and also calorie restriction [106-108]. SIRT7 was found to be expressed in rDNA transcription sites during cell cycle and by means of its interaction with a rDNA transcription factor, Upstream Binding Factor (UBF), it becomes associated with Nuclear Organizer Regions (NORs) during mitosis [100]. This way, SIRT7 activated the rDNA transcription [100, 104]. SIRT7 is mostly expressed in metabolically active tissues like the liver and it directly interacts with RNA Polymerase I (RNA Pol I) [104]. During mitosis, rDNA transcription could be repressed by the cyclin-dependent kinase (CDK) 1-cyclin B pathway due to the phosphorylation of RNA Pol I machinery components. This repression could be reversed by the dephosphorylation [109, 110]. SIRT7 was found to be phosphorylated in HeLa cells during mitosis via the CDK 1-cyclin B pathway and its activation was found to be essential to stimulate rDNA transcription when exiting mitosis [100].

1. 8. 2. Cytoplasmic Sirtuins

SIRT2 is another sirtuin homologue that plays role in neurogenesis. Apart from deacetylating histones, α-tubulin and NF-κB, it is essential for myelination and regulation of microglia-induced neurotoxicity and microglial survival [111].

1. 8. 3. Mitochondrial Sirtuins

Mitochondrial sirtuins are small and range from 30-40kDa. They are of major importance in linking PTMs and mitochondrial homeostasis and are crucial in the regulation of cell

signaling, apoptosis, metabolism, ATP production, core proteins of Electron Transport Chain (ETC) and of key enzymes of the acetate cycle [40, 112-114]. Sirtuins are considered to be the central protein deacetylases in mitochondria, owing to the fact that NAD^+-independent protein deacetylase activity is absent in mitochondria according to studies carried out with histone peptide as a substrate [115].

The ADP ribosyltransferase SIRT4 is known to down-regulate the insulin secretion by inhibiting the Glutamate Dehydrogenase (GDH) activity [116]. The absence of SIRT4 activity leads to increased insulin secretion [116]. Also, SIRT4 provides substrate for ATP synthase by binding the adenine nucleotide trans-locator (ANT) [117].

Sirt5 gene comprises multiple repetitive elements. The fact that this gene is located in a chromosomal region related to malignant diseases suggests a role for SIRT5 in the the formation of chromosomal breaks [118]. SIRT5 deacetylates the mitochondrial inter-membrane space (IMS) protein, cytochrome c, which is a crucial player in oxidative metabolism [119]. This deacetylation seems possible in the light of experiments showing import of SIRT5 both into matrix and IMS of mitochondria [119]. SIRT5 activates carbamoyl phosphate synthetase (CPS1), an essential enzyme catalyzing the rate-limiting first step of urea cycle for the removal of ammonia [112] and prevents hyperammonanemia by deacetylating the urea cycle enzymes upon fasting [120].

Having the most robust mitochondrial deacetylase activity, SIRT3 is the primary regulator of lysine-acetylation in mitochondria [121]. SIRT3 is initially translated as an inactive precursor in the cytoplasm. It is then imported to the mitochondria, where it is proteolytically cleaved, leading to its activation [72]. Full protein deacetylase activity of the SIRT3 requires the cleavage of SIRT3 peptide from the precursor protein at the N-terminus upon import into mitochondria [115]. Arg99 and Arg100, which are conserved among species, are the two residues necessary for this cleavage [115, 122]. By acting as a tumor suppressor, SIRT3 regulates longevity in humans and its absence was implicated in tumor progression in cancer cells in a ROS-dependent manner [25]. Protein hyperacetylation in mitochondria was observed in the absence of SIRT3 activity and this contributed to the onset of age-related insulin resistance [123]. SIRT3 is pivotal in cell survival as it controls the formation and opening of mitochondrial permeability transition

pore (mPTP) through deacetylation of a key pore component, Cyclophilin-D (CypD) [124]. CypD deacetylation catalyzed by SIRT3 resulted in the detachment of Hexokinase II (HKII) from mitochondria. As a mediator of mPTP, HKII directs cell metabolism towards increased rates of glycolysis with a concurrent decrease in OXPHOS rates when bound to mitochondria [124]. By increasing LDH concentration in culture medium and leading to cellular membrane damage in HCC cells, Sirt3 overexpression triggered apoptosis in HepG2 cells [125].

Mitochondrial proton gradient, which occurs as a result of mitochondrial respiratory chain activity, is also crucial in the regulation of cancer cell metabolism. The inactivation of ATP synthesis dependent on mitochondrial proton gradient, referred to as the mitochondrial uncoupling, is controlled by the uncoupling proteins (UCPs) [126, 127]. Mitochondrial uncoupling provides metabolic shifting, allowing the utilization of non-glucose carbon sources in order to maintain the mitochondrial activity [128, 129]. This shortage of glucose required for OXPHOS contributes to metabolic shift towards the enhanced glycolysis by cancer cells [130-132]. SIRT3 was known to be required for the expression of uncoupling protein UCP1.

Shift away from liver glycolysis through SIRT3 activation under calorie restriction did not only increase respiration for ATP synthesis, but also for the conversion of amino acids into glucose and urea [133].

SIRT3 plays a role in reducing age-associated oxidative damage to macromolecules and the mitochondrial glutathione antioxidant defense system is regulated by SIRT3 [134]. Two direct mechanisms, namely Isocitrate Dehydrogenase 2 (IDH2) and Superoxide Dismutase 2 (SOD2), relate SIRT3 to decreased ROS production. IDH activity generates NADPH from $NADP^+$ by catalyzing the conversion of isocitrate to α-ketoglutarate in citric acid cycle taking place in mitochondria [124]. IDH2 deacetylation by SIRT3 increases its activity, enhancing the glutathione antioxidant defense system [124, 135]. Calorie restriction showed to be effective in decreasing IDH2 acetylation in the liver of wild-type mice while this effect was absent in SIRT3 KO mice [124]. SOD2 mutations are known to lead to human cancers [136]. Independent studies linked the prevention of ROS-mediated oxidative damage to SOD2 deacetylation by SIRT3 [137-139].

Two important enzymes having regulatory functions in mitochondrial metabolism, acetyl-CoA synthetase 2 (AceCS2) and GDH, are direct substrates of SIRT3. AceCS2 is responsible for converting acetate into acetyl-CoA to be used in the citric acid cycle [113]. It is activated through deacetylation and localizes to mitochondria. Under conditions of low nutrient availability and calorie restriction, AceCS2 enables the free acetate to be used instead of pyruvate for the generation of acetyl-CoA [119]. SIRT3 activates the acetyl-coenzyme-A synthetase by deacetylating it and facilitates the entry of acetate into TCA cycle [140]. The activation of both AceCS2 and GDH under these circumstances provides a metabolic shift from liver glycolysis. Diverse mitochondrial functions; such as metabolism, ATP generation, response to oxidative stress have been shown to be regulated by SIRT3, which interacts with OXPHOS complexes CI and CII, increasing their activities and maintaining mitochondrial energy homeostasis [114, 141]. Decreased ATP levels are observed in liver of SIRT3 KO mice as a result of these interactions [114]. CI and CII are found to be hyperacetylated in mice lacking SIRT3. SIRT3 absence was shown to induce Warburg effect, by increasing the rate of glycolysis [73]. Loss of SIRT3 activity hinders OXPHOS activity and mitochondrial oxygen consumption while its overexpression can reduce the cellular ROS levels by 40% [121]. This implies that SIRT3 is important in metabolic reprogramming under nutritional stress [124].

Nutrient starvation induces an elevation in NAD^+ levels leading to the increased activity of SIRT3. Loss of SIRT3 activity has been linked to metabolic reprogramming in a way to support tumorigenesis and cell proliferation [93]. Cells with increased glycolysis have increased levels of NADH and decreased levels of NAD^+, resulting in the inactivation of sirtuins [93]. SIRT3 KO mice undergone calorie restriction lacked the protection against oxidative damage and showed poor antioxidant capacity [135]. Moreover, these mice showed high genomic instability, increased sensitivity to the oncogenic transcription and high ROS levels. These findings may help to explain why human tumors exhibit reduced levels of SIRT3 [94].

1. 9. Metabolic Reprogramming

Cells can alternate between energy-generating pathways depending on their cellular needs. The possibility for a given cell to achieve this alteration between glycolysis and OXPHOS is described by a process known as 'metabolic flexibility'. This metabolic shift is observed in certain cancers such as hepatocytes [142]. Tumor cells bare the capacity to reprogram their metabolism to rapidly adapt to genetic or environmental changes [25, 142, 143]. Various reasons can account for such a process to take place in cancer cells such as the demand of cellular proliferation, high or low glucose availability, hypoxia, mitochondrial dysfunction or reduced mitochondrial content as seen in HeLa cells and breast cancer cells [143, 144].

Hypoxia can occur due to limited tumor vascularization. In this case, tumor cells can benefit from the increased glycolysis [145]. Comparing the effects of hypoxia and SIRT3 activity, Finley et al. demonstrated that the absence of SIRT3 led to similar metabolic reprogramming observed under hypoxic conditions [146]. Moreover, the expression levels of HIF1α target genes, Glut1 and HKII, were both increased due to SIRT3 KO and to hypoxia [147]. Enhanced glycolysis due to metabolic shifting results in lactate accumulation, which provides a survival advantage to tumor cells by means of creating an acidic environment [148]. The catalytic subunit of the mitochondrial ATP synthase (b-F1-ATPase) is downregulated in tumor cells undergone to a metabolic shift, demonstrating a reduced mitochondrial activity and ATP production, which may lead to enhanced glycolysis [36, 149]. These high rates of glycolysis in tumor cells can be confirmed by means of 18F-deoxyglucose positron emission tomography (FDG-PET) [145, 150].
Increased rates of glycolysis, lactate production and macromolecular biosynthesis are the characteristics of proliferating cells. Instead of getting completely oxidized, the majority of glucose-derived carbon is converted to lactate in cells displaying the Warburg effect [151]. Their nutrient uptake in order to perform biosynthetic processes exceeds cellular bioenergetic needs [152]. Normally, extracellular stimulation is required for signal induction. However, cancer cells are generally independent of these physiological stimuli owing to the mutations they incorporate that induce instructional signaling pathways downstream of growth factor receptors [153, 154]. This way, they can increase their nutrient uptake from the environment and direct their excess metabolites to biosynthetic

pathways. The fact that the levels of glycolysis intermediates, glucose-6-phosphate (G6P) and ribose-5-phosphate were increased in SIRT3 KO cells while a decrease was observed in those of TCA cycle supported the idea that the excess glucose up-taken from the environment was directed to the biosynthetic pathway, pentose phosphate pathway (PPP), through metabolic shifting [146].

Induction of glycolysis and high lactate production is significantly controlled by the activation of the heterodimer HIF1, which is composed of HIF1α and HIF1β subunits [155-157]. HIF1 activity favors glycolysis over OXPHOS by enhancing pyruvate dehydrogenase kinase 1 (PDK1) activity, which restricts the entry of glycolytic carbon into TCA cycle through the phosphorylation of pyruvate dehydrogenase (PDH) complex [154, 158, 159]. The activity of HIF1 is dependent on SIRT3 activity and ROS production, which are known to stabilize HIF1α [146, 160, 161]. These regulations lead to metabolic shifts towards glycolysis.

HIF1 is a crucial mediator of glycolytic enzymes; however, induction of other genetic factors such as signaling kinases, oncogenes or tumor suppressor genes are also of great importance in the upregulation of glycolysis, leading to the Warburg effect [162-167]. Activation of pathways involving these genes induce stress resistance, angiogenesis and increased glycolysis in cancer cells resulting in the reprogramming of their metabolism [147, 168-170]. Oncogenes including ras, c-myc, akt are shown to be involved in the induction of glycolytic enzymes while the tumor suppressor gene, p53, mediates mitochondrial respiration [165, 171-174]. The expression of transcriptional factor myc is dependent on growth factor stimulation. Upon activation, myc controls cell cycle entry and cellular proliferation [167]. Oncogenic induction of c-myc significantly affects glycolysis [167]. The serine-threonine kinase Akt is known to mediate glycolysis upon activation through phosphorylation [36]. Induced expression of Akt promotes enhanced glycolysis and prevents apoptosis [175, 176].

AMP-activated protein kinase (AMPK) is an evolutionary conserved protein kinase complex mediating cellular energy homeostasis in response to metabolic stress [177, 178]. Metabolic stimulations altering cellular ATP levels such as nutrient limitation or hypoxia activate AMPK [167, 179]. AMPK activation further leads to the phosphorylation and the activation of p53, which is a crucial mediator of the metabolic homeostasis between

OXPHOS and glycolysis [174, 180, 181]. By regulating a suppressor of glycolysis, TP53-induced glycolysis regulator (TIGAR), and the cytochrome *c* oxidase subunit (COXII), activation of p53 induces ATP generation through OXPHOS [174, 182].

Recent studies have shown that reducing the glycolysis flux in cells can increase the mitochondrial oxidative phosphorylation under conditions of fixed energy demand [183]. Moreover, an increase in the efficiency of OXPHOS is also achieved, hampering cancer progression by decreasing the cell proliferation and invasiveness [184]. Thus, inducing metabolic flexibility while ameliorating mitochondrial functioning should arrest tumor progression and restore apoptosis.

1. 10. Caloric Restriction and Sirtuins

Acetylation levels of proteins are dependent on the cellular nutritional status. Thus, are highly sensitive to calorie restriction [82]. A crucial mechanism in carcinogenesis is the crosstalk between sirtuins and nutritional starvation. By means of starvation, cells avoid unnecessary energy consumption for synthesis and growth. Instead they use the limited resources for survival. Starvation provides an advantage to healthy cells by protecting them while sensitizing tumor cells to drug treatment [93].

This study is a pioneer in demonstrating SIRT3 activity to be triggered by NaB treatment following starvation. The reduction in cancer cell proliferation observed in this study demonstrates that HEP3B tumor cells are sensitive to calorie restriction.

2. EXPERIMENTAL PROCEDURES

2. 1. Cell Culture

HEP3B, human hepatoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco) and 1% penicillin – streptomycin (Gibco) at 37 ^{o}C and 5% CO_2.

2. 2. Boron Treatment

HEP3B cells were treated with sodium pentaborate (Boren) in low glucose (1 g/L) Dulbecco's modified Eagle's medium (Gibco) for 72 hours and starved for serum for 24 hours.

2. 3. Protein Amount Measurement

Treated cells were lysed in RIPA Lysis Buffer (SantaCruz). Protein amounts of cell lysates were determined by using the BCA Protein Assay Kit (Pierce). The BCA assay is a colorimetric assay, which detects bicinchoninic acid (BCA) to quantify the amount of total protein in a given sample. The kit consists of a 4% cupric sulfate solution and an alkaline solution containing BCA. Following the reduction of Cu^{+2} to Cu^{+1} by the given protein in the alkaline medium, Cu^{+1} and two molecules of BCA chelate, forming a purple-colored product that can be easily detected at 562 nm. The amount of protein is correlated with the absorbance value read at this wavelength. Analyzed lysates were supplemented with 1% (v/v) Protease Inhibitor Cocktail (Sigma-Aldrich), 1% (v/v) Deacetylation Inhibition Cocktail (SantaCruz), 1% (w/v) PMSF (Roche Applied Science) and 1% sodium orthovanadate (Five Photon Biochemicals).

2. 4. Mitochondrial Isolate Preparation

Mitochondria were isolated from $8x10^7$ HEP3B cells treated with 15 μg/ml NaB for 72 hours, by employing a protocol modified from *Cimen, H. et al.* [141]. Cell pellets were

resuspended in Isolation Buffer (26 mM Sucrose (Carlo Erba), 50 mM Tris-HCl (pH:7.6) (MP-Biomedicals), 40 mM KCl (Merck), 20 mM MgCl (Merck), 0.8 mM EDTA (Merck), 6 mM BME (Merck)) supplemented with 0.1% (w/v) PMSF (Roche). After separating the cytosolic fraction, the remaining pellets were resuspended in Wash Buffer (50 mM Tris-HCl (pH:7.6), 40 mM KCl, 20 mM MgCl, 1 mM DTT) supplemented with 0.1% (w/v) PMSF. Samples were then centrifuged twice at 12000 g, +4 ^{o}C for 10 min. The supernatants were discarded and mitochondrial pellets were stored at -80 ^{o}C for analysis.

2. 5. Immunoblotting

Protein samples harvested from HEP3B cells were loaded onto SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (PVDF). 20 μg protein was loaded for each sample to be analyzed. For acetylome profiling, the blot was probed with an acetylated lysine monoclonal antibody (Cell Signaling) at a 1:1000 dilution, Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) mouse monoclonal antibody (Abcam) and Heat-Shock Protein 60 (HSP60) mouse monoclonal antibody (Cell Signaling) at 1:5000 dilutions. To analyze the oxidative phosphorylation enzyme complexes, the blot was probed with Total OXPHOS Rodent WB Antibody Cocktail (Abcam) and HSP60 mouse monoclonal antibody (Cell Signaling) at 1:5000 dilutions. Secondary antibody for acetylation was anti-rabbit IgG (Sigma-Aldrich) at a 1:2500 dilution and anti-mouse IgG (Sigma-Aldrich) at a 1:5000 dilution for GAPDH, HSP60 and OXPHOS profiling. Imaging was performed using the BIO-RAD ChemiDoc^{TM} XRS+ Molecular Imager with Lab^{TM} Software.

2. 6. Intracellular NAD^+ / NADH Ratio Measurement

Intracellular NAD^+/NADH Ratio of NaB treated HEP3B cells were determined by using the NAD^+/NADH Assay Kit (Abcam). The kit contains a specific enzyme mix that creates a cyclic reaction in which the oxidized form of nicotinamide dinucleotide, NAD^+, and its reduced form NADH, are constantly being produced and metabolized. The kit is designed to specifically detect NADH and NAD, so the intracellular ratio of NAD^+ to NADH can be calculated accordingly. For this assay, HEP3B cells were cultured and treated in 6-well plates. $1{,}5x10^6$ cells were harvested for each sample and the assay was performed

immediately after cell harvesting. The amount of NADH was detected at 450 nm, using BIO-TEK ELx800 Microplate Reader.

2. 7. Cell Viability Assay

The viability of treated cells was investigated by using the CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega). The assay colorimetrically determines the number of viable cells that proliferate in culture medium by using a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and an electron coupling reagent phenazinemethosulfate (PMS). The viability is measured at 490 nm, through the formation of a formazan product resulting from the cellular bioreduction of MTS by the aid of the intermediate electron acceptor PMS. For the absorbance measurements, BIO-TEK ELx800 Microplate Reader was used. Results were calculated by taking the average of absorbance values of each sample run in triplicate. Data were normalized based on absorbance results obtained on the initial day of experiment.

2. 8. Cellular ROS Detection

Cellular ROS detection was performed in the presence of a cell permeant reagent 2',7' – dichlorofluoresceindiacetate (DCFDA) by using the DCFDA Cellular ROS Detection Assay Kit (Abcam). DCFDA is a fluorogenic dye that is used to measure ROS activities within a cell. As a cell permeant reagent, DCFDA diffuses into the cell where it is deacetylated to a non-fluorescent compound by cellular esterases. This compounds is further oxidized into a highly fluorescent compound, 2',7' – dichlorofluorescein (DCF) by ROS, which can be easily detected at a maximum excitation and emission spectra of 495 nm and 529 nm respectively. HEP3B cells were cultured and treated in 6-well plates. $5x10^4$ cells were harvested for each sample to be run in BDFACSCalibur™ Flow Cytometer.

2. 9. SIRT3 Activity Assay

The effect of 15 μg/ml NaB on SIRT3 deacetylase enzymatic activity was determined by using the SIRT3 Deacetylase Fluorometric Assay Kit (CycLex). The activity of the NAD^+-dependent histone deacetylase (HDAC) SIRT3 is measured in the presence of a HDAC inhibitor, Trichostatin A. The kit contains a substrate peptide which comprises of a fluorophore and a quencher that are coupled to its amino and carboxyl terminals, respectively. Following the simultaneous activities of added protease and SIRT3, the quencher is separated from the peptide, releasing the part containing the fluorophore. This reaction results with a fluorescent emission that can be detected at an emission ranging between 340-360 nm and an excitation ranging between 440-460 nm. The measurements were done using the BC BIOTEK FLx800 TBID fluorometric micro-plate reader.

2. 10. Statistical Analysis

The statistical significance of each experimental result was analyzed by running an unpaired t-test from averaged data obtained from three independent experiments, using the GraphPad Software. $p<0.05$ is taken as significant from three-independent experiments.

3. RESULTS

3. 1. NaB treatment decreases proteome acetylation

HEP3B cells were initially treated with 10 μg/ml and 100 μg/ml NaB to display the acetylation with increasing NaB concentrations. Treatment with 10 μg/ml NaB resulted in an increase in acetylation while a 27% decrease was observed with 100 μg/ml NaB treatment in whole cell lysate proteins (Figure 3.1). In order to determine the lowest possible treatment concentration, NaB concentrations between 0-20 μg/ml were analyzed. HEP3B cells were treated with 0, 10, 12.5, 15, 17.5, 20 μg/ml NaB for 72 hours. The cells were serum starved for 24 hours prior harvesting. According to immunoblotting analyses, a significant decrease was observed in 15 μg/ml NaB treated sample (Figure 3.2. (A) and (B)).

For further analysis, 15 μg/ml NaB was chosen as the effective treatment dose. HEP3B cells were treated with 15 μg/ml NaB in low glucose DMEM containing 1 g/L glucose, 10% FBS and 1% penicillin – streptomycin for 72 hours and serum-starved for the last 24 hours. Cells were harvested, lysed and proteins were separated by 10% SDS-PAGE and immunoblotted on PVDF membrane (Figure 3. 3. (A)). Analysis showed that 15 μg/ml NaB treatment significantly decreased proteome acetylation by 19% ($p<0.05$) (Figure 3.3. (B)).

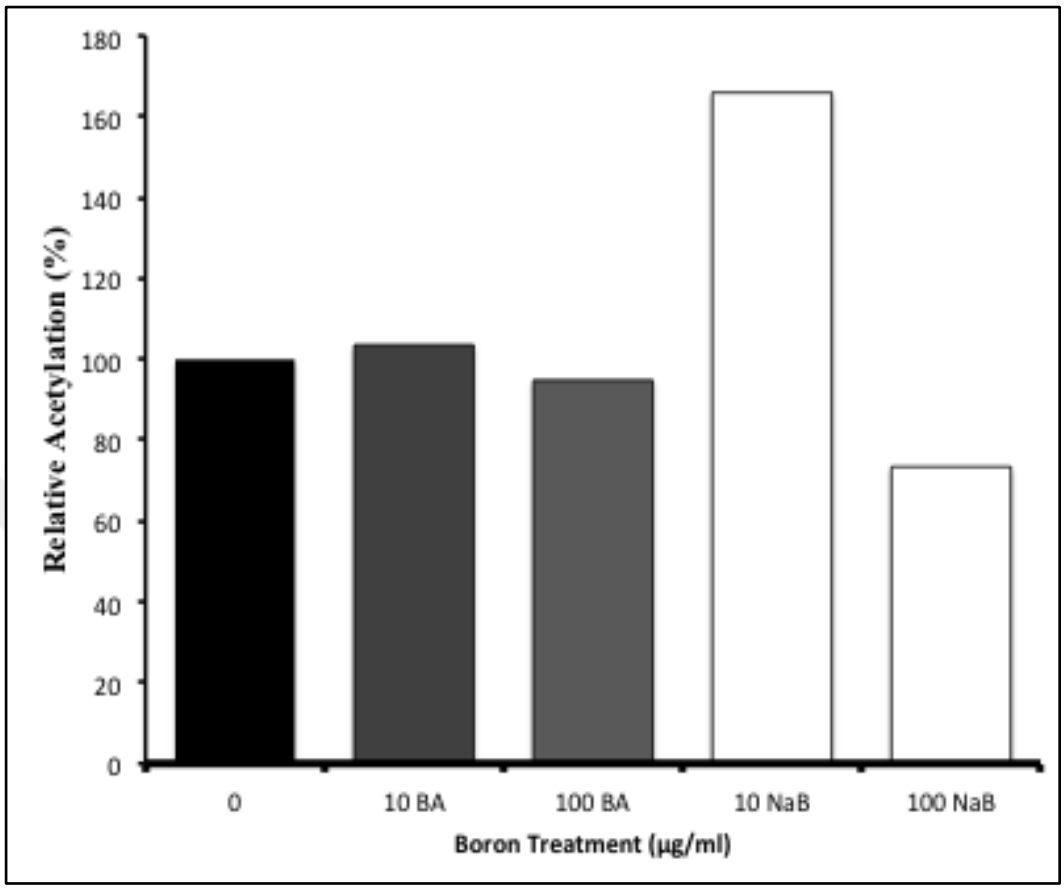

Figure 3.1. NaB treatment is more effective in decreasing proteome acetylation when compared to BA treatment. Relative ratio of acetylation level to control (β-actin) in whole cell lysate proteins from HEP3B cells treated with either boric acid (BA) or sodium borate (NaB). Percent relative acetylation of whole cell lysate protein samples are calculated by normalizing the obtained quantitative acetylation data. Each quantitation is done initially by using the control sample (0 µg/ml) as reference. The amount of acetylation signal of each sample is then divided by the amount of respective control signals.

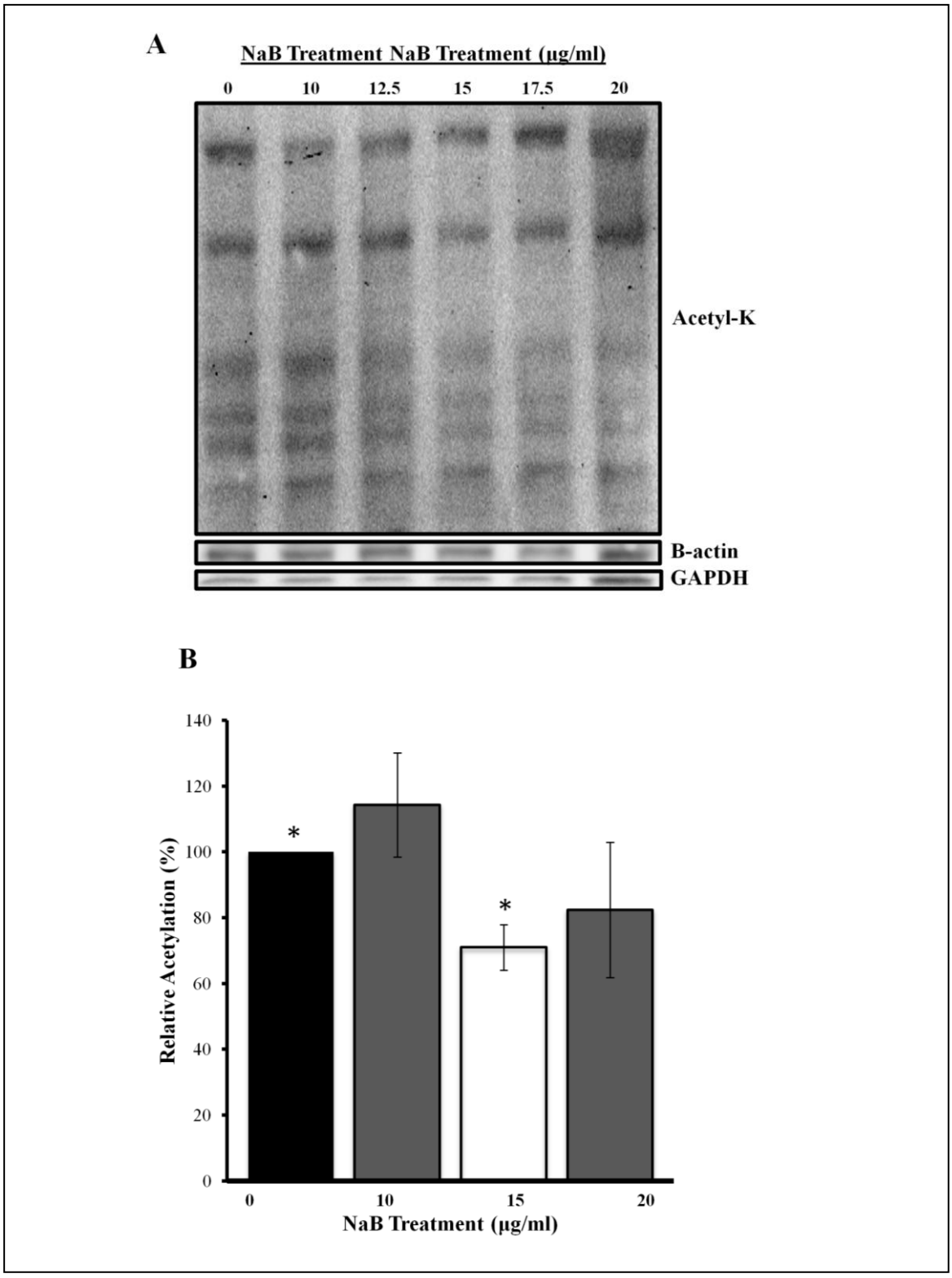

Figure 3.2. NaB treatment decreases proteome acetylation. (A) Acetylated lysine monoclonal antibody (1:1000), β-actin mouse monoclonal antibody 1:5000) and GAPDH

mouse monoclonal antibody (1:5000) were blotted onto the same PVDF membrane. GAPDH and β-actin antibodies were used as loading controls and were applied together, as an antibody cocktail. The membrane was incubated overnight at 4 ^{o}C for acetylation and control antibody signals, separately. Acetylation and GAPDH signals of control and NaB treated whole cell lysate proteins. (B) Relative ratio of acetylation level to control (β-actin) in whole cell lysate proteins from HEP3B cells treated with NaB. Percent relative acetylation of whole cell lysate protein samples are calculated by normalizing the obtained quantitative acetylation data. Each quantitation is done initially by using the control sample (0 μg/ml) as reference. The amount of acetylation signal of each sample is then divided by the amount of respective control signals. According to statistical analysis obtained from three independent experiments, a significant decrease of 29% in overall proteome acetylation was observed in 15 μg/ml NaB treated whole cell lysate protein samples (***** denotes for $p<0.05$).

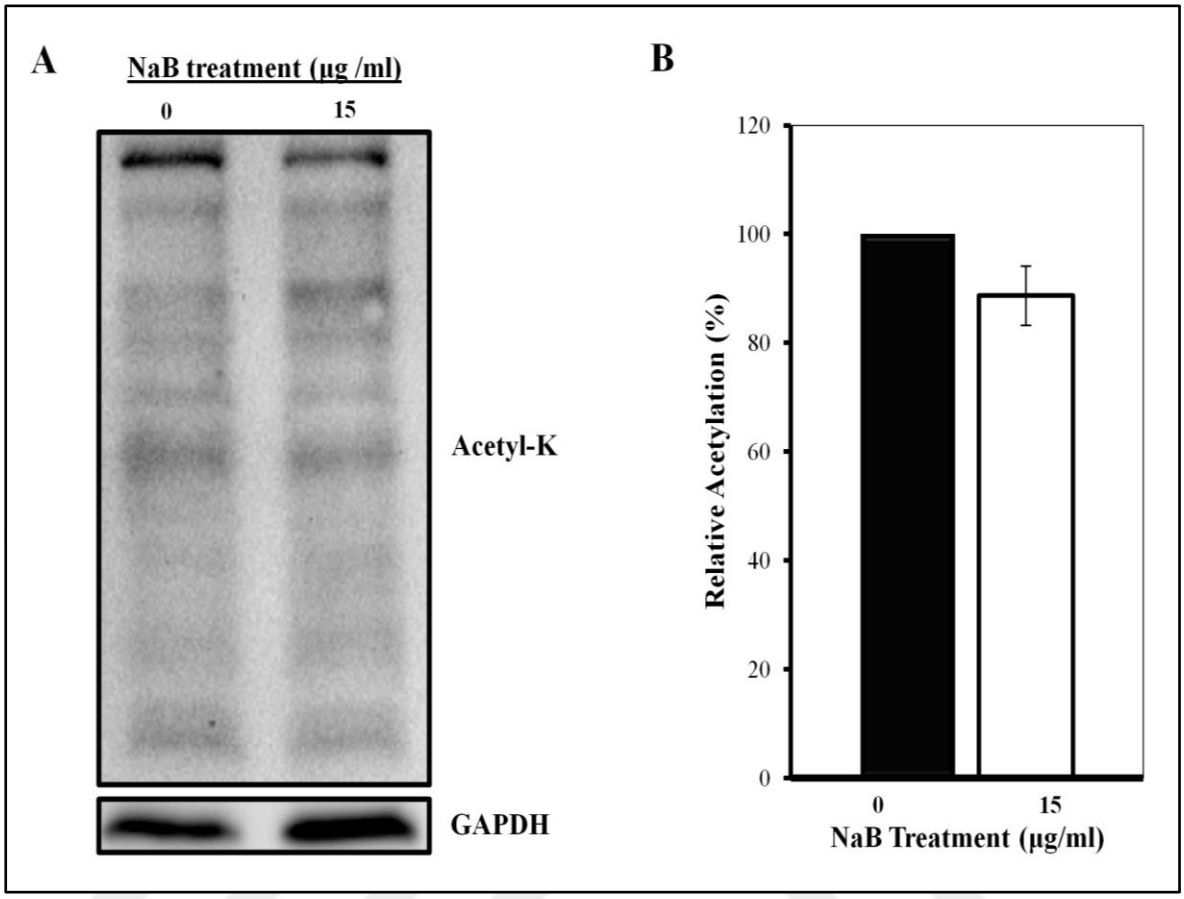

Figure 3.3. Proteome acetylation significantly decreases upon 15 µg/ml NaB treatment. (A) Acetylation and GAPDH signals of control and 15 µg/ml NaB treated whole cell lysate proteins. Acetylated lysine monoclonal antibody (1:1000) and GAPDH mouse monoclonal antibody (1:5000) were blotted onto the same PVDF membrane. GAPDH antibody was used as loading control. The membrane was incubated overnight at 4 ^{o}C for acetylation and control antibody signals, separately. (B) Relative ratio of acetylation level to control (GAPDH) in whole cell lysate proteins. Percent relative acetylation of whole cell lysate protein samples are calculated by normalizing the obtained quantitative acetylation data. Each quantitation is done initially by using the control sample (0 µg/ml) as reference. The amount of acetylation signal of each sample is then divided by the amount of respective control signals. According to statistical analysis obtained from three independent experiments, a significant decrease of 19% in overall proteome acetylation was observed in 15 µg/ml NaB treated whole cell lysate protein samples ($p<0.05$).

3. 2. Cellular NAD^+/NADH ratio is increased upon NaB treatment

Cellular NAD^+ / NADH ratio were determined by separately measuring NAD total (NADt) and NADH amounts in cultured HEP3B cells harvested upon 15 μg/ml NaB treatment for 72 hours in combination with starvation applied for the last 24 hours. In order to measure the NADH amounts, samples were heated at 60 ^oC to decompose NAD. NAD^+ / NADH ratios were then calculated by subtracting NADH from NADt and dividing the outcome by NADH itself. According to the results obtained at 450 nm, 15 μg/ml NaB treatment decreased NAD^+ level by 14% and NADH level by 53% alone. The cellular NAD^+ / NADH ratio; however, doubled in cultured HEP3B cells ($p<0.05$) (Figure 3.4.).

3. 3. Mitochondrial proteins are deacetylated upon NaB treatment

In order to determine whether the deacetylase activity observed in whole cell lysate samples are related to mitochondrial proteins, mitochondria were isolated from 15 μg/ml NaB treated HEP3B cells and lysed for protein analysis (Figure 3.5.). Samples were separated by 10% SDS-PAGE. Immunoblotting analysis revealed a statistically significant decrease by 11% in mitochondrial proteome acetylation ($p<0.05$) (Figure 3.6. (A) and (B)).

3. 4. Protein synthesis is decreased in OXPHOS machinery upon NaB treatment

For the analysis of proteins related to energy metabolism, the amounts of enzyme complexes involved in the oxidative phosphorylation pathway were investigated upon the NaB treatment. According to immunoblotting results following SDS-PAGE separation, protein synthesis in OXPHOS complex enzymes significantly decreased. A 17%, 24%, 22%, 11% and 13% reduction was observed in CI (NDUFB8), CII (SDHB), CIII (UQCRC2), CIV (MTCO1), and CV (ATPSA), respectively ($p<0.05$) (Figure 3.7.). Further analysis on CIV confirmed that the synthesis of this mitochondrial enzyme decreased significantly by 11% (Figure 3.8. (A) and (B)).

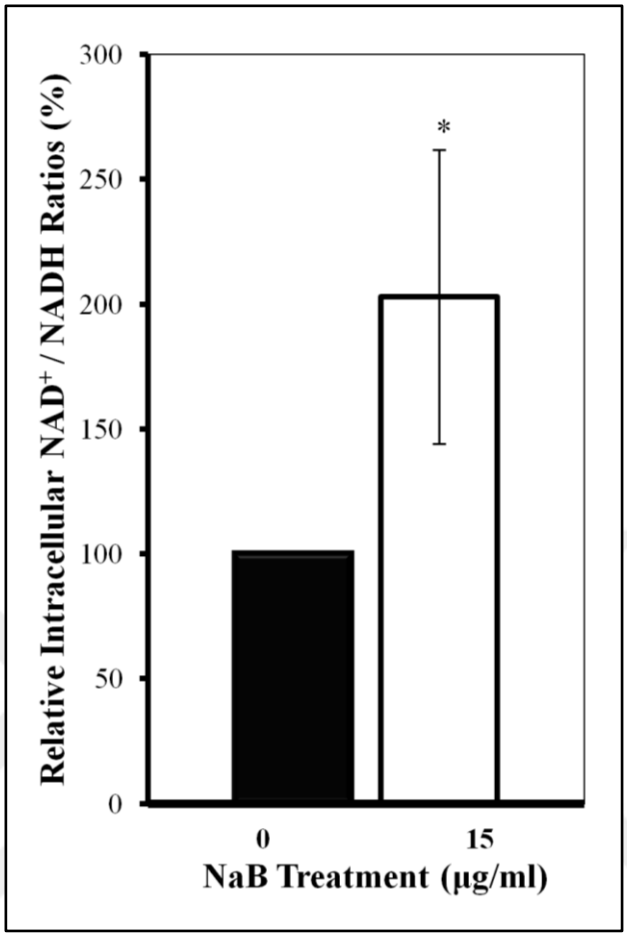

Figure 3. 4. Cellular NAD^+ / NADH ratio is increased upon NaB treatment. Intracellular NAD^+ / NADH ratios of cultured HEP3B cells treated with NaB. Cellular NAD^+ / NADH ratio were determined by separately measuring NAD total (NADt) and NADH amounts in cultured HEP3B cells harvested upon 15 µg/ml NaB treatment for 72 hours in combination with starvation applied for the last 24 hours. NAD^+ / NADH ratios were then calculated by using NADH and NADt amounts. According to the results obtained from three independent experiments, a 100% increase was observed in the 15 µg/ml NaB treated HEP3B cell sample (* denotes for $p<0.05$).

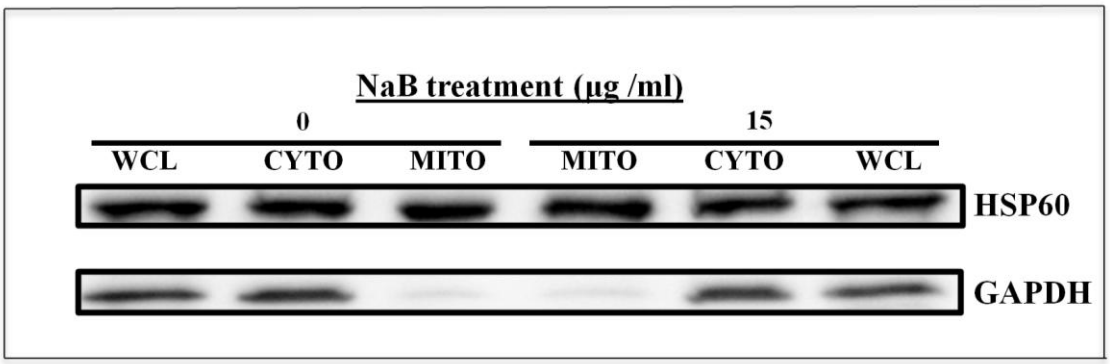

Figure 3.5. Mitochondria are isolated from whole cell lysates. GAPDH and HSP60 signals of control and 15 µg/ml NaB treated whole cell lysate (WCL), cytosolic fraction (CYTO) and mitochondrial (MITO) proteins. GAPDH mouse monoclonal antibody (1:5000) and HSP60 mouse monoclonal antibody (1:5000) were blotted onto the same PVDF membrane, as control antibodies. The membrane was incubated in an antibody cocktail containing both antibodies, overnight at 4 ^{o}C. Results obtained from mitochondrial HSP60 antibody signal and cytoplasmic GAPDH antibody signal show that the mitochondria of 15 µg/ml NaB treated samples and control samples were successfully isolated.

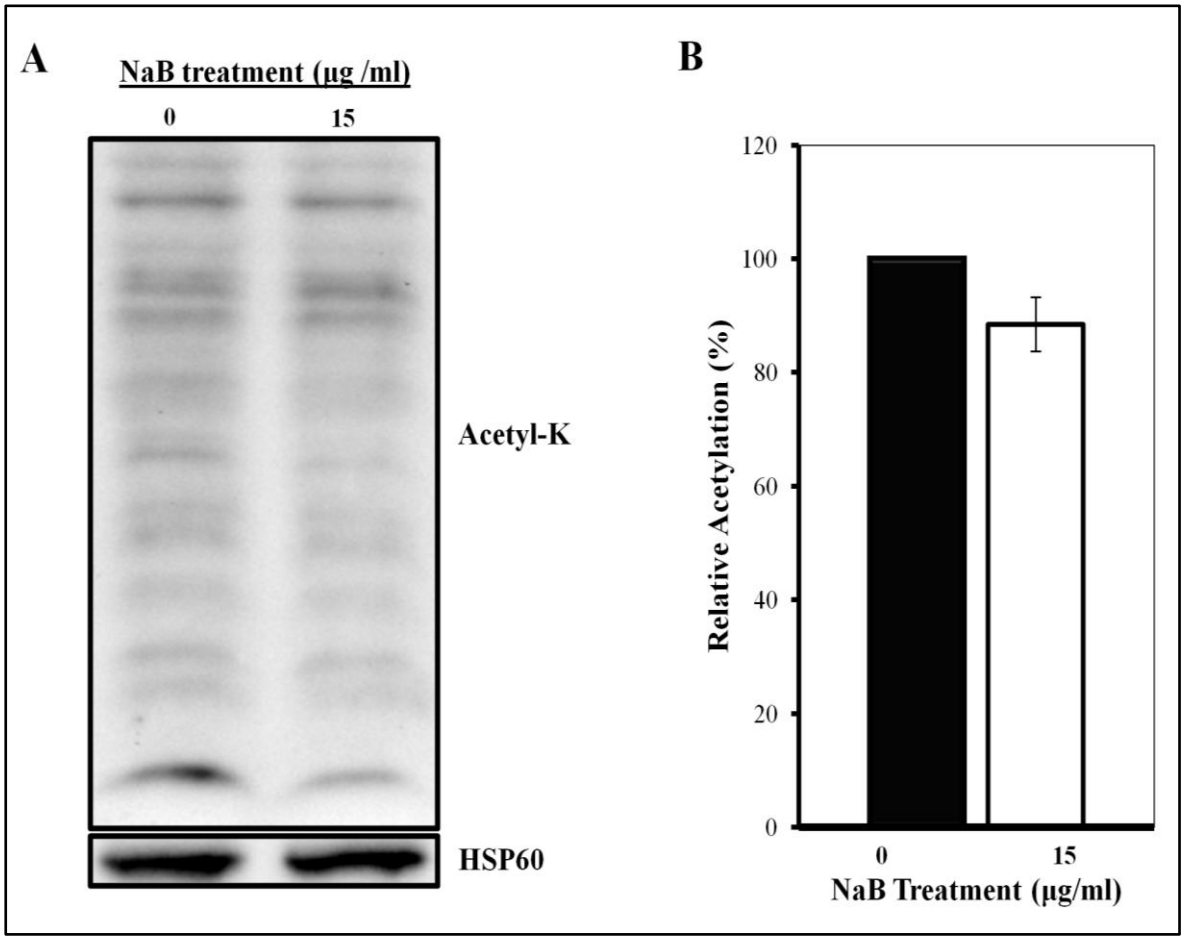

Figure 3.6. Mitochondrial proteins are deacetylated upon NaB treatment. (A) Acetylation and HSP60 signals of control and 15 µg/ml NaB treated mitochondrial proteins. Acetylated lysine monoclonal antibody (1:1000) and HSP60 mouse monoclonal antibody (1:5000) were blotted onto the same PVDF membrane. HSP60 antibody was used as loading control. The membrane was incubated overnight at 4 ^{o}C for acetylation and control antibody signals, separately. (B) Relative ratio of acetylation level to control (HSP60) in mitochondrial proteins from HEP3B cells treated with NaB. Percent relative acetylation of mitochondrial protein samples are calculated by normalizing the obtained quantitative acetylation data. Each quantitation is done initially by using the control sample (0 µg/ml) as reference. The amount of acetylation signal of each sample is then divided by the amount of respective control signals. According to statistical analysis obtained from three independent experiments, a significant decrease of 12% in overall proteome acetylation was observed in 15 µg/ml NaB treated whole cell lysate protein samples ($p<0.05$).

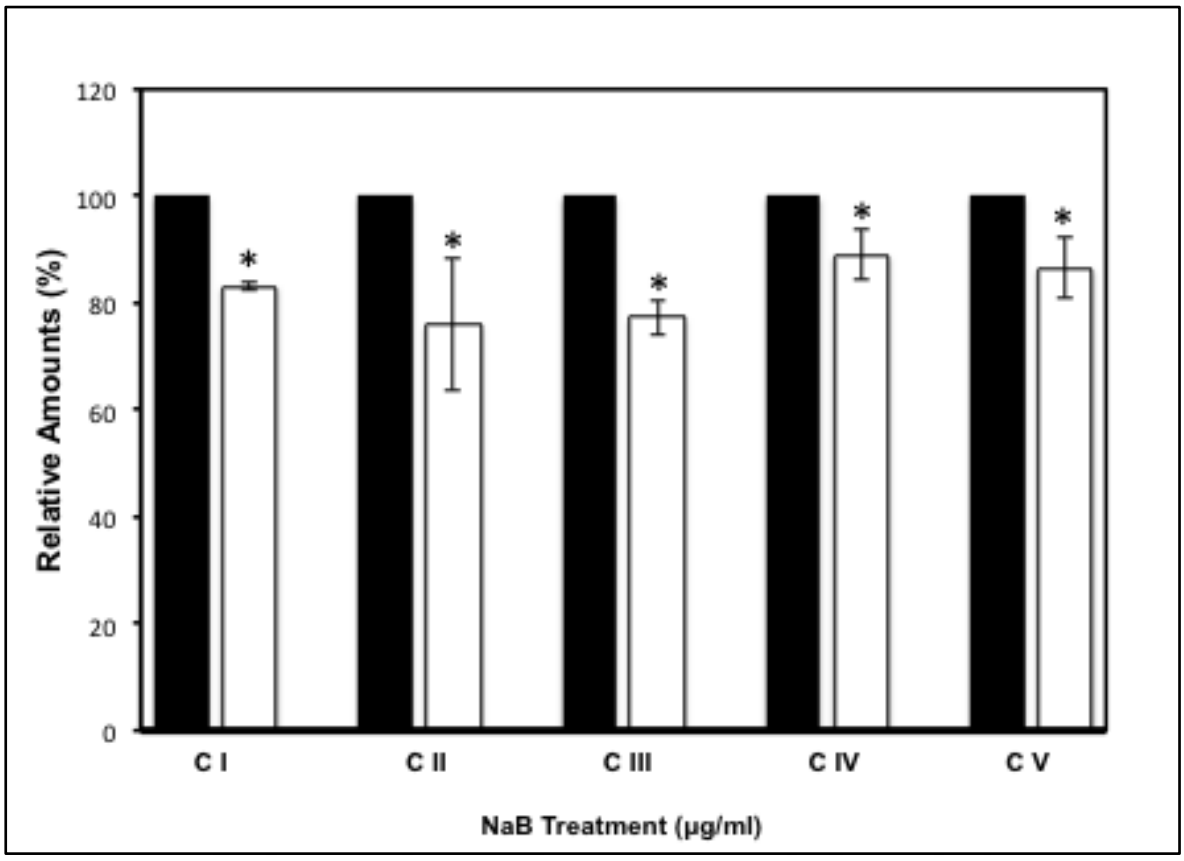

Figure 3.7. Protein synthesis is decreased in OXPHOS machinery upon NaB treatment. Relative ratios of OXPHOS Complex Enzyme levels to control (HSP60) in mitochondrial proteins from HEP3B cells treated with NaB. Total OXPHOS rodent antibody cocktail (1:5000) and HSP60 mouse monoclonal antibody (1:5000) were blotted onto the same PVDF membrane. HSP60 antibody was used as loading control. The membrane was incubated overnight at 4 ^{o}C for OXPHOS and control antibody signals, separately. Percent relative amounts of mitochondrial OXPHOS subunits are calculated by normalizing the obtained quantitative data. According to statistical analysis obtained from three independent experiments, a significant decrease of 17%, 24%, 22%, 11% and 13% was observed in the synthesis of OXPHOS subunits, in 15 µg/ml NaB treated mitochondrial protein samples (* denotes for $p<0.05$).

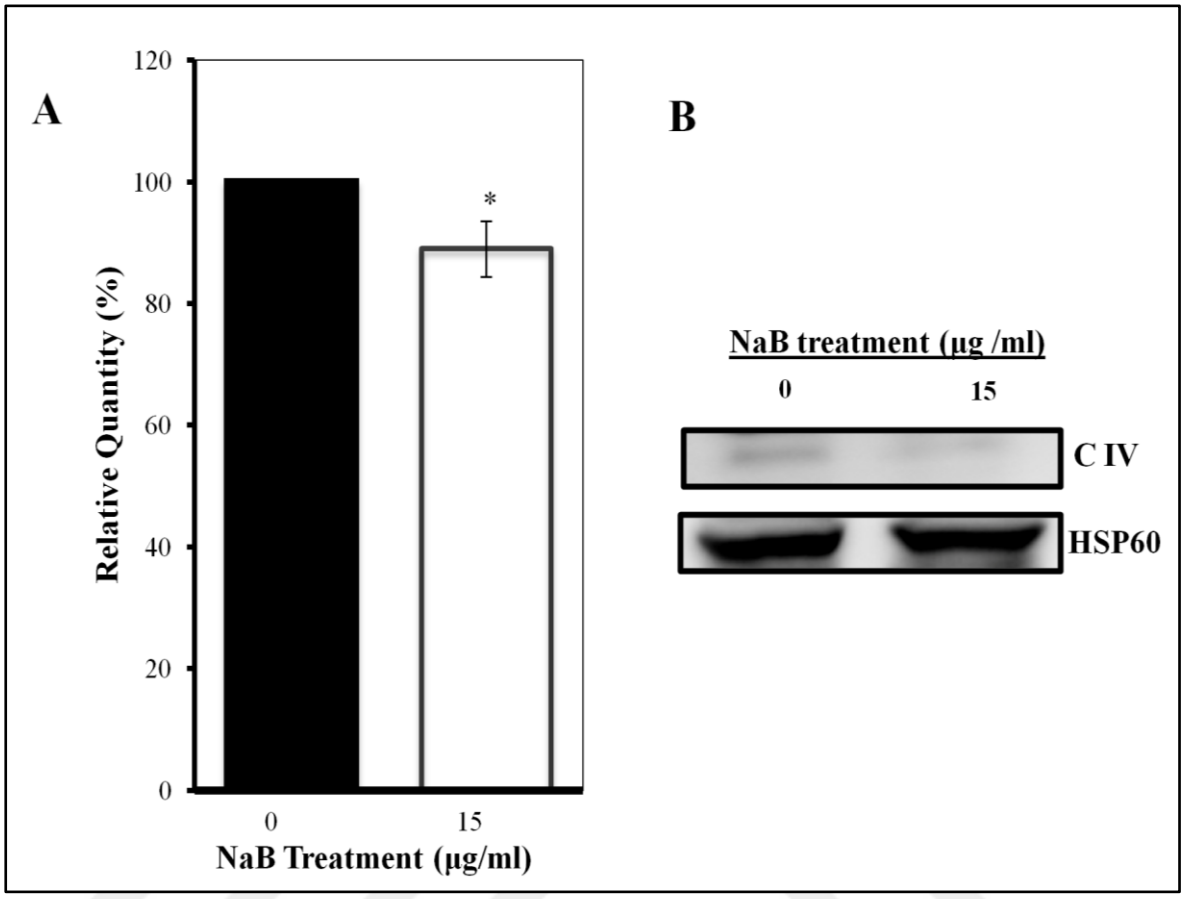

Figure 3.8. Protein synthesis is significantly decreased in CIV of OXPHOS machinery. (A) Relative ratio of Complex IV level to control (HSP60) in mitochondrial proteins from HEP3B cells treated with 15 µg/ml NaB ($p<0.05$). (B) Cytochrome c oxidase subunit 1 (CIV) and HSP60 signals of control and 15 µg/ml NaB treated mitochondrial proteins Total OXPHOS rodent antibody cocktail (1:5000) and HSP60 mouse monoclonal antibody (1:5000) were blotted onto the same PVDF membrane. HSP60 antibody was used as loading control. A significant decrease of 11% in the amount of C IV subunit of OXPHOS machinery was observed in 15 µg/ml NaB treated mitochondrial protein sample compared with control (0 µg/ml NaB) sample. (* denotes for $p<0.05$).

3. 5. NaB treatment reduced HEP3B cancer cell proliferation

Cellular proliferation of HEP3B cells was determined in the presence of the inner salt (MTS) and the intermediate electron acceptor PMS. 1000 cells/well were inoculated into 96-well plate. The NaB treatment was initiated after day 1 measurement of cell viability. Serum starvation in combination with the 15 μg/ml NaB treatment was initiated after day 3 measurement of cell viability. The results obtained through 4 days show that cancer cell proliferation was reduced by 33%, 20%, 32% on day 2, 3, 4 respectively, upon 15 μg/ml NaB treatment ($p<0.05$) (Figure 3.9.).

3. 6. Cellular ROS levels are reduced upon NaB treatment

Cellular ROS activity was measured depending on the activity of cellular esterases that deacetylate DCFDA to a non-fluorescent compound, which is then oxidized to a highly fluorescent compound, 2', 7' – dichlorofluorescin (DCF) by ROS. The fluorescence intensity of samples was measured at an excitation of 485 nm and an emission of 535 nm. According to the fluorescence data obtained from flow cytometer, 15 μg/ml NaB treatment for 72 hours in combination with 24 hours of starvation decreased cellular ROS levels by 73% ($p<0.05$) in HEP3B cells (Figure 3.10.).

3. 7. NaB treatment enhanced SIRT3 deacetylase activity

SIRT3 enzymatic activity was determined by measuring its deacetylase activity on a Fluoro-substrate peptide added to the reaction mixture. The fluorescence intensities of samples were measured at an excitation of 360 nm and an emission of 460 nm. Specific activity of SIRT3 was calculated by using the fluorescence intensity values obtained in the initial 20 min of reaction. Data show that 15 μg/ml NaB treatment enhances SIRT3 deacetylase activity by 31% ($p<0.01$) (Figure 3.11.).

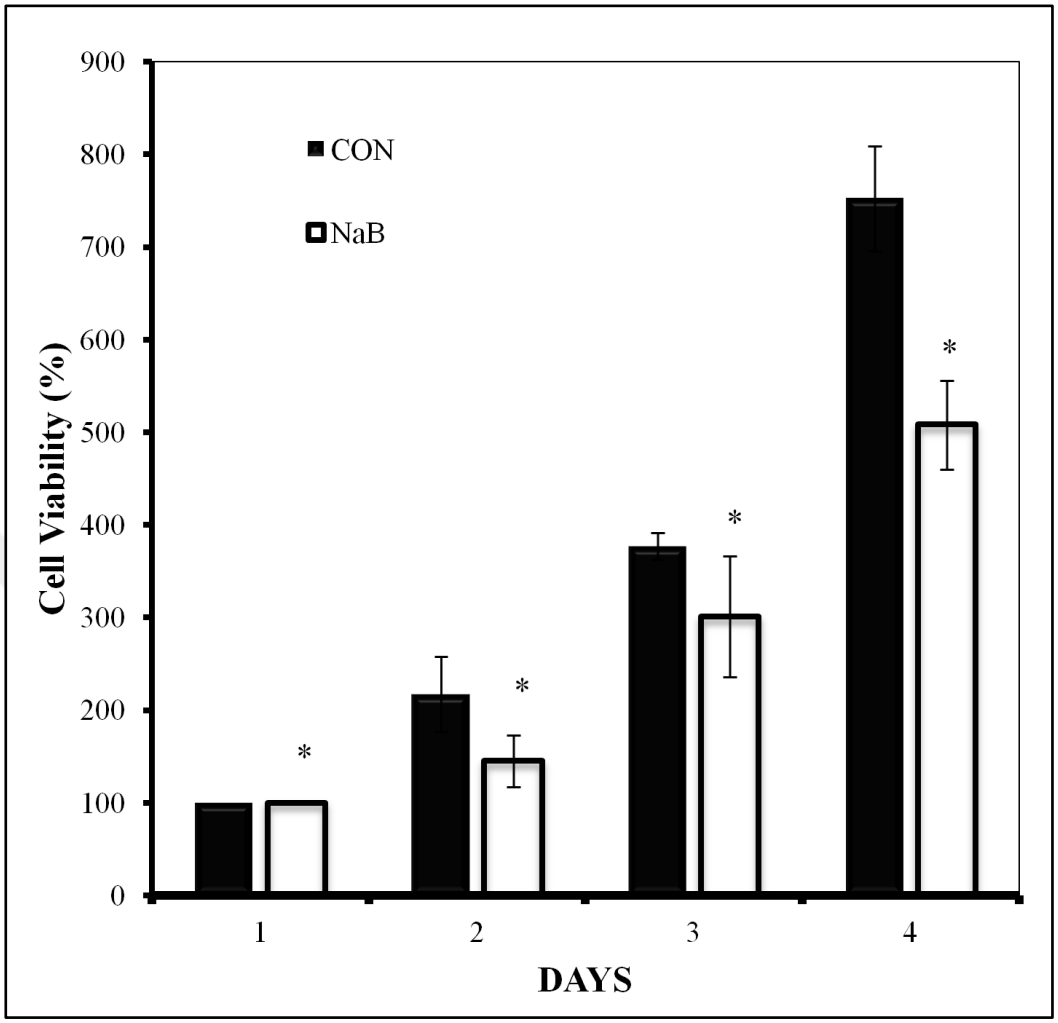

Figure 3.9. NaB treatment reduced HEP3B cancer cell proliferation. Percent cell viability measured through metabolic activity of cultured HEP3B cells treated with 15 μg/ml NaB. 1000 cells/well were inoculated into 96-well plate in high glucose DMEM media containing 10% FBS and 1% penicillin-streptomycin. 15 μg/ml NaB treatment in low glucose DMEM media containing 10% FBS and 1% penicillin-streptomycin was initiated on day 2, prior measurement. Starvation in combination with 15 μg/ml NaB treatment, in serum-free low glucose DMEM media was initiated after day 3 measurement. Significant decreases of 33%, 20%, 32% in cellular proliferation rate was observed on day 2, 3 and 4, respectively (* denotes for $p<0.05$).

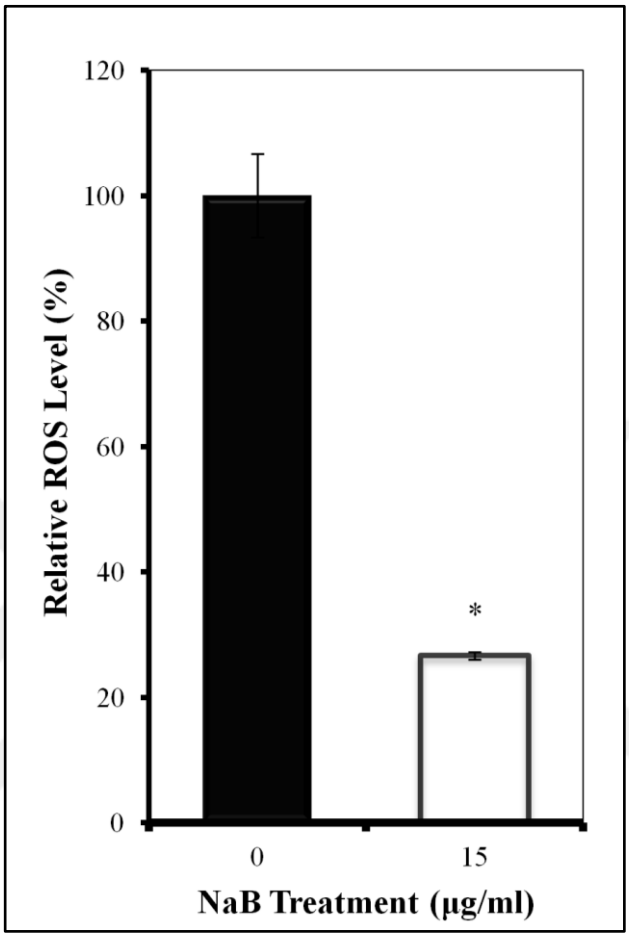

Figure 3.10. Cellular ROS levels are reduced upon NaB treatment. Percent decrease in ROS level in HEP3B cells treated with 15 µg/ml NaB compared to cells without treatment. $5x10^4$ HEP3B cells treated in 6-well plate were harvested and analyzed in flow cytometer. According to the results of three independent experiments, 15 µg/ml NaB treatment for 72 hours in combination with 24 hours of starvation decreased cellular ROS levels by 73% (* denotes for $p<0.05$).

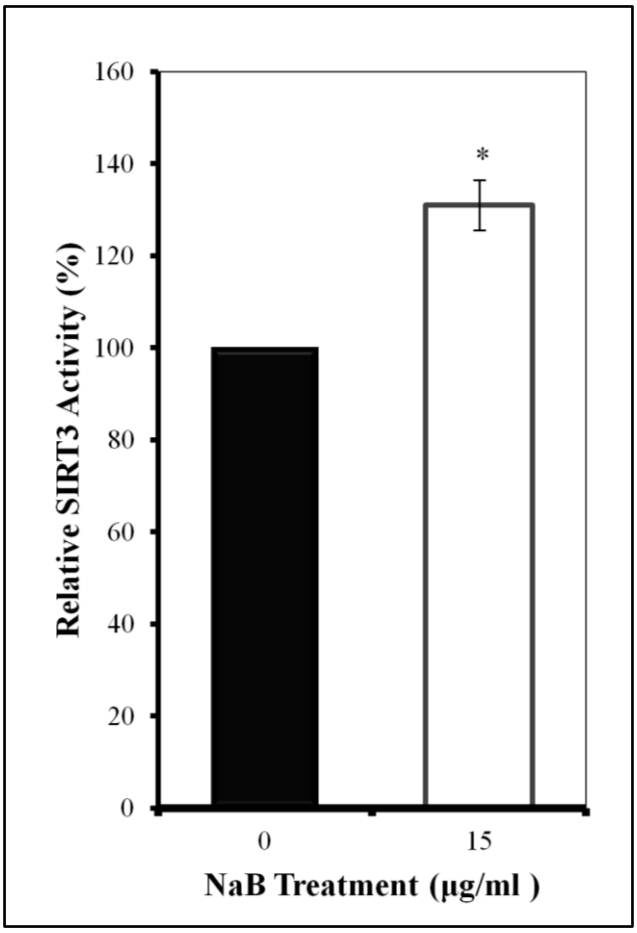

Figure 3.11. Relative SIRT3 Activity is increased upon NaB treatment. SIRT3 enzymatic activity was determined by measuring its deacetylase activity on a Fluoro-substrate peptide added to the reaction mixture. Specific activity of SIRT3 was calculated by using the fluorescence intensity values obtained in the initial 20 min of reaction, according to the data obtained from a fluorometric Microplate reader. The results showed that SIRT3 specific activity significantly increased by 31% in HEP3B cells treated with 15 µg/ml NaB compared to cells without treatment (***** denotes for $p<0.01$).

4. DISCUSSION

Mitochondria are cellular centers for energy homeostasis. Metabolic pathways in addition to the major production site of energy in the form of ATP are localized to mitochondria, which possess their own genomic DNA, polymerases, and ribosomes in order to carry out transcriptional and translational processes. Mitochondrial DNA mutations are very common due to the lack of histone proteins. Harboring most complex metabolic reactions, any problem encountered in these organelles during these reactions leads to accumulation of ROS, metabolic impairment, and finally to the reprogramming of targeted gene expression.

Most cancer cells are devoid of functional mitochondria. They exhibit high ROS generation, reduced mitochondrial enzyme activity, decreased levels of OXPHOS-derived ATP, and increased acetylation of proteins regulating metabolic gene regulation. These functional impairments in mitochondria result in the Warburg effect, which is depicted with enhanced glycolytic rate.

Metabolic gene regulation is actively controlled by post-translational modifications (PTM). Acetylation is the major PTM regulating mitochondrial gene expression. Activation of mitochondrial protein target genes can be modulated through acetylation-deacetylation mechanisms. SIRT3 is the main mitochondrial sirtuin functioning as NAD^+-dependent deacetylase. The stress-induced activity of SIRT3 is central in maintaining mitochondrial homeostasis through gene regulation. Activation of SIRT3 upon nutritional stress is well known to be dependent on intracellular level of NAD^+. Being a vital cofactor of various metabolic pathways, the intracellular levels of NAD^+ can be modulated through factors such as calorie restriction and chemical interactions. Sodium borate (NaB) is known to interact with NAD^+ via interactions involving hydroxyl groups of NaB that complex with that of NAD^+ [185-187]. This binding is well known to be strengthened by the capacity of NaB to bind the ribose moiety of NAD^+.

In this study, the impact of NaB-NAD^+ interaction on the activity of the stress-responsive mitochondrial deacetylase SIRT3 was investigated. Sodium pentaborate pentahydrate

(NaB) was used to alter the intracellular levels of NAD^+ via chemical interaction. This interaction, together with nutritional stress which is known to activate mitochondrial deacetylation [35], would lead to the modulation of SIRT3 deacetylase activity. Given the importance of protein functioning in coordinating metabolic pathways, the cellular energy hubs, mitochondria, were targeted by this combined treatment strategy. It was aimed to reprogram the metabolism of HEP3B human HCC cells by means of NaB treatment in order to reverse the Warburg effect by restoring proper mitochondrial functioning.

Immunoblotting analyses were performed to investigate the acetylome of the proteins harvested from HEP3B cells. A broad range analysis was initially performed with 10 μg/ml and 100 μg/ml NaB concentrations. The results showed that the proteome acetylation was increased with 10 μg/ml treatment while being decreased with higher NaB concentrations (Figure 3.1). The concentration range was narrowed down to 0-25 μg/ml NaB, accordingly. Immunoblotting analyses performed on six independent sample groups revealed a significant decrease of 29% in the proteome acetylation of 15 μg/ml NaB treated sample (Figure 3.2 (A) and (B)). Though remaining lower than that of control sample, the overall acetylation level was found to increase step by step with increasing NaB concentrations. This result may be due to the ideal protein structure achieved via the interaction between NaB and NAD^+ hydroxyl groups in 15 μg/ml NaB concentration. Borate is capable of reacting with hydroxyl groups to form borate monoesters with a tetrahedral configuration and a negative charge [186]. The binding of NaB to NAD^+ through ribose moieties further have impact on certain metabolic processes, because NAD^+ serves as a cofactor for oxidoreductase enzymes. This interaction possibly alters the intracellular levels of NAD^+, further leading to alterations in the activities of NAD^+ dependent deacetylases. To make sure that this result was related to NAD^+ levels in the cells, intracellular NAD^+ / NADH ratio was investigated next. As 15 μg/ml NaB was considered to be the ideal treatment concentration, experiments were further carried out using this NaB concentration as the effective dose (Figure 3.3). The three independent analyses showed that the intracellular NAD^+ / NADH ratio in HEP3B cells was increased by 100% upon 15 μg/ml NaB treatment applied in combination with 24 hours of starvation (Figure 3.9). This result confirmed that the decrease in overall protein acetylation observed in whole cell lysate proteins was in fact related to NaB-NAD^+ interaction and brought up the possibility of an improved cellular redox state achieved through functioning of metabolic proteins. This is

possible due to the alterations in metabolic reactions as a result of boron binding to substrate compounds [188]. Boron is known to inhibit the activities of glycolytic enzymes *in vitro*, and this way, it controls energy-substrate utilization [189]. Analyses were directed towards the proteome of the cellular center of energetic pathways, mitochondria.

In order to analyze the mitochondrial proteome, mitochondrial isolation was initially performed. Immunoblotting analyses carried out using specific antibodies of the mitochondrial protein HSP60 and the cytosolic protein GAPDH served as a confirmation of a reliable isolation process. While HSP60 signals were observed in the proteins from whole cell lysates, the cytosolic fractions and the mitochondrial fractions obtained from HEP3B cells, GAPDH signals were almost completely absent in the mitochondrial fractions (Figure 3.4). This confirms that the mitochondria of HEP3B cells were efficiently isolated. Next, acetylation of the mitochondrial proteome was investigated by immunoblotting analysis upon 15 μg/ml NaB treatment. The results from three independent analyses revealed a significant decrease of 11% in the overall mitochondrial protein acetylation (Figure 3.5). Thus, the NaB treatment showed to be effective in reducing the mitochondrial proteome acetylation, as expected. This post-translational alteration might have an impact on the synthesis of mitochondrial proteins. The acetylation / deacetylation states of lysine residues in mitochondria are especially important in regulating protein synthesis and functioning by means of altering gene expressions and enzymatic activities [72, 73]. According to proteomic studies, peculiar lysine acetylation promotes cancer by inducing macromolecular synthesis; thus increased rate of cellular proliferation [22, 70, 71, 74]. The mitochondrial protein synthesis would further provide an insight on mitochondrial biogenesis. Studies relying on MS analysis demonstrate a link between metabolic stress and the acetylation levels of metabolic enzymes, which play role in energetic pathways [77, 78, 82]. In light of these data, a significant reduction in mitochondrial proteome acetylation would reduce the synthesis of proteins especially related to ATP generation in energetic pathways. Given their vital roles in energy generation, the analyses on mitochondrial protein synthesis were preferentially carried out on the proteins involved in the mitochondrial OXPHOS pathway. The amounts of NDUFB8 (CI), SDHB (CII), UQCRC2 (CIII), MTCOI (CIV) and ATPSA (CV) subunits of the OXPHOS complex enzymes were determined by immunoblotting, using rodent OXHOS antibody cocktail. According to the analysis, a 17%, 24%, 22%, 11% and 13%

decrease was observed in the amounts of CI, CII, CIII, CIV and CV proteins, respectively (Figure 3.6). The reductions were significant and were observed in three independent studies. An overall reduction in the synthesis of proteins responsible for ATP generation indicates a reduction in mitochondrial biogenesis. This reduction would have an impact on the rate of cellular proliferation as cancer cells are able to proliferate at higher rates by means of increased macromolecular synthesis [74]. A reduction in the cellular proliferation rate would be expected in the light of these results. The rate of HEP3B cell proliferation was investigated via MTS assay. According to the results obtained, significant decreases of 33%, 20% and 32% in cellular proliferation rate were observed on day 2, 3 and 4 upon 15 μg/ml NaB treatment, respectively (Figure 3.8). Together with the results on the synthesis of OXPHOS subunit enzymes, a reduction in mitochondrial biogenesis was confirmed.

This finding could indicate a functional restoration of the mitochondria by means of increasing mitochondrial quality by retaining and by making use of only the functional parts of the mitochondrial proteome. Boron has shown to be very effective against damages caused by oxidative stress, especially in liver. Boron pre-treatment can down-regulate the activity of a pro-oxidant enzyme, xanthine oxidase, by 60%, which is severely induced in fulminant hepatic failure (FHF), leading to enhanced generation of ROS. The administration of boron resulted in decreased production of ROS and the amelioration of tissue damage [60]. Moreover, boron supplementation has shown to replenish the depleted hepatic glutathione levels, restoring the oxidative balance in the tissue [190]. Boron was also effective in decreasing glucose levels while increasing the levels of alanine and pyruvate, demonstrating to have a significant impact on Krebs cycle as well as glucose-alanine cycles [190]. The same study showed that boron increases the levels of creatine, a compound that ensures ATP / ADP homeostasis through creatine-phosphocreatine energy buffer system. This, in turn, acts to compensate for the damages caused by cellular dysfunction. For the investigation, levels of ROS, the major factor causing mitochondrial damage was investigated in HEP3B cells. Cellular ROS detection revealed a significant decrease of 73% in the ROS levels of 15 μg/ml NaB treated HEP3B cells (Figure 3.10). This result verified that the mitochondrial functioning was indeed restored upon treatment.

Lastly, in order to certify that the mitochondrial deacetylase enzyme, SIRT3, was in fact responsible for this functional restoration and metabolic reprogramming, the specific activity of SIRT3 was determined in the presence of a protease (Lysylendopeptidase) that

released fluorophore-attached fragments from a substrate peptide. The intensity of the fluorescent signals coming from these fragments was the measure of SIRT3 activity. The results showed that SIRT3 deacetylase activity was significantly enhanced by 31% upon 15 μg/ml NaB treatment (Figure 3.11).

All together, these results indicate that NaB treatment in combination with calorie restriction is effective in inducing SIRT3 activation through NAD^+ interaction. This combined treatment strategy involving NaB treatment and calorie restriction is shown to be successful in achieving metabolic regulation through modifying mitochondrial protein acetylation, paving the way for future investigations aiming to uncover the impacts on tumor apoptotic mechanism.

5. FUTURE DIRECTIONS

This study indicates that metabolic reprogramming is possible through simultaneous actions of NaB treatment and stress induction on human hepatocellular carcinoma. For this reason, it is applicable to improve NaB based products to enhance the efficiency of treatments against hepatocellular carcinoma.

Several promising tumor biomarkers including alpha-fetoprotein isoform L3 (AFP-L3) have been discovered in Hepatocellular carcinoma (HCC) [17] and molecular pathways such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), and mammalian target of rapamycin (mTOR) have recently been associated with HCC pathogenesis and are considered to be potential targets for therapy. The anti-angiogenic multikinase inhibitor ‘sorafenib’ is the first authorized therapy to be applied, which shows significant achievements in survival against HCC [191]. Other targeted therapies under assessment include mTOR inhibitors, sunitinib, brivanib, linifanib, and ramucirumab [191]. Metabolomics, which is the study of metabolites that are regulated by genes and proteins, is indispensable in the analysis of HCC occurrence, development, metastasis and recurrence [192-195]. Metabolic profiling on HCC by LC-MS analysis revealed significant alterations in carbohydrate, amino acid, and lipid metabolisms in addition to the major differences being observed in bile acids, steroids, and fatty acids [195]. Metabolic-profile based risk predictors are recently being identified; however, further studies including ethnic and confounding factors are required [195].

Studies on therapies targeting certain molecular pathways related to HCC, which are currently under assessment, will help eliminate the obstacles on the way through survival against HCC. As metabolic profiling is becoming of critical importance in disease assessment, future studies shall focus on drug delivery systems targeted to specific HCC biomarkers discovered by means of mass spectrometry analysis. These reliable systems may incorporate NaB in order to increase drug efficiencies on targeted approach to metabolic disorders.

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