NOTTINGHAM TRENT UNIVERSITY

Role of sustained nutrient regimes in metabolism and epigenetics of diabetic and cancer cell models

By

Akashdeep Singh

A thesis submitted in partial fulfilment of the requirements of Nottingham Trent University for the degree of Doctor of Philosophy

May 2019

ABSTRACT

Central metabolism plays an intrinsic role in the regulation of gene expression, as cells adapt their epigenetic profile in response to nutrient availability. Moreover, epidemiological studies revealed a strong correlation between various pathologies, such as metabolic syndromes and cancer with nutrient status. To address the molecular mechanisms of this link, a cancer model (HeLa) and β-cell model (INS-1) were exposed to a sustained acetate and galactose nutrient regimes. Under this treatment, assessment of gene expression and epigenetic profiles, both from *IDH2* and *insulin* gene in INS-1 cells, and *IDH2* and *NIX* in HeLa cells showed active regulation of DNA promoter methylation. Moreover, using a targeted dCAS9-Tet system, *IDH2* epigenetic regulation was induced by promoter hydroxymethylation. In addition, acetate treatment led to the accumulation of DNMT3a protein in INS-1 cells, a process which was also enhanced by inhibiting proteasomal activity. Moreover, galactose led to reduction in the DNMT3a protein in HeLa cells.

Functionally, acetate-treated INS-1 cells showed a significant reduction in mitDNA content, mtCOI expression, total α-ketoglutarate/ATP contents and increased histone acetylation levels, resulting in reduced insulin content. Whereas, galactose-treated HeLa cells showed a significant increase in mitDNA content, mtCOII expression, total α-ketoglutarate and reduction in histone acetylation, resulting in reduced proliferation and increased apoptosis. Overexpression of IDH2 effectively reverted the effects acetated-induced effect in INS-1 cells, as shown by DNMT3a protein abundance, histone acetylation and ATP content. Our results also show that the alterations in the nutrient availability of INS-1 cells, such as elevated acetate levels, led to a modified epigenetic control of *IDH2* and *insulin* gene, subsequently altering cellular functions. Moreover, cell cycle analysis showed that IDH2 acetylation but not expression is regulated in cell cycle, and acetate (in the absence of glucose) can be sufficient in surpassing the nutrient sensitive restriction point during cell cycle. Collectively, these observations provide a mechanistic link between nutrient availability and epigenetic control in diabetes, cancer and cell cycle models, and highlight the rationale for interventions in this process as part of therapeutics strategies in cancer and diabetes.

CONTENTS

PUBLICATIONS AND CONFERENCE PROCEEDINGS

Submitted for publication:

Akashdeep Singh, Silas Odom, Daniel Kirk, Mark D. Turner and Sergio Colombo. **Acetate promotes IDH2 and insulin downregulation by promoter methylation in INS-1 cells.**

Science and Technology Annual Research Conference 2017

Best PhD presentation award

Akashdeep Singh, Mark D. Turner and Sergio Colombo. **Role of nutrients in epigenetics: Modulation of IDH2 in a diabetic model.**

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my Godfather, grandparents, parents, siblings and partner for their support and guidance.

I would like to also thank my director of studies and supervisor, Dr Sergio Colombo for giving me opportunities, experience and skills. I would not have made this journey through without his guidance and support. His knowledge greatly contributed to building a strong base for my career. I would like to thank my co-supervisor Dr Mark Turner, and my independent assessor Dr Cristina Montiel-Duarte for the mentorship and development opportunities. NTU VC Scholarship team for funding this research. Finally, acknowledging my laboratory colleagues and technicians for their support and knowledge.

LIST OF FIGURES AND TABLES

ABBREVIATIONS

2-HG	2-hydroxyglutarate
5-caC	Carboxylcytosine
5-fC	Formylcytosine
5-ghmC	Glucosylated cytosine
5-hmC	Hydroxymethylcytosine
5-mC	Methylated cytosine
ACLY	ATP Citrate Lyase
ACSS	Acyl-CoA synthetase
ADP	Adenosine diphosphate
APC/C	Anaphase-promoting complex/cyclosome
APC/C-CHD1	Anaphase-promoting complex/cyclosome-CDH1
ATP	Adenosine Triphosphate
BER	Base excision repair
BMAL1	Brain and Muscle ARNT-Like 1
cAMP	Cyclic adenosine monophosphate
CDK	Cyclin dependant kinase
COX	Cytochrome c oxidase
CpG	Cytosine-Phosphate-Guanine
CRE	cAMP responsive element
CSC	Cancer stem cells
DNMT1	DNA Methyltransferase 1
ES	Embryonic Stem
ETC	Electron transport chain
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FFAR2	Free fatty acid receptor 2
GB	Genome browser
GFP	Green fluorescent protein
GLP-1	Glucagon like peptide-1
GLS1	Glutaminase 1
GLT	Glucolipotoxicity
GLUT	Glucose transporter
HAT	Histone acetyltransferases
HDAC	Histone deacetylases

hESC	Human embryonic stem cells
HK	Hexokinases
HIF	Hypoxia induced factor
H3	H3
IDH	Isocitrate dehydrogenase
JBP1	J base deoxygenase 1
KI	Potassium Iodide
mAb	Monoclonal Antibody
MBD	Methyl binding domain
MBP	Methyl-CpG-binding proteins
MeCP2	Methyl CpG binding protein 2
mitDNA	Mitochondrial DNA
MODY	Mature onset of diabetes of the young
mtCOI	Cytochrome C oxidase subunit 1
mtTFA	Mitochondrial transcription factor A
NCZ	Nocodazole
NFR	Nuclear respiratory factor
OXPHOS	Oxidative phosphorylation
p53	Tumour protein 53
Pdx1	Pancreatic and duodenal homeobox 1
PEI	Polyethylenimine
PFK1	Phosphofructokinase-1
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, isoform 3
PGC1α	Peroxisome-proliferator-activated-receptor gamma co-activator
PI	Propidium Iodide
PPARα	Peroxisome proliferator-activated receptor alpha
pRB	Retinoblastoma
ROS	Reactive oxygen species
R-point	Nutrient sensitive restriction point
SAH	S-adenosylhomocysteine
SAM	S-adenyl methionine
SCF	Skp1/cullin/F-box
SIRT	Sirtuins
SNP	Single nucleotide polymorphisms
SNP	Single nucleotide polymorphisms
SRA	Set and RING finger-associated domain
T2D	Type 2 diabetes

TDG	Thymine DNA glycosylase
TET	Ten Eleven Translocation-Methylcytosine Dioxygenase enzymes
TSS	Transcription start site
UHRF1	Ubiquitin Like with PHD And Ring Finger Domains 1
XIST	X-inactive specific transcript
ZBTB4	Zinc Finger and BTB Domain Containing 4
α-KG	α-Ketoglutarate

CHAPTER 1: INTRODUCTION

The trans-generational epigenetic inheritance has emerged as a new area of research and challenged the central dogma of biology stating that the genetic code is the sole basis for biological inheritance. The area of epigenetics has provided new insights into the underlying biological mechanisms that can be involved in transmitting traits to following generations (Zakhari 2013; Rissman, Adli 2014). Understanding the cues leading to epigenetic changes in normal and cancer cells remains an essential factor to be further investigated. It was previously established that metabolism and epigenetic modifications are directly linked, and epigenetic changes can cause several pathologies, including diabetes, cancer, neuronal disorder and cardiovascular diseases (Hanahan, Weinberg 2011). Many of these conditions are associated with altered DNA methylation status of several genes (Lin, Wang 2014). DNA methylation and histone acetylation are important processes in epigenetic transcriptional regulation, and each cell type has a differential epigenetic pattern that correlates with its function. For example, epigenetic reprogramming of the cells leads to DNA promoter demethylation of pluripotency genes during pluripotency induction or somatic cell nuclear transfer (Reichetzeder et al. 2016). Previous studies have shown that genes involved in development have a considerable overlap with genes involved in various diseases, and these genes were also altered in adult renewal system with majority being regulated by epigenetics (Mohammad, Baylin 2010). In human body the genetically homogeneous cells are divided into different tissue types, each displaying heterogeneous qualities and functions. This heterogeneity is due to the differential gene expression profiles, that are initiated during early stage of development and differentiation, thereafter, maintained during mitosis. This heterogeneity is also referred to as epigenetic profiles, which is heritable without incorporating any permanent genetic changes in the cell (Jaenisch, Bird 2003). Epigenetics influences gene expression profiles which can show effect prenatally and after birth, leading to long term health outcomes. These changes can be driven by diet, exercise and environment; raising from a complex combination of factors (Katada et al. 2012).

The central dogma of biology, suggested by Sir Francis Crick, connected the transformation and control of biological information, i.e. DNA to RNA to proteins (Crick 1957). The fundamentals of this dogma are correct; however, recent discoveries of non-coding RNA and epigenetics revolutionised and provided layers of transformation and control of biological information (Figure 1.1). The basis of central dogma states that the majority of the genome is non-coding (at one point referred to as ‘Junk’ DNA). However, as the evolution progressed, these non-coding regions increased in more complex multicellular

organisms, such as homo sapiens, in comparison to other species (Ganesan 2018). Moreover, genotypically identical monozygotic twins are not physically identical, and these phenotypic differences arise with time and growth. Monozygotic twins have played an important role in studies conducted to understand the connection between environment and epigenetics. Differential gene expression has been observed as the twins age, which correlated with epigenetic changes and therefore suggesting epigenetic modification can naturally arise in individuals in addition to the inherited patterns (Aquino et al. 2018).

1.1. Epigenetics

The mechanisms of environmental adaptation in eukaryotes is mediated by active epigenetic modifications. The genome of cells remains constant, whereas, the epigenome is dynamic, therefore, it responds to the nutritional and environmental changes. These epigenetic changes play a pivotal role in health and disease. This dynamic nature of epigenetics can essentially be reversed with beneficial outcomes in the disease without editing the genomic sequence (Ganesan 2018). Epigenetic silencing of genes is commonly found in many cancers (Ohm et al. 2007). Our current understanding of epigenetics originates mainly from cancer cell models, therefore, studies conducted in other disease models are essential to fully elucidate the mechanism involved in epigenetic regulation. There are three main types of epigenetic modifications, including DNA methylation, chromatin modifications and RNA-mediated processes (Table 1.1). The well-established epigenetic aberrations in health and disease are histone acetylation and DNA methylation, which can regulate the accessibility of DNA in eukaryotic cells by modulating chromatin accessibility (Sterner, Berger 2000). Silencing of tumour suppressors associated with hypermethylated DNA promoter regions, is commonly found in many cancers. DNA methylation occurs mostly at the Cytosine-Phosphate-Guanine (CpG) sites of gene promoter regions. Roughly $3x10^7$ CpG islands have been identified in the human genome, providing a wide range of possible methylation patterns that can control gene expression and therefore increase the potential epigenetic information content of the cell (Edwards et al. 2010). Clinical trials targeting epigenetics started in 1967 with 5-azacytidine, meanwhile lacking the knowledge of drug's mechanism of action on DNA methyltransferases (Ganesan 2018). The Food and Drug Administration (FDA) has already approved 'Epi-drugs' targeting the epigenetics for the treatment of some cancers, e.g. Vorinostat is used in the management of cutaneous T cell lymphoma by targeting histone acetylation (Bubna 2015).

DNA methylation has also played a crucial role in epigenetic targeted interventions. DNA methylation has long been considered the central player of the epigenetic network, therefore, studies focusing on DNA methylation has contributed most significantly towards our understanding of epigenetics (Zhang 2015).

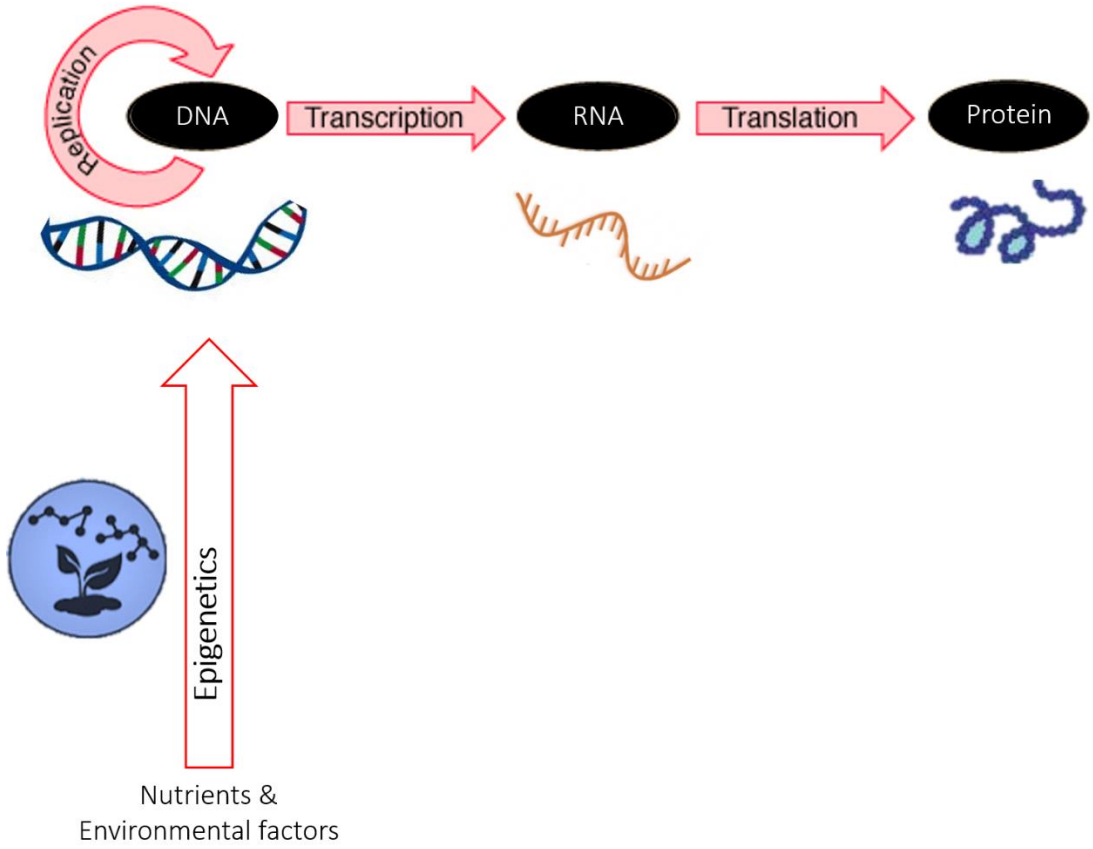


Figure 1.1: The relationship between central dogma of biology and epigenetics. The biological information encoded in DNA can be decoded to RNA and amino acid sequence in the proteins, which was originally proposed by Sir Francis Crick (1957). Beyond the central dogma, epigenetics has emerged as an essential heritable form of biological information and a key regulator of the genetic biological information. Epigenetics is an important factor in the flow and inheritance of biological information, which can greatly add to our understanding of how nutrients and environmental factors effect this molecular form of data.

Table 1.1: Epigenetic modifications and their role in gene regulation

Epigenetics	Modification	Consequence
DNA methylation	5th position of the cytosine residues are covalently modified with the addition of a methyl group donated from S-adenosylmethionine, resulting in production of 5-methylcytosine in a DNA methyltransferase mediated reaction (Newell-Price et al. 2000).	**Gene repression** by inhibiting transcription factor binding, repressor complex recruitment and chromatin remodelling (Lim, Maher 2010).
DNA hydroxy methylation	5-hydroxymethylcystosine is generated from 5-methylcytosine by Ten eleven translocation-methylcytosine dioxygenase enzyme mediated oxidation. 5-hydroxymethylcytsoine can be converted to 5-formylcystosine and 5-carboxylcytosine as additional intermediates in an passive demethylation process (Ponnaluri et al. 2017).	**Gene expression** by passive demethylation of cytosines, regulating co-transcriptional splicing and possibly chromatin modulation (Ponnaluri et al. 2017).
Chromatin modulation	**Histone acetylation:** Lysine residues on the protruding histone tails can be acetylated and deacetylated by Histone acetyltransferases and Histone deacetylases, respectively. Other forms of chromatin modulation includes but are not limited to: sumoylation, ubiquitylation, methylation, phosphorylation, ADP ribosylation, non-covalent proline isomerisation and deamination (Gibney, Nolan 2010).	**Gene expression** by acetylation and **gene repression** by deacetylation of histones mediated by structural chromatin modulation, disruption or recruitment of proteins binding to chromatin (Gibney, Nolan 2010).
Small non-coding RNAs	Usually derived by RNAse III-family enzymes mediated cleavage, resulting in mainly Short interfering RNAs and micro RNAs (Agarwal, Weinstein 2018).	**Gene repression** by drosha and/or dicer dependant pathway (Agarwal, Weinstein 2018).
Long non-coding RNAs	RNA molecules with more than 200+ nucleotides, that can serve as decoy, signal, scaffold, short peptides and enhancer RNAs (Fang, Fullwood 2016).	**Controversial transcription regulation.** Responsible for gene expression and repression in response to various stimuli (Fang, Fullwood 2016; Gibney, Nolan 2010).

1.1.1. DNA Methyltransferases

Methylation of cytosines is the most common DNA covalent modification known in eukaryotes, that has been shown to play an important role in development and disease. Following the discovery of DNA methylation in eukaryotes, Holliday et al. (1975) speculated that methyltransferase enzymes need to be present in cells at all times to maintain the DNA methylation status. Initially it was speculated that two methyltransferases 'E1' and 'E2' need to exist simultaneously, where the first enzyme (E1) methylates one strand and the second enzyme (E2) completes and maintains the DNA methylation during the cell cycle. These theories have now been validated, by showing that the maintenance of DNA methylation is carried out by DNMT1, 3a and 3b. DNMT1 is most abundant during the entry into S phase and is targeted to the hemimethylated sites on the parental strand. This feature allows the correct methylation pattern to be incorporated onto the new strand, which also requires the methyltransferases DNMT3a and DNMT3b activity (Shen et al. 2014; Kishikawa et al. 2003). DNMT3L is also a member of the DNA methyltransferase family and is highly expressed in germ cells. DNMT3L lacks the catalytic domain (Kobayashi et al. 2012), however despite the absence of catalytic domain, DNMT3L acts as a cofactor regulating DNMT3a and DNMT3b activity (Zhang et al. 2010). DNMT2 targets cytosine-28, which is located at the transfer-RNA in the anticodon loop and therefore does not methylate DNA (Aquino et al. 2018).

DNMT3a and DNMT3b, as the central mediators of *de novo* DNA methylation, were shown to play a key role in cellular identity and differentiation (Yan et al. 2011). The term *de novo* methylation was initially referred to developmental associated methylation process observed in embryonic cells and fertilised gametes, where newly synthesised DNA strands or not previously methylated strands were subjected to DNA methylation (Chen et al. 2003). The remodelling of DNA methylation is carried out during the fertilisation in zygotes and primordial germ cells. *De novo* methylation determines the cell-specific DNA methylation profile that defines the functionality, and therefore may play a key role in the development of disease (Robertson et al. 1999; Yan et al. 2011). Moreover, mouse model of DNMT3a and DNMT3b knockouts have been shown to block *de novo* methylation in embryonic stem (ES) cells and early embryos, resulting in severe phenotypes and death (Okano, Bell, Haber, Li, et al. 1999). DNMT3a is a 130 kD protein located at 2p23 with several domains performing differential function in the methylation process (Robertson et al. 1999). *DNMT3a* encodes for two variants (DNMT3a1 and DNMT3a2) via alternative

splicing, DNMT3a2 is the smaller isoform found in human and mouse, and expressed predominantly in ES cells in the testes and ovaries (Chen et al. 2002). The DNMT3a2 is transcribed from the isoform initiated at the downstream intronic promoter, which results in the loss of N-terminal. The N-terminal of DNMT3a plays a regulatory role, allosterically controlling the catalytic activity of the protein (Jeltsch, Jurkowska 2016). DNMT3a mediated reaction involves several modifiers, that interacts with different domains of the methyltransferase to perform DNA methylation (Figure 1.2). Therefore, it is important to understand the underlying mechanisms and variabilities that regulate the structure and modifications of DNMT3a. The ATRX/DNMT3-DNMT3L (ADD) domain of DNMT3a is guided by the binding with histone tails; this binding can be disrupted by di/trimethylation or acetylation of K4 and phosphorylation of T11, S10 or T3 (Zhang et al. 2010). In addition, ADD domain plays a key role inducing autoinhibition of DNMT3a, and regulates the interaction with unmodified histone tails (e.g. histone H3 peptides unmodified in position K4 (H3K4me0)) in order to upregulate the enzymatic activity of DNMT3a (Guo et al. 2015). H3K27me3 has also been shown to be associated explicitly with the hypermethylated regions of DNA (Jeong et al. 2014). Collectively, these findings suggest the interaction between DNMT3a and histones is complex and require further elucidation.

Unlike DNMT1, that has high preference to hemimethylated DNA, DNMT3a and DNMT3b do not differentiate between unmethylated or hemimethylated DNA. Despite the genetic and biochemical similarities between DNMT3a and 3b, both exert different cellular functions. DNMT3a targets single copy genes and retrotransposons for methylation. Whereas, DNMT3b, targets repetitive DNA in pericentromeric satellite regions (Chen et al. 2003; Okano, Bell, Haber, Li 1999; Kaneda et al. 2004). DNMT3a was shown to be expressed at different levels in various tissue types, whereas, DNMT3b is expressed at much lower levels in majority of tissues, except for testis, bone marrow and thyroid (Xie et al. 1999). The *de novo* methylation is critical for the pluripotent cells to differentiate and carry out specific organelle related functions. Moreover, germline mutations in *DNMT3a*, have been shown to be associated with a number of pathologies (Figure 1.2) including developmental disability, intellectual disability, distinctive skeletal features and Acute myeloid leukaemia (AML) (Tatton-Brown et al. 2014). Almost one third of AML with normal cytogenetics display *DNMT3a* mutations, which have also been reported in myelodysplasia (Nikoloski et al. 2012). The predominant mutation reported is a point mutation targeting the Arg882 position in DNMT3a. According to predictive modelling of DNMT3a protein structure, this

mutation affects the DNA binding site, and therefore causes aberrant DNA methylation in the cell (Tatton-Brown et al. 2014).

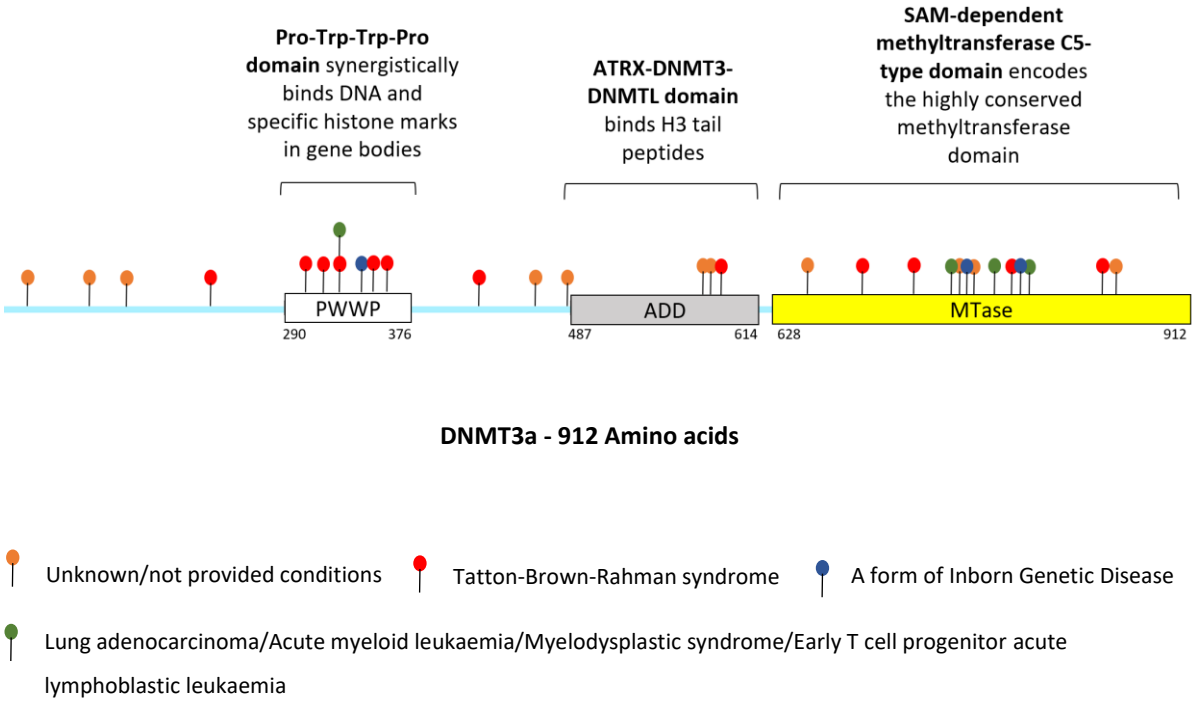


Figure 1.2: The structure of DNMT3a, and disease associated (pathogenic) mutations in the protein. The sequence and domains of DNMT3a protein and domains was obtained from NCBI (NCBI 2019)(created using SnapGene viewer), and the mutations reported in patients obtained from publicly available ClinVar database (NCBI 2019), classified as Pathogenic or Likely Pathogenic according to ACMG guidelines. Mutations identified as Unknown Significance or intronic mutations were not included in this figure. The mutations that have been associated with disease observed in patients spanned the entire protein, suggesting a highly functionally important structure. Additionally, multiple diseases were shown to be associated with DNMT3a, suggesting a diverse and crucial role of the protein. (PWWP: Pro-Trp-Trp-Pro domain, ADD: ATRX-DNMT3-DNMTL domain, MTase: SAM-dependent methyltransferase C5-type domain).

1.1.2. Methylation and Demethylation of DNA

The methylation of cytosine involves a reaction catalysed by DNA methyltransferases, where a methyl group is transferred from S-adenosyl methionine (SAM) to the fifth carbon on cytosine base within the CpG island (Moore et al. 2013). In the mammalian genome, most of the housekeeping and tissue-specific genes correspond to promoter regions with the CpG islands (Cohen et al. 2011). The methylation of cytosine residues is carried out at the 5^{th} position of the pyrimidine base cytosine (5-mC) (Taleat et al. 2015). The CpG islands are distributed unevenly across mammalian genomes, with an increasing number of genes showing 5-mC in the promoter regions. Methylation of these islands provokes long-term but reversible changes leading to transcriptional repression of the associated genes (Jones, Takai 2001). Moreover, this epigenetic feature is well conserved among most animals, plants and fungi kingdoms (Feng et al. 2010). The inactivation of mammalian X chromosome in females is mediated by epigenetics (Panning 2008). X-inactive specific transcript (*Xist*), a non-coding RNA, was shown to regulate X-inactivation. *Xist* gene promoter was found to be methylated at the inactive chromosome in ES cells of females, and this *de novo* methylation was shown to be modulated by DNMT3a (Sado et al. 2004). Another notable example of gene expression regulation by DNA methylation is the imprinting of DNA, which is dependent on paternal and maternal. During development, differential DNA methylation controls the expression of alleles in imprinted genes, resulting in the selection of either maternal or paternal copy of the gene to be expressed. Centromeres and other repeat regions may also contain methylation that can support chromosol and genome stability (Bhagavan et al. 2015).

DNA methylation is a genetically and chemically stable modification; however, it can be reversed by the maintenance-proteins. In order to reverse the methylation of cytosine, the methyl group from 5-mC is passively or actively removed (Figure 1.3). For example, ten eleven translocation-methylcytosine dioxygenase enzymes (TET) mediates 5-mC conversion in to hydroxymethylcytosine (5-hmC) (Wen et al. 2014). However, the function of this modification is currently not well understood. In addition to transcription regulation, DNA methylation can provide structural support for the centromeres and gene splicing control (Moarefi, Chédin 2011). The active method of demethylation has been suggested to be mediated by TET proteins by using α-Ketoglutarate (α-KG) as a substrate through three sequential oxidative reactions converting 5-mC to 5-hmC to formylcytosine (5-fC), and finally to carboxylcytosine (5-caC) (Wu, Zhang 2011). TET also mediates the reaction

that oxidises 5-mC into 5-hmC by hydrolysing α-KG in Fe^{2+} dependant reaction (Etchegaray, Mostoslavsky 2016). Penn et al. (1972) initially detected 5-hmC in mammals using 2D chromatography of DNA components, UV spectra in alkaline and acid solution. The analysis of tissues collected from brain and liver of mouse and frog demonstrated that 15% to 17% of cytosines were modified to 5-hmC. Moreover, high levels of 5-hmC were also been detected in ES cells (Huang et al. 2010). Additionally, evidence to support the findings of TET was provided by a computational approach to search for homologs of J base deoxygenase 1 (JBP1) and JBP2 in trypanosome species. JBP1 and JBP2 are involved in the conversion of thymine into base J (in trypanosomes). J base acts as methylated cytosines and is associated with gene silencing (Yu et al. 2007). TET proteins 1,2 and 3 were shown to have 5-mC hydroxylase activity, and both hemimethylated and methylated CpG islands can serve as a substrate for TET mediated demethylation (Wu, Zhang 2011). 5-fC and 5-caC levels are difficult to detect due to their low abundancy; however, both types of cytosine modifications have been detected by Ito et al. (2011) in the DNA of mouse ES cells. 5-fC and 5-caC may also be excised by thymine DNA glycosylase (TDG) and thereafter replaced by base excision repair (BER) protein with an unmodified cytosine (Aquino et al. 2018).

Evolutionary studies showed that CpG island functions was conserved amongst various species, suggesting a crucial role of CpG islands (Illingworth et al. 2010). The methylation and demethylation of CpG islands have been shown to modulate gene expression by multiple mechanisms, such as the interaction of DNA promoter region with histone methylation, resulting in permissive transcription chromatin state around the methylated promoter region (Inoue, Zhang 2011). Additionally, the regions on DNA harbouring CpG rich sites tend to serve as binding motifs for transcription factors, and the methylation of these sites is correlated with lower binding affinity (Prestridge, Burks 1993). Furthermore, the methylated CpG islands controls the compactness can adjust the ~~stiffness~~ found in double helix structure of DNA, which can alter nucleosome positioning and therefore regulate the binding of transcription factors as well as reducing the access and activity of RNA polymerases (Segal, Widom 2009). Methylated CpG islands have also been shown to facilitate methyl-CpG-binding proteins (MBP) leading to the recruitment of chromatin modifiers, resulting in gene repression (Joulie et al. 2010). Moreover, protein with different structural domains can contain CpG binding domains e.g. methyl binding domain (MBD), set and RING finger-associated domain (SRA) and zinc finger family domain. MBD is part of methyl CpG binding protein 2 (MeCP2), MBD1, MBD2 and MBD4. SRA is found in ubiquitin like with PHD and ring finger domains 1 (UHRF1) and UHRF2 regulatory protein, and zinc

finger domains in zinc finger and BTB domain containing 4 (ZBTB4) and ZBTB33 regulatory protein (Joulie et al. 2010). SRA and zinc finger domains allow the binding of these regulatory proteins onto methylated CpG islands and therefore controlling the gene expression. However, mice lacking proteins comprised of MBD and zinc finger domains, such as MeCP2, MBD2 and ZBTB33, did not hinder embryonic development suggesting a complex mode of expression controlled by methylated CpG islands that need to be further elucidated (Martín Caballero et al. 2009). The methylation pattern of specialised cells has been shown to play an important role in the regulation of differential gene expression. In contrast to the well documentation of CpG islands and their association with *de novo* methylation, the cues that designate the *de novo* methylation of specific CpG islands are not well-documented (Edwards et al. 2017). Therefore, further investigations are required with emphasis on the regulation of *de novo* methylation and methyltransferases to elucidate the mechanisms involved in health and disease.

A global decrease in DNA methylation accompanied by variability in the methylation pattern is termed epigenetic drift. Epigenetic drift has been evident in the ageing population. Moreover, monozygotic twin also demonstrated discordant methylation prolife with age. Based on the DNA methylation profile, the epigenetic models can be used to predict an individual's age. Interestingly, all tissue types demonstrated an epigenetic age except for embryonic and induced pluripotent stem cells, where the DNA methylation variability was zero; therefore demonstrated no sign of epigenetic ageing (Aquino et al. 2018). In stem cells, the predominant methyltransferase that maintains DNA methylation is DNMT3a (Jeltsch, Jurkowska 2016). Epigenetic ageing is associated with adverse changes in gene expression, transcriptional process, downstream protein structure and cellular function. Epigenetic ageing was associated with increased risk of age-related diseases, such as diabetes and cancer. The underline reason for this epigenetic ageing is due to the regulatory mechanisms of the DNA methyltransferases. The loss of regulatory mechanism due to epigenetic aging, can distort cellular ability to respond to environmental cues resulting in loss of epigenetic modulation (Aquino et al. 2018).

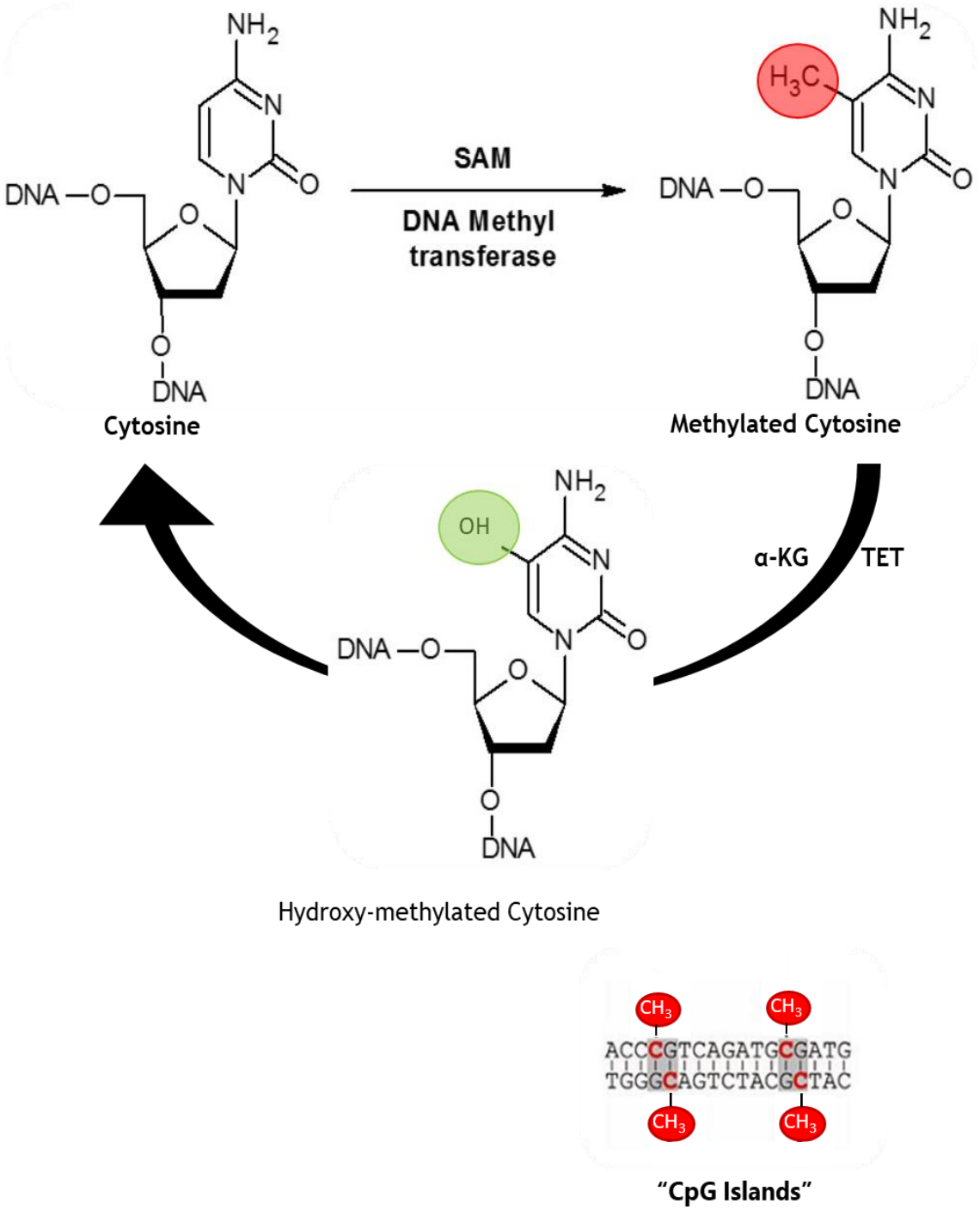


Figure 1.3: The methylation and demethylation of cytosine, carried out by DNMTs and TETs respectively. Methylation on the 5th position of cytosine base is carried out by DNMTs using S-adenosylmethionine (SAM) as a methyl donor, resulting in the production of 5-methylcytosine; cytosines targeted for methylation are generally located next to guanine and therefore forming a cytosine-phosphate-guanine island in the double stranded DNA. 5-methylcytosine can be oxidised by TET group of enzymes using α-ketoglutarate (α-KG) in the reaction and converting the methylated cytosine to 5-hydroxymethylcytosine, which is first and important step involved in the demethylation process, subsequently leading to unmethylated cytosine. These two forms of modified cytosines, i.e. 5-methylcytosine and 5-hydroxymethylated cytosine are the most stable forms of modifications of cytosine.

1.1.3. DNA methylation and pathology

In simple non-mammalian multicellular organisms, the gene expression profiles of different specialised cells are highly similar. However, in mammals, the gene expression profiles vary more than 50% amongst different tissue types. These differences are mainly induced and regulated by epigenetic modifications, such as DNA methylation, to provide the tissue its identity and function (Bergman, Cedar 2013). Additionally, population based epigenetic epidemiologic studies have shown that aberrant DNA methylation plays an important pathological role in a growing number of diseases, such as diabetes (Jin, Liu 2018). Yang *et al.*, (2003) provided one of the first substantial evidence indicating the role of DNA methylation in diabetes caused by aberrant genomic imprinting. Yang group showed that DNA methylation regulated the imprinting control (IC) region of insulin-like growth factor 2 (IGF2/H19), thereby, distorting the binding of zinc finger CCCTC-binding factor (CTCF) resulting in the downregulation of IGF2/H19 expression. The mechanisms underlying epigenetic transgenerational inheritance involves genomic imprinting. Aberrations of imprinting can result in a number of pathologies, including Prader-Willi syndrome, Beckwith-Wiedemann syndrome and types of cancers (Jin, Liu 2018).

Genome-wide methylation analysis in cancer patients have confirmed that defective DNA methylation is associated with the onset symptoms of the disease (Jelinic, Shaw 2007). Additionally, changes in the expression of DNMTs and TETs have been shown to play an important role in the pathogenesis of cancer (Cui et al. 2002). Jin and Liu (2018) stated that after the development of cancer, epigenetic modifications can cause the observed heterogeneity in patients. This heterogeneity plays an important role in drug resistance to conventional therapeutics in cancer. Moreover, autoimmune diseases, such as rheumatoid arthritis, were shown to display changes in the DNA methylation of peripheral blood mononuclear cells, where the response to anti-rheumatic drugs was also dictated by the DNA methylation profile of the patient (Liu et al. 2013; Glossop et al. 2017). Systemic lupus erythematosus, another autoimmune disease, was shown to display aberrant DNA methylation profile, and included a number of genes associated with auto-antibody production (e.g. NLRC5 gene) (Chung et al. 2015).

World Health Organisation (WHO 2019) and Centres for Disease Control and Prevention (Center for Disease Control and Prevention 2018), estimated the economic burden of cancer and diabetes to be amongst the top four diseases. The role of DNA methylation in cancer and diabetes is poorly understood. DNA methylation mediated changes, including

hypermethylation of promoter regions, global DNA hypomethylation, loss of imprinting and chromatin remodelling, contribute to the development of cancer (Estécio, Issa 2011; Gopalakrishnan et al. 2008; Yamada et al. 2005). Locus specific DNA methylation of promoter region is common in cancers, and is associated with tumour suppressor genes silencing (Gopalakrishnan et al. 2008). The aberrant DNA methylation was suggested as one of the underlying causes of pancreatic cancer, and was detected in early stages of precancerous cells development (Peng et al. 2006). Tumour initiating cells arising from stem cells, progenitor cells or dedifferentiated cells are suggested to be generated by epigenetic changes. This transformation occurs by epigenetic or genetic inactivation of tumour suppressor genes resulting in the loss of highly regulated self-renewal system and consequently initiating tumour progression (Gopalakrishnan et al. 2008).

Diabetes arises from a combination of epigenetic and genetic factors. Type 2 diabetes (T2D) is the most common type of the diabetes mellitus. Genome-wide DNA methylation studies of pancreatic islets showed aberrant DNA methylation in T2D patients, which was associated with differential gene expression profile (Dayeh et al. 2014). Obesity is another factor that correlates with increased risk of T2D, and was shown to induce aberrant DNA methylation on hypoxia-inducible factor 3 in adipose tissue (Dick et al. 2014). Moreover, CpG islands on the promoter regions containing single nucleotide polymorphisms (SNP), resulted in aberrant locus specific DNA methylation and were associated with elevated plasma triglyceride levels, consequently, increasing the risk of T2D (Toro-Martín et al. 2016). Nitert et al. (2012) investigated the DNA methylation status of muscle cells obtained from individuals that do not harbour the disease but have a family history of T2D, the outcome demonstrated differential DNA methylation profile and exhibited reduced expression of more than 60 genes, relative to control subjects without T2D or its history. The majority of these downregulated genes were also shown to be differentially methylated in T2D patients, suggesting a major role of DNA methylation in the inheritance of T2D. These findings combined suggest that the pathological role of DNA methylation is not yet clear; however, more recent studies provided evidence implicating DNA methylation in various pathologies, including cancer and diabetes.

1.1.4. Histone acetylation

DNA associate with histones to form nucleosomes. A nucleosome consists of eight histone proteins, two of each: H2A, H2B, H3 and H4, wrapped in 147 bp of DNA with high specificity. H1 is the histone linker protein that connects the nucleosomes to DNA, providing structural support, thus playing an essential role in the structure of chromatin (Gibney, Nolan 2010; Speranzini et al. 2016). Chromatin structure plasticity and dynamics play an important role in defining the cell's function and therefore its role in a multicellular organism (Speranzini et al. 2016). Histones are predominantly positively charged proteins, which supports the binding of negatively charged DNA (Gibney, Nolan 2010). Transcription factors regulate gene expression by binding to specific regions on DNA sequence around the histones. The association between histone, transcription factors and DNA can be determined by the interactions between histone amino acids and DNA (Speranzini et al. 2016). Collectively, in the context of interactions between DNA and histones, these findings suggest that investigations carried out on epigenetics of the cells need to address both, DNA methylation and histone modification.

The discovery of histone acetylation was solely associated with transcriptional activation. However, subsequent investigations showed that histone acetylation can also mediate DNA repair (Gibney, Nolan 2010; Verdone et al. 2005). The flexible histone tails can serve as a primary target for epigenetic modifications. In the process of histone acetylation, the N^{ε}-acetylation of lysine residues acquires the acetyl group, thereby neutralising the positive charge and distorting the electrostatic interaction between histones and DNA, resulting in an open-chromatin formation leading to increased DNA accessibility (Figure 1.4) (Dawson, Kouzarides 2012). Sirtuins (SIRT) are deacetylases that were originally found to regulate the life cycle in *Caenorhabditis elegans,* and also shown to play a critical role in the histones deacetylation process in mammals (Zou et al. 2017). Loss of SIRT1 was shown to increase histone acetylation, resulting in modified expression of numerous genes. However, SIRTs, are not histone specific and can deacetylate other proteins. For example, SIRT1 can also deacetylate the tumour suppressor p53, and brain and muscle ARNT-like 1 (BMAL1), which regulates mammalian circadian clock (Stünkel, Campbell 2011). In addition to SIRTs, histone acetylation status is regulated by coordinated actions of histone acetyltransferases (HAT) and histone deacetylases (HDAC). HDACs are very similar to SIRTs, and function by modulating the PTM of lysine residues on histone (Su et al. 2016). Site specific acetylation and deacetylation have been highly associated with chromatin modulation and

transcriptional competency (Keating, El-Osta 2015). There are seven known SIRT isoforms, SIRT 1 to 7 with nuclear, cytoplasmic and mitochondrial localisation with a possibility of intercompartment movement. This subcellular localisation likely dictates the specific function and targets of these deacetylases (Flick, Lüscher 2012).

The histone tails are prone to other PTMs including methylation, ubiquitination, phosphorylation, deamination, sumoylation and ADP (Adenosine diphosphate) ribosylation (Table 1.1). The histones mainly consist of basic amino acids such as lysine and arginine, which increases the possibility of PTMs, and consequently structural regulation mediated by epigenetic modifications (Gibney, Nolan 2010). Acetylation of histones is also regulated by the intracellular acetyl-CoA levels; however, the precise mechanism and fundamentals are not known. More recently, Mews et al. (2017) demonstrated a positive correlation between decreased and increased acetyl-CoA levels, and histone deacetylation and acetylation, respectively.

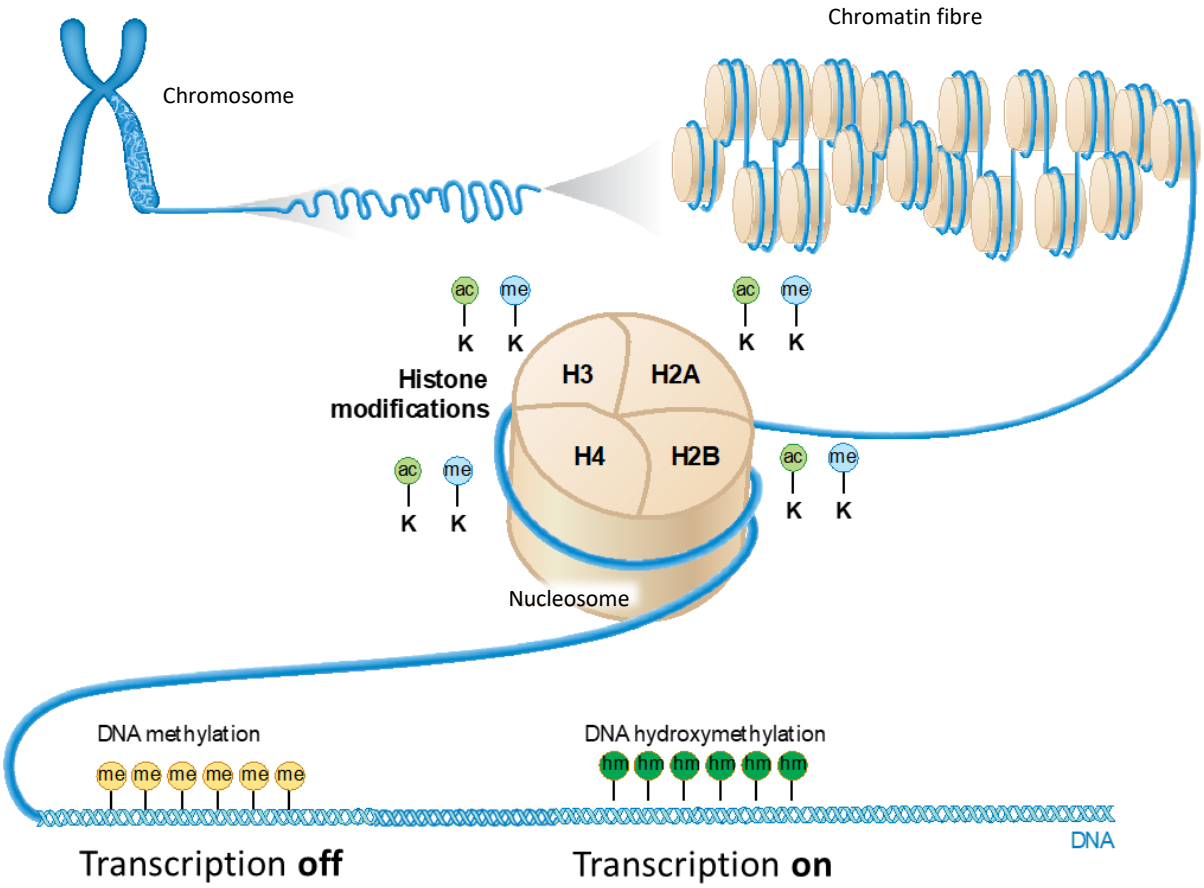


Figure 1.4: Schematic representation of the key epigenetic modifications. A model of chromatin structure from chromosome to double helix DNA is illustrated above. Acetylation of histones results in chromatin structural changes from a closed to an open conformation, increasing the DNA accessibility, mediated by the removal of positive charge in the process of acetylation, which interferes with the interaction between negatively charged DNA. Histone acetylation is carried out by HATs on the lysine residues located at the N-terminal tail, which can be deacetylated by HDACs. Acetylation and deacetylation of histones results in opposing outcomes, i.e. open and closed chromatin conformation, respectively. Histone methylation, another histone modification is targeted towards the gene transcription depending on the target amino acid. The rate of DNA methylation at the promoter region of the gene is inversely correlated with transcription, and the rate of hydroxymethylation is positively correlated with transcription.

1.2. Nutrients and epigenetics

Nutrient availability mediates the cellular adaptability, in order to sustain multiple cellular processes, including viability and proliferation. This adaptability requires the activation of specific transcriptional and metabolic programming, allowing cells to respond to the environmental changes (Sebastián, Mostoslavsky 2017). Lifestyle and environmental factors, such as exercise, diet, smoking and alcohol consumption, have been shown to influence epigenetics. Exercise combined with low-fat diet was shown to affect the metabolism, resulting in delayed ageing (Aquino et al. 2018). Additionally, epigenetic changes from short term caloric restriction diet displayed a profound effect on DNA methylation. Hahn et al. (2017) profiled global DNA methylation changes and their correlation with gene expression in response to diet restriction, i.e. reduction in intake of food without reaching malnutrition. The study demonstrated that age related DNA methylation changes were substantially reduced in individuals with reduced food intake, and the DNA methylation profiles remained unaffected. These findings outline the connection between epigenetics and ageing, and the ability of nutrients to impact epigenetics, and subsequently, health and disease. Furthermore, ageing mice showed a loss of DNA methylation and imbalance between methylated and unmethylated promoter regions of several genes (Kim et al. 2016). Mathers et al. (2008) stated that the differences in epigenetic markings may explain discrepancies observed in inter-individual disease susceptibility and response to nutritional interventions. DNA methylation can be regulated by nutrients, either globally or in a locus-specific manner. The nutrition status of a cell can affect key metabolic intermediates and therefore gene expression (Bhagavan et al. 2015). For examples, DNA methylation can be controlled by regulating the substrate SAM for DNMTs and therefore limiting the rate of enzymatic activity. During DNA methylation, DNMTs utilises SAM, methyl (CH_3) donor derived from methionine cycle, to methylate cytosine at the 5^{th} position. The methylation of cytosines at the CpG islands is influenced by the metabolic pathway of methionine, in addition to other components of this metabolic pathway, such as folic acid, vit B6 and vit B12 (Bhagavan et al. 2015). In addition, the different metabolic pathways dictating the availability of methyl donor (SAM) can consequently result in DNA methylation changes. Metabolic pathways, such as folate-mediated one-carbon metabolism and serine pathway, can also regulate methionine cycle, subsequently modulating the production of SAM (Maddocks et al. 2016). Moreover, the serine pathway regulated by histone modification, can sequentially mediate cofactor for

DNA methylation (Ding et al. 2013). In addition to regulating the cofactors used by DNMTs or TETs, and targeting the enzymes that regulate these cofactors (Zhang 2015). Malnutrition can mediate locus-specific DNA methylation changes, hypomethylation or hypermethylation, depending on target gene (Straten et al. 2010). An example of locus-specific regulation by nutrient mediated DNA methylation exists in the renal carcinoma cells, where epigallocatechin-3-gallate (nutrient found in green tea) decreased DNA promoter methylation at ~~the~~ tissue factor pathway inhibitor 2, leading to apoptosis and decreased proliferation (Ding et al. 2009; Zhang 2015). Curcumin, the major functional component of turmeric, which is commonly used in south east Asia, was found to induce global DNA hypomethylation in leukaemia cell line by inhibiting DNMT1, via covalent binding to the protein and therefore blocking the catalytic thiolate C1226 (Liu et al. 2009). S-adenosylhomocysteine (SAH) is the by-product of the methylation reaction carried out by DNMTs. Following DNMT mediated reaction, SAH can be converted into homocysteine and recycled back into the methionine cycle and subsequently converted into methionine. The ratio between SAM and SAH is maintained tightly, because SAH is a potent inhibitor for DNA methylation (Etchegaray, Mostoslavsky 2016). Moreover, TET mediated DNA demethylation uses vitamin C as a cofactor. Vitamin C enhances the activity of TET by interacting with its catalytic domain (Etchegaray, Mostoslavsky 2016). Chung et al. (2010) correspondingly reported that high vitamin C levels in human embryonic stem cells (hESC) can lead to global DNA demethylation. Chung group suggested that some of demethylated genes were involved in the differentiation process of hESC and reprogramming of fibroblasts into iPSCs.

Acetyl-CoA, an important metabolic intermediate, is the universal substrate for histone and protein acetylation, that is also a key metabolite of catabolic and anabolic reactions. Acetyl-CoA is mainly derived from glycolysis and TCA cycle, and can be generated from acetate, β-hydroxy acids and β-ketoacyl-CoA (Etchegaray, Mostoslavsky 2016). ATP Citrate Lyase (ACLY), an enzyme regulating the conversion of citrate into acetyl-CoA, has been shown to directly affect the acetylation of histones. Moreover, silencing of ACLY was shown to decrease histone acetylation levels (Wellen et al. 2009). Cai et al. (2011) correspondingly demonstrated that the availability of nutrients and intracellular acetyl-CoA levels in yeast correlated with the acetylation of histones and increased growth-associated gene expression. Other metabolites, such as butyrate, have been shown to regulate acetyl-CoA production, subsequently increasing the intracellular concentration of this metabolite and

regulating epigenetics. Butyrate, a short-chain fatty acid, is produced in large quantities by colon microbiota, mediated by diet (Etchegaray, Mostoslavsky 2016).

A number of metabolic intermediates are required for epigenetic modifications, therefore dietary intake is essential in determining the metabolic rate and intermediates, consequently modulating cellular epigenetics (Figure 1.5) (Keating, El-Osta 2015). Central metabolism and epigenetics can be modified by altering the source of nutrients, such as glucose, acetate, galactose and curcumin (Liu et al. 2009; Dott et al. 2014; Jacob et al. 1997). Galactose, a highly homologue metabolite to glucose, has been shown to enhance mitochondrial activity (Palmfeldt et al. 2009). Acetate, have also been shown to regulate energy metabolism and therefore functions of specific tissues (Jacob et al. 1997). In stem cells, the nutritional utilisation is different depending on whether the cell is in a differentiative, proliferative or quiescent state (Cavallucci et al. 2016). One of the key characteristics of stem cells includes its plasticity that is provided by the active epigenetic modifications, giving the cell its heritable developmental cues and response to the environment accordingly (Atlasi, Stunnenberg 2017). The epigenetic changes, in most cases are reversible without genetic interventions and therefore are attractive targets for future therapies (Allis, Jenuwein 2016).

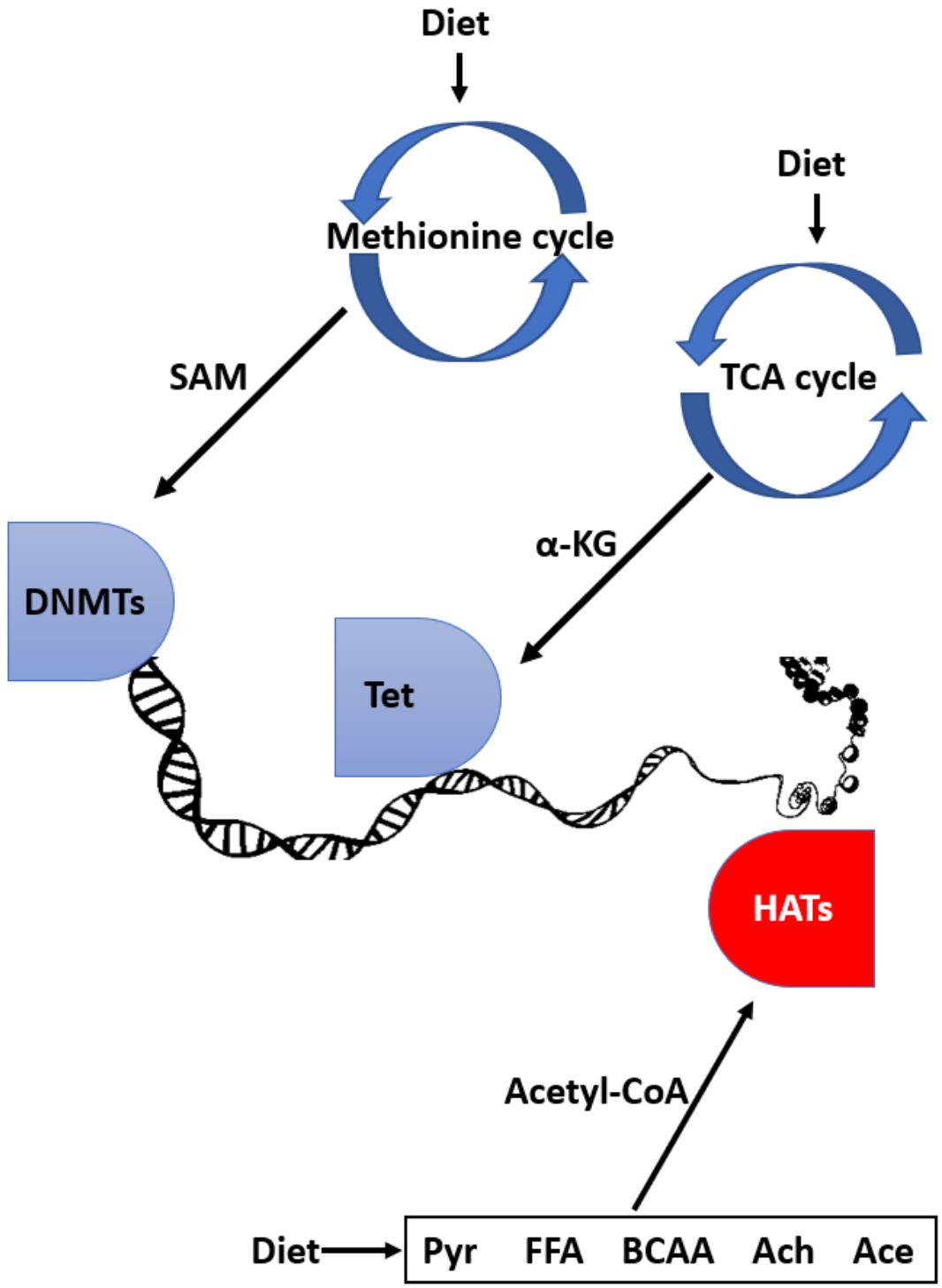


Figure 1.5: Nutrients can modify epigenetics and regulate transcription. Fatty acids, carbohydrates and amino acid metabolism can produce metabolic intermediates including acetyl-CoA; co-factor in the histone acetylation reaction. Acetyl-coA is mainly generated from oxidative metabolism of free fatty acids (FFA), glycolytic pyruvate (Pyr), branched amino acids (BCAA), Acetate (Ace) and Acetylcholine (Ach) (Pietrocola et al. 2015). Methionine, an essential amino acids found in a variety of dietary intakes can also produce S-Adenosylmethionine (SAM); a co-factor in DNA methylation reaction (Orgeron et al. 2014). α-ketoglutarate (α-KG), a co-factor in TET mediated demethylation reaction can be obtained from the TCA cycle, that has been shown to be modulated by variety of dietary intake. (DNMTs: DNA Methyltransferases, TET: Ten Eleven Translocation, HATs: Histone acetyltransferases)

1.3. Nutrients and Pathology

Historical events such as the 1944-1945 winter Dutch famine in the Netherlands (this was a 5-month period of extreme food shortage) and its implications on disease in later life have demonstrated the importance of diet in health and disease, especially during development. A study conducted by Painter et al. (2005) on subjects that were born during or immediately following this period showed lower birth weight that was associated with the development of T2D, cardiovascular diseases and obesity. Other studies have also associated the risk of cardiovascular diseases, hypertension and T2D of the offspring to mother's nutrition intake during her childhood. Moreover, the nutrition of grandmother during pregnancy has been shown to dictate the health of grandchild (Kaati et al. 2002). Collectively, these studies indicate the direct connection and long-term effect between nutrition and disease. Birth weight has been associated with the development of pathologies in adult life, and growing evidence suggests that ~62% of birth weight variations can result from an intrauterine environment including nutrients (Guilloteau et al. 2009). Conversely, 18% to 20% variation of the birth weight was shown to reside to the maternal and paternal genetic variants (Holt 2002). It is not just the nutrition intake but the time of nutrition during pregnancy that was also shown to contribute to different pathological outcomes, whereas, the nutrition restriction during early gestation results in cardiovascular disease risk, and later gestation results in increased risk for T2D (Guilloteau et al. 2009). In addition to nutrients, hormones can also largely regulate metabolism. However, hormones in large physiological quantities, are restricted from crossing over to fetus through the placenta. Therefore, the nutrients in maternal circulation, not the hormones, dictates the fetus metabolism (Guilloteau et al. 2009). These findings support the evidence of pathologies arising from changes in nutrient and metabolism. In addition to the prenatal, the postnatal effect of nutrients has also been associated with metabolic reprogramming and pathological changes. For instance, a high protein diet in new born rats resulted in insulin resistance and modified the expression of glucose transporters (Robert et al. 2009). Also, the high carbohydrate diet in neonatal rats resulted in hyperinsulinemia (Srinivasan et al. 2000).

Reprogramming of energy metabolism are one of the hallmarks of cancer and diabetes (Hanahan Weinberg 2011, Feng et al. 2016). Diabetes arises from intricate interaction between diet and genetic factors, yet the mechanism of this interplay is to be fully understood (Park et al. 2015). Metabolic deregulation is directly linked to diabetes end-

point effects, including insulin secretion (Friedrich 2012; Suhre 2014). Moreover, Warburg's observations also connect the metabolism to end-point effects observed in cancer cells, such as converting glucose mainly into lactate even in the presence of sufficient amounts of oxygen (Warburg et al. 1927). However, this metabolic feature of cancer cells has also been observed in normal proliferating T cells, suggesting an intrinsic property of proliferation (Colombo et al. 2010). Cell metabolism, such as glycolysis and glutaminolysis, is strongly co-ordinated in proliferating cells. Anaphase-promoting complex/cyclosome (APC/C) and Skp1/cullin/F-box (SCF) have been shown to regulate the degradation of cell-cycle proteins as well as glycolysis promoting proteins, such as 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, isoform 3 (PFKFB3) and the glutaminolysis pathway (e.g. Glutaminase 1, GLS1) (Moncada et al. 2012). Furthermore, the glycolysis promoting PFKFB3 enzyme is necessary for progression into S phase of the cell cycle, in a point which has previously been described as 'nutrition sensitive' (Moncada et al. 2012). In addition, sustained supply of nutrients (e.g. fatty acid and high levels of glucose) to β-cells from pancreas, leads to functional changes in insulin secretion. The utilisation of nutrients by β-cells is an essential component of their sensing system mediated glucose homeostasis. Changes in the sensing system can lead to a global alteration of the hormonal secretion, resulting in intrinsic pathophysiological consequences (e.g. diabetes development). The impact of nutrients on cellular function have provided the rationale for its characterisation, and ultimately, with the concept that interventions on these links could be used as potential therapeutic targets for cancer and diabetes.

Epigenetic-metabolomic interplay has been associated with normal physiological functions and various pathologies. For example, Purushotham et al. (2009) showed that a combination of high-fat diet and knock out of SIRT1 in mice, results in impaired fatty acid oxidation and ketogenesis leading to inflammation, ER stress and hepatic steatosis. In the same study, mice lacking SIRT1 displayed no abnormal weight gain on chow diet (grain-based diet); whereas, western diet increased the weight significantly in comparison to control mice. These findings highlight the importance of diet in the development of pathophysiological risks. In tumorigenesis, cellular metabolism and oncogenic mutations are directly or indirectly linked, resulting in metabolic reprogramming (Pavlova, Thompson 2016). The ability of cancer cells to acquire nutrients from a nutrient-deficient environment to generate biomass and maintain viability has been the focus of research for decades (Pavlova, Thompson 2016). The combination of nutrient utilisation and metabolic reprogramming leads to the phenomenon termed 'The Warburg effect' (Warburg et al.

1956). The Warburg effect state that cancer cells primarily rely on aerobic glycolysis instead of mitochondrial oxidative phosphorylation (OXPHOS), and obtain around 50% of their ATP by metabolising glucose to lactic acid (Heiden et al. 2009).

1.3.1. Mitochondrial and IDH2 regulation

Mitochondrial dysfunction is a key modification in cancer that plays a significant role in the emerging research of cancer therapies. Furthermore, common diseases including neurodegenerative disorders, obesity and cardiomyopathies, are linked to dysfunctional mitochondria (Nunnari, Suomalainen 2012). The number of mitochondria present in aerobic eukaryotes varies depending on the developmental stage and functional activity. In addition to the number of mitochondria, the shape and size also differ depending on the cell, where mitochondria can undergo fission or fusion to accommodate the volume and size requirements. The nuclear genome encodes the genes required for fission and fusion, therefore a nuclear-mitochondrial communication plays a key role in metabolic regulation (Bhagavan et al. 2015). Nuclear respiratory factor (NFR) transcriptional activity on the peroxisome proliferator-activated receptor alpha (PPARα) and mitochondrial transcription factor A (mtTFA) synchronises the mito-nuclear regulation, which are accordingly controlled by the 'master regulator of mitochondria', peroxisome-proliferator-activated-receptor gamma co-activator (PGC1α). NRFs increase the expression of mtTFA and number of other nuclear encoded subunits of TCA cycle and the complexes of electron transport chain (ETC). Thereafter, mtTFA binds to the enhancer located upstream of mitochondrial DNA (mitDNA) and increases mitochondrial activity. Mitochondrial complexes, PGC1α expression and targeted transcription factors in healthy subjects (with no indication of mitochondrial defects) showed a high correlation with maximal oxygen uptake (Rosca, Hoppel 2013). PGC1α expression and promoter activity can be increased by reactive oxygen species (ROS), resulting in increased ETC associated gene expression and cyclic adenosine monophosphate (cAMP) mediated mitochondrial and cytosolic ROS-detoxifying system. These findings indicate that moderate ROS production by mitochondrial biogenesis is important. However, ROS generated by complex I and III was shown to damage cellular components, such as DNA, lipids and proteins (Nunnari, Suomalainen 2012). In addition to the hypoxia response for adaptive stress signalling, ROS has been shown to regulate differentiation and proliferation of cells (Hamanaka, Chandel 2010).

Mitochondria regulates cellular NADH, amino acids, enzymatic co-factors, lipid synthesis, pyrimidine biosynthesis, fatty acid β-oxidation pathway and acetyl-CoA production (Figure 1.6). Furthermore, mitochondria plays an important role in metal metabolism, which is the central part of the Fe-S cluster and heme production and is essential for DNA repair and oxygen carrier machinery (Lill, Mühlenhoff 2008). The energy requirements of the cell are also met by the oxidation of proteins, lipids, carbohydrates, therefore, yielding reduced co-enzymes including $FADH_2$ and NADH. ETC of the mitochondrial respiration utilises the oxidised $FADH_2$ and NADH using four enzymatic complexes (complex I, II, III and IV). Complex IV, also known as the terminal complex of mammalian cytochrome c oxidase (COX), consists of 13 subunits, where cytochrome c oxidase subunit 1 (mtCOI), 2(mtCOII) and 3(mtCOIII) are encoded by the mitDNA forming the functional core of complex IV (Capaldi 1990). The energy in mitochondria is captured in the form of ATP, generated by a step-wise cycle combining oxygen consumption with ETC and OXPHOS. OXPHOS utilises 13 proteins encoded by the mitDNA, that are maternally inherited (e.g. mtCOII). MitDNA is another central component of mitochondria that is modulated in cancer (van Gisbergen et al. 2015). MitDNA is a double stranded circular form of 16.6 kb long DNA. The initiation of mitDNA replication and expression is regulated from the D-loop region that contains the transcription promoter and an origin of replication (Seidel-Rogol, Shadel 2002). MitDNA copies are present in human cells in large quantities ranging from 100-10,000 copies/cell. The modulated copy number of mitDNA in the cells has been shown to contribute to pathologies, including encephalopathies and neuropathies by impairing mitochondrial respiration (Reznik et al. 2016). Ishikawa et al. (2008) correspondingly reported that ‘swapping' mitDNA from highly metastasised cancer cells with cells of lower ability to metastasise, results in the reversion of the metastatic ability.

Mitochondria consist of two membranes, the outer and inner. The outer membrane is highly permeable to smaller solutes (<5 kDa). However, the inner membrane has lesser permeability, in which case the mitochondrial carriers play an important role in transportation. Mitochondrial carriers are the nuclear-encoded family of transport proteins, that regulate the flux of nucleotides, metabolites and coenzymes across the mitochondrial membrane. Because of cellular compartmentisation, metabolites need to be transported in and out of mitochondria for many metabolic reactions. Metabolites, such as citrate, malate and ATP, need to be exported out of the mitochondria to be utilised in other reactions, including the conversion of citrate into acetyl-CoA which serves as an important precursor in epigenetic modifications. Citrate carrier has also been shown to play a

significant role in histone acetylation (Palmieri, Pierri 2010). The citrate carrier is a significant component of the citrate-malate and oxoglutarate shuttles, playing a vital role in the intermediary metabolism by connecting lipogenesis and carbohydrate catabolism, in addition to epigenetics. The role of citrate carrier is to transport citrate, that is mainly derived from the breakdown of sugar sources out of mitohondria. This transportation is carried out via a voltage-dependant anion channel in a passive diffusion manner. This reaction catalysed by citrate carrier as either citrate-citrate or citrate-malate exchange (Gnoni et al. 2009). In both directions of transport (i.e. from and to mitochondria), the rearrangement of the bound form is faster than of the free form. In addition, the dissociation constant for the internal (mitochondrial) substrate is higher than of the external citrate. As a result, the citrate transport rate by citrate carrier is larger when the transport is carried out with the exchange of another substrate, e.g. malate (Palma et al. 2005).

Currently, there is no treatment available for mitochondrial related disorders, but symptom-based interventions are being used in patients. In most cases, complexities arising in mitochondrial disorders are tissue-specific and largely heterogeneous. For example, mitochondrial aminoacyl-transfer RNA synthetases defects can give rise to clinically variable conditions, including ovarian dysfunction, cerebral white matter disease, hearing loss and/or cardiomyopathies. This heterogeneity indicates the diverse role of mitochondria in each cell and tissue type. Mitochondria have been traditionally known for energy production; however, the heterogenous role of mitochondria in several diseases suggest a more diverse role within the cell that extends beyond energy production (Nunnari, Suomalainen 2012).

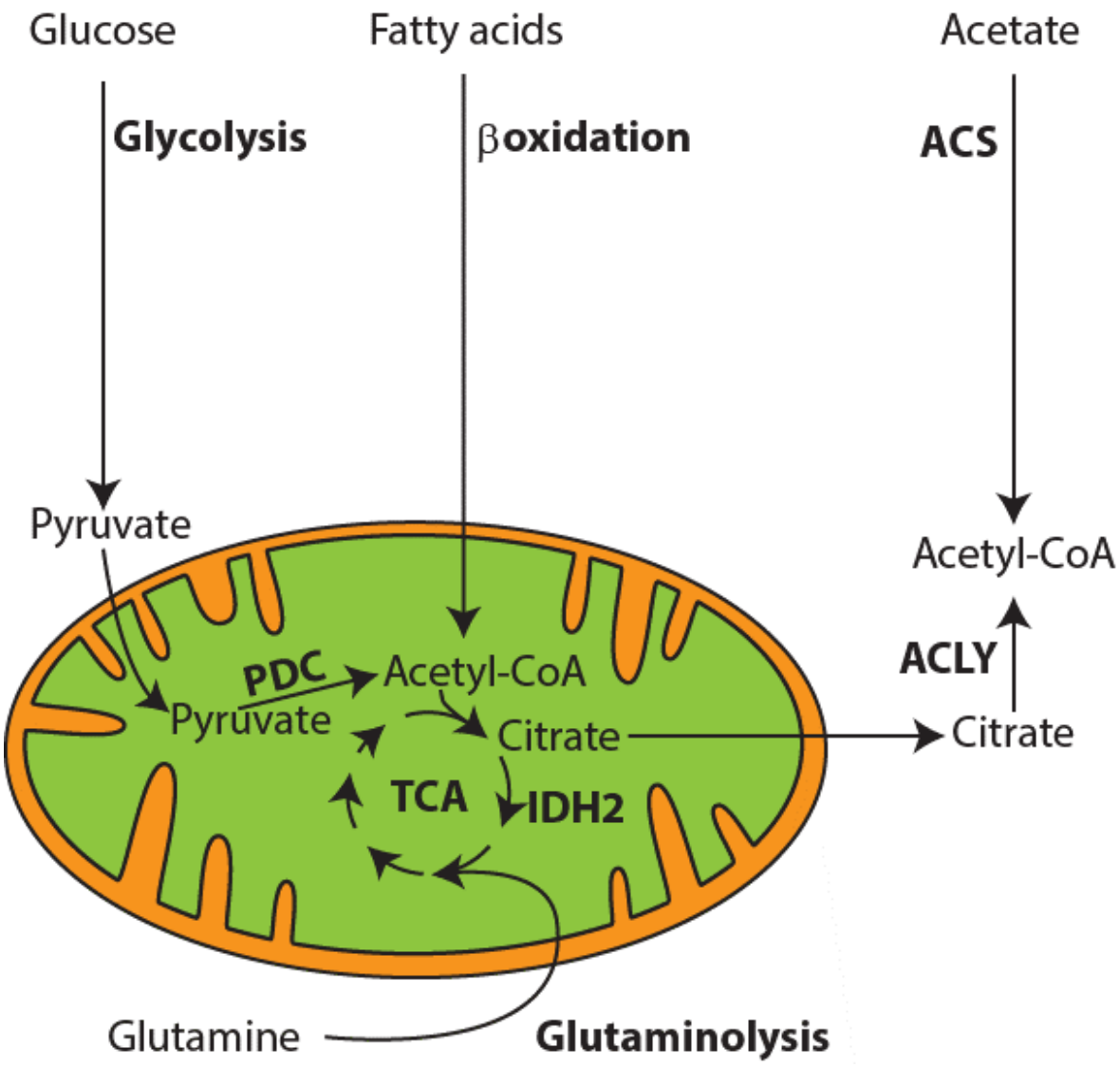


Figure 1.6: Schematic of the mitochondrial and central metabolism of cell. Glycolysis and oxidative phosphorylation are tightly linked together. In glycolysis, glucose is converted to pyruvate thorugh a number of steps (providing a net gain of 2 ATPs), which is then transported into the mitochondria. Pyruvate Dehydrogenase Complex then converts the pyruvate into acetyl-CoA. Fatty acid β-oxidation of fatty acids and acetyl-CoA esters is performed thorough a number of enzymatic reaction leading to the production of acetyl-CoA. Acetyl-CoA generated from glycolysis and β oxidation can be further oxidised by the TCA cycle, or converted into citrate and exported out of mitochondria before being utilised by IDHs: which leads to an increase in the pool of cytosolic acetyl-CoA, where citrate is converted to acetyl-CoA by ACLY conversion. Moreover, the pool of cytosolic actyl-CoA can also be directly increased by nutrients, e.g. acetate. (PDC: Pyruvate Dehydrogenase Complex, ACS: Acetyl-CoA synthetase, ACLY: ATP citrate lyase)

1.3.1.1 The role of IDH2 in mitochondria and disease

Mitochondrial-generated metabolites regulate cellular activity. In mitochondria, the metabolites are processed using the TCA cycle to produce energy through the electron carriers such as NADPH. ETC then utilises this energy in the generation of ATP. In addition, an essential part of the TCA cycle is the Isocitrate dehydrogenase (IDH) mediated reactions. Human IDHs different isoforms are encoded by *IDH1* gene located at 2q33.3, *IDH2* gene at 15q26.1, *IDH3A* gene at 15q25.1 and *IDH3B* gene at 20q13 (Al-Khallaf 2017; Bhagavan et al. 2015). The gene expression levels of either IDH1/2 vary depending on the tissue types, therefore, suggesting two distinct pathways in the TCA cycle that regulate a cell specific function. Muscle, heart and lymphocytes have shown higher IDH2 expression levels. Interestingly in T-cells IDH1 is undetectable, and only the IDH2 is expressed in resting and activated state (Al-Khallaf 2017). IDH2 supplies NADPH that is required for glutathione production against oxidative damage in mitochondria, therefore, is a key factor in the antioxidation process during oxidative stress (Jo et al. 2001). IDH2 consists of 18 beta sheets and 14 alpha helices forming homodimer (Ceccarelli et al. 2002). IDH2 is an NADP-dependant mitochondrial isocitrate dehydrogenase and is considered a tumour suppressor protein in many cancer types (YI et al. 2016). Mutations in IDH2 have been associated with multiple cancer types (e.g. R172K, R172M, R172W associated with Gliomas, R140Q, R172K associated with Myeloid malignancies, R140G, R172G, R172K, R172T with Angioimmunoblastic T-cell lymphoma, R172W with Cholangiocarcinoma and R172S with Enchondromas) (Dang et al. 2016; Yang et al. 2012). IDH2 functions as part of TCA cycle, catalysing the conversion of isocitrate to α–KG by oxidative decarboxylation (Reitman et al. 2010) (Figure 1.7). In IDH2, the interaction between Ser95 and Asp279 blocks the active site access, coversley, Mg^{2+} -isocitrate complex, located at the active site, can increase the catalytic rate of IDH2 mediated reaction. Additionally, IDH2 mediated catalysis is one of the irreversible reactions (due to the large negative free energy change) in TCA cycle, therefore it is allosterically regulated by ADP and ATP. In this reaction, ATP inhibits, and ADP positively regulates the reaction (Pelosi et al. 2016). IDH2 dehydrogenates isocitrate to oxalosuccinate and then decarboxylates to α–KG, in the process producing NADH (Akram 2014). NADPH is involved in many vital cellular functions, including fatty acid synthesis and cholesterol biosynthesis, in addition to the defence against oxidative stress (Yang et al. 2012). The isocitrate converted by IDH2 (NADP dependent enzyme) into α-KG to produce NADPH continues into the TCA cycle for other reactions, but the remaining isocitrate, that

is not catalysed by IDH2, is converted back into citrate through the reversible mitochondrial aconitase enzyme. The citrate from the mitochondria is then transported into the cytosol, where ACLY converts the citrate into acetyl-CoA to be utilised in cholesterol synthesis, fatty acid synthesis and epigenetic changes (Schug et al. 2015). Thus, IDH2 is thought to be important for cell growth due to its effect on many cellular processes and involvement in the TCA cycle.

IDH2 in primates, bats and cetaceans was shown to have a parallel evolution, because primates and cetaceans have larger brains in comparison to other species and bats locomotion of flight requires a high amount of energy. This parallel evolution indicated the importance of IDH2 in meeting the higher energy demand in different species (Ai et al. 2014). Moreover, a study carried out in *Saccharomyces cerevisiae* showed that disruption of IDH2 resulted in its incapability to utilise acetate, glycerol or lactate (Cupp, McAlister-Henn 1991). Collectively, it is unequivocal that nutrients and metabolism are inlfuencing epigenetics. This active relationship has been recognised and refered to as nutriepigenomics or nutritional epigenomics. Substainal evidence have been provided with DNA methylation analysis directly and actively correlating with the gene expression. However, locus specific DNA methylation studies are required to further extend our limited knowledge on the connection between nutrients, metabolism and the 'Readers' and 'Writers' of epigenetics (i.e. DNMTs).

Previous work from our group (Dr M Turner – Secondary supervisor) showed that *HMGCR* expression was downregulated in INS-1 cells treated with high levels of glucose and lipids (GLT). This *HMGCR* downregulation was associated with a reduction in insulin secretion. GLT and acetate have previously been shown to associate with diabetes. Moreover, glucose, lipids and acetate are known regulators of intracellular acetyl-CoA levels (Bagnati et al. 2016). In addition to being an important metabolic intermediate, acetate, contributes to unfavourable outcome in diabetes (Hameed et al. 2015). Herein, to characterise the role of acetate in the development of diabetes, INS-1 cells in acetate nutrient regime were investigated. Following the findings from INS-1 model, HeLa cells were characterised under the galactose nutrient regimes.

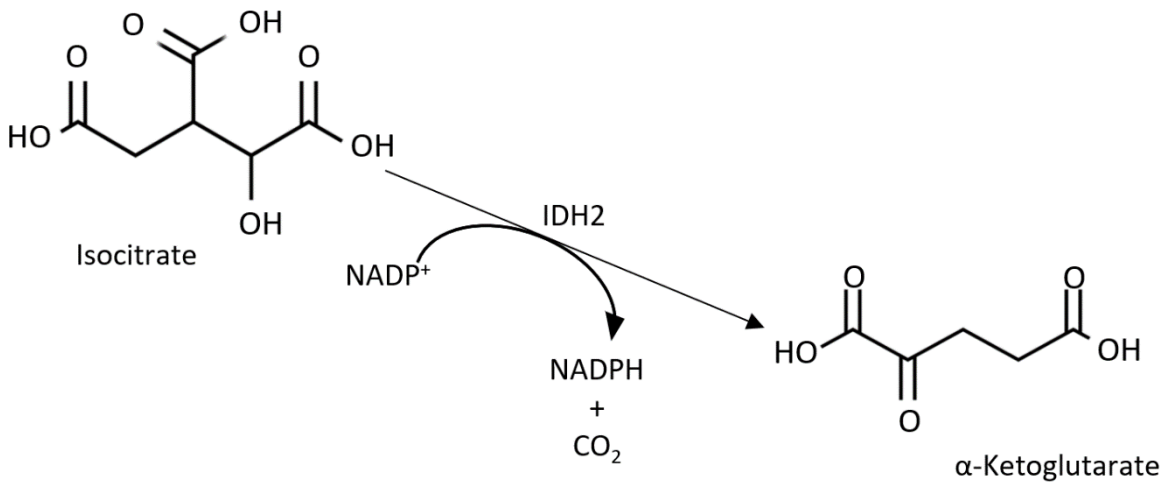


Figure 1.7: IDH2 mediated conversion of isocitrate into α-Ketoglutarate. IDH2 is located in the mitochondrial matrix, catalysing the reaction in TCA cycle. The enxymatic reaction catalysed by IDH2 converts Isocitrate to α-Ketoglutarate, with concomitant production of CO_2 and NADPH from $NADP^+$.

1.4. Aims and objectives of the thesis

1.4.1. Aims

- Assessing the role of acetate and galactose on epigenetics of selected genes (e.g. *IDH2, insulin, NIX*) in a diabetic and cancer model, respectively
- Elucidating the effect of acetate and galactose on mitochondrial function and functional consequence of the cellular models (e.g. insulin synthesis, apoptosis and proliferation)
- Characterising the modulation of cell cycle and associated proteins by acetate nutrient regime

1.4.2. Objectives

- Identifying potential mitochondrial associated genes regulated by acetate and galactose
- Studying the potential role of IDH2 in regulating metabolism in terms of lactate, ATP content and mitochondrial function
- Investigating the role of acetate and galactose in DNA methylation, histone acetylation and protein abundance of DNMT3a
- Evaluating the role of promoter DNA methylation in regulating *IDH2, insulin* and *NIX*
- Characterising the effect of nutrient regimes on DNMT3a
- Evaluating effect of genetic interventions in diabetic and cancer cellular models, including the effect of *IDH2* overexpression and dCAS9-TET mediated DNA promoter hydroxymethylation of *IDH2*
- Evaluating the role of differential nutrient regime on insulin content in the diabetic model, and proliferation and apoptosis in cancer model
- Evaluating the effect of acetate on regulating key metabolic proteins during cell cycle

CHAPTER 2: MATERIALS AND METHODOLOGY

2.1. Cell culture

Three different cell lines were utilised in this research: HeLa, HEK293 and INS-1 cell lines. These cell lines were passaged at regular intervals in T25cm^2, T75cm^2 or T175cm^2 tissue culture flasks with DMEM (Life Technologies UK) (HeLa, HEK293) or RPMI-1640 (Life Technologies UK) (INS-1) and maintained at 37°C with 5% CO_2 in humidity. Cells were collected and counted using haemocytometer and 1% trypan blue (Life Technologies UK) for protein/DNA/RNA extraction. Insulin secreting cell line (INS-1 cells) established from an x-ray induced rat transplantable insulinoma (Asfari et al. 1992) (kindly provided by Dr M Turner) were cultured in RPMI-1640 (11mM Glucose + 10% Fetal bovine serum, and 1% v/v sodium pyruvate supplemented). Human embryonic kidney cell line (HEK293 cells) transformed with sheared AD5 DNA (Thomas, Smart 2005) and HeLa cells were cultured in DMEM (25mM Glucose + 10% Fetal bovine serum supplemented).

Upon reaching 80-85% confluency, the cells were passaged. To passage, firstly the medium was removed, and cells were washed with prewarmed PBS (Life Technologies UK) twice for the removal of any residual FBS (Fetal bovine serum) (Life Technologies UK). Afterwards, 1mL of trypsin-EDTA (for T75cm^2) (Life Technologies UK) was added to the cells to ensure the complete coverage of culture flask surface area. The cells were then incubated at 37°C for 5 minutes and gently agitated. Following observations by Olympus inverted light microscope, 10 mL of media was added to the cells to neutralise trypsin-EDTA, followed by transfer to sterile 50 mL flacon tube and centrifugation at 200 x g for 5 minutes. Finally, the supernatant removed, pellet reconstituted, and cells passaged at a ratio of 1:3 (INS-1 cells) and 1:4 (HeLa and HEK293 cells). Cells were passaged for a maximum of 12 passages.

Cells were stored/preserved using Synth-a-freeze media (Life Technologies UK) following manufacture's instruction at a cell density of 2 x10^6 cells in cryovials. The cryovials were stored in -80°C freezer in a cryogenic freezing container overnight and then transferred to vapour phase of liquid nitrogen. To revive from frozen stock the cells were thawed at 37°C and added to pre-warmed media. Cells were then centrifuged and reconstituted in fresh media and replaced in a T75cm^2 flask.

2.1.1. Acetate and Galactose treatments

For acetate nutrient regime, INS-1 cells were cultured in T25cm^2 flasks for 24 hours prior to subjecting with 5mM acetate. Sodium acetate (Sigma Aldrich) was prepared as 500mM stock and filtered using 0.2µm pore size filter. Thereafter, acetate was added to cultured cells at a concentration of 5mM for 48 hours in RPMI-1640 media. For galactose nutrient regime, HeLa cells were cultured in T25cm^2 flasks for 24 hours prior to subjecting with 25mM galactose DMEM media (Thermofisher-glucose negative media supplemented with galactose). HeLa cells were incubated in galactose nutrient regime for 2 or 5 days, and cells collected using the methodology described above (section 2.1). Following the nutrient regimes, cells were harvested for total DNA, RNA and protein, as instructed by the manufacturer (Qiagen),for downstream analysis.

2.1.2. Cell transfection

pcDNA3.1 IDH2v5 plasmid was co-transfected with green fluorescent protein (GFP) using Polyethylenimine (PEI) as per manufacturer's guidelines (Figure 2.1). Prior to transfection, cells were seeded into 6 well plate at density of 3x10^5 cells/well and incubated in RPMI (INS-1) or DMEM (HEK293) media for 24 hrs to achieve 40% confluency. Cells were then transfected at 1:3 ratio of plasmid DNA:PEI (Sigma Aldrich) diluted in 250 µl Opti-MEM (Thermofisher) without serum. PEI/plasmid DNA mixture was then added to the cells and incubated at 37°C for 24 hours. Thereafter, cells were subjected to the selection process with 400 µg/mL G418 (Sigma Aldrich) for an additional 7 days, before being subjected to experiments.

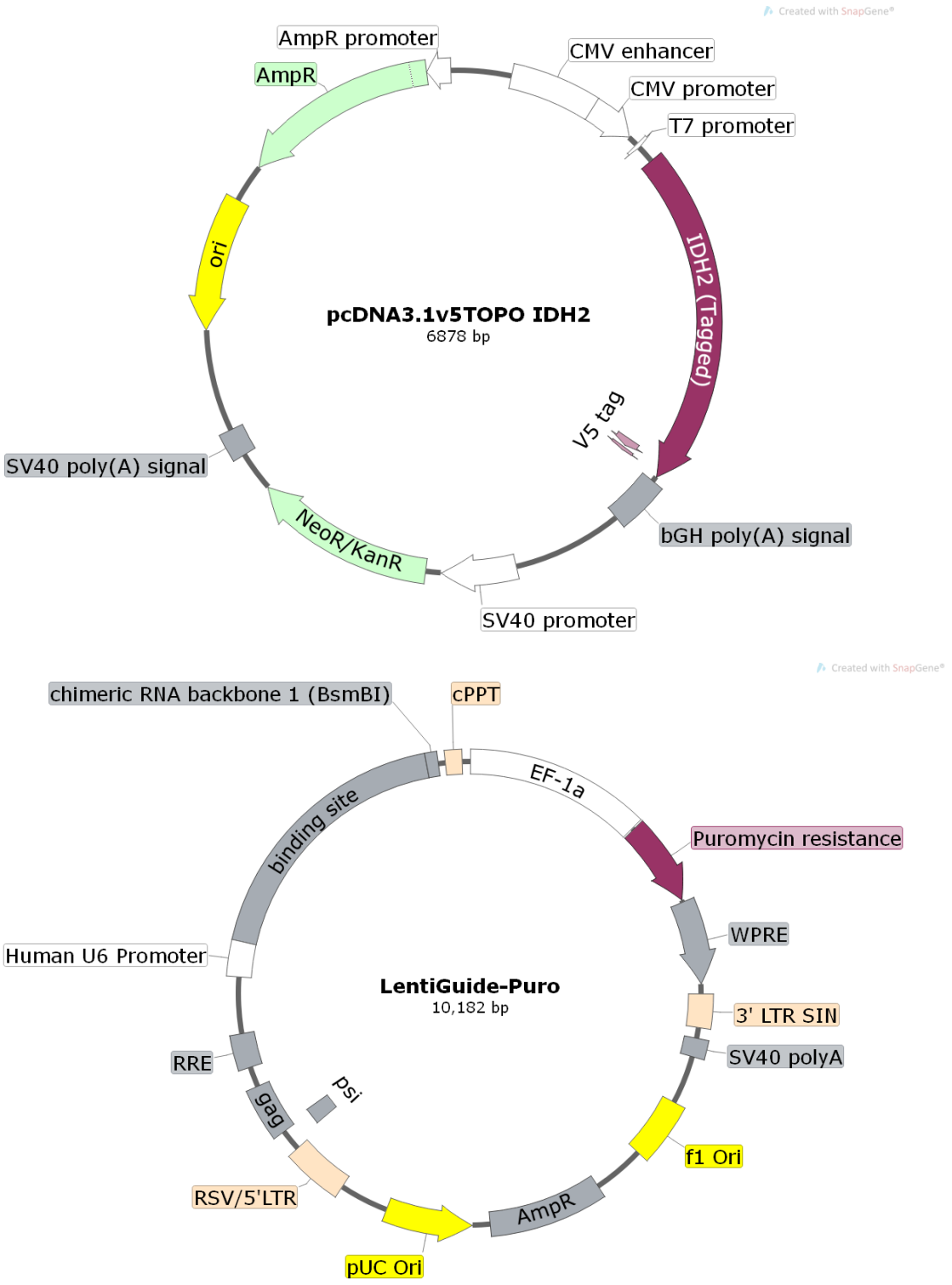


Figure 2.1: Plasmid maps indicating restrictions digest enzyme sites in pcDNA3.1 IDH2v5 and LentiGuide-Puro.

2.2. Gene expression: cDNA synthesis

Typically, the cells were grown at density of 2.5 x 10^6 under the nutrient regimes, and were used to obtain RNA, extracted using an RNAeasy minikit (Qiagen) following the manufacturer's instructions. RNA concentration and quality were analysed by Nanodrop (Thermofisher). Total RNA (1µg) was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kits (Applied Biosystem) in combination with StepOneplus 7500 (Applied Biosystem), with thermal cycler steps consisting of 10 minutes at 25°C, 2 hours at 37°C and final 5 minutes at 85°C, followed by storage at -80°C until further use.

2.3. Primer design for mRNA expression and DNA promoter analysis

The primers used in this research for mRNA expression and DNA promoter analysis were designed using NCBI primer blast, IDT primer quest and Zymoresearch seeker primer designer software. The parameters for a set of primers included optimum Tm with a maximum difference of 1°C, primer length to be ideally between 15 to 25 nucleotides and optimum amplicon size between 80bp and 140bp (except for insulin mRNA, mitDNA and DNA methylation analysis primers(Figure 2.2), where the size span was extended to 220bp). Primers were designed to target different exons, and at least one primer from the pair to span an exon-exon junction (except for primers used in DNA methylation analysis (Table 2.2)). Primers with GC content of 55% or more (exception for primers used in Bisulfite combined sequencing analysis), and maximum Self complementarity of ≤6.00, maximum Self 3'complementarity of ≤2.00 were selected (Table 2.1). The designed primers were generated by Sigma Aldrich with Synthesis scale of 0.05, Desalt purification, Dry format and diluted to 100µM in dH_20 for final use in PCR and qPCR.

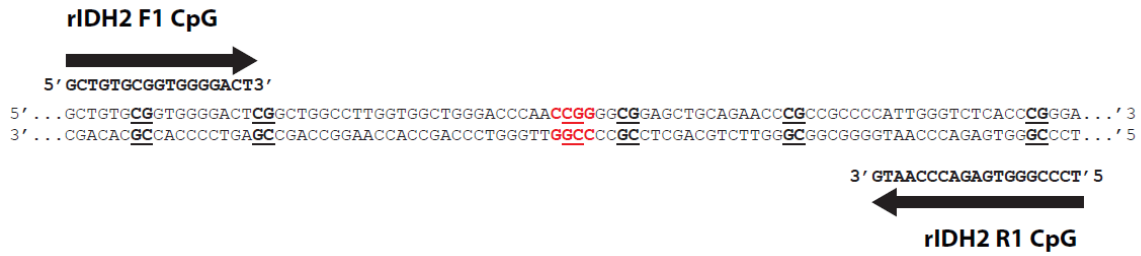


Figure 2.2: Schematic example of rat *IDH2* targeted primer for DNA methylation analysis. The underscored nucleotides represent CpG islands found in the region. In red, the CCGG domain harbouring an internal CpG island, and susceptible to digestion by HpaII and/or MspI. Arrows indicate primer binding regions.

Table 2.1: Human and rat gene expression primer sequences

Primer target	Primer sequence	Amplicon
hIDH2 mRNA NM_002168.3	AGACCGACTTCGACAAGAATAAG GACTGCACATCTCCGTCATAG	127
hBnip3 mRNA NM_004052.3	TAGCTCCAAGAGCTCTCACT CTCTCCAATGCTATGGGTATCTG	97
hGAPDH mRNA NM_001256799.2	CTAGCTGGCCCGATTTCTC GATGGCAACAATATCCACTTTACC	122
hTet2 mRNA NM_001127208.2	AGGTTTGGACAGAAGGGTAAAG CGAACCACCCACTTAGCAATA	104
hLDHA mRNA NM_005566.4	AGATTCCAGTGTGCCTGTATG ACCTCTTTCCACTGTTCCTTATC	108
hmitDNA* NC_012920.1	CACCCAAGAACAGGGTTTGT TGGCCATGGGTATGTTGTTA	108
hnuc B2-mg* NM_004048.3	TGCTGTCTCCATGTTTGATGTATCT TCTCTGCTCCCCACCTCTAAGT	86
hNix mRNA NM_004331.3	GGAAGTCGAGGCTTTGAAGAA GTTTAGGGTGTCTGAAGTGGAA	101
rDNMT3a mRNA NM_001003958.1	ACAGAGAAACCCAAGGTCAAG GGCTCCCACATGAGATACAAA	115
rHMGCR F NM_013134.2	AAGAGTCGCTGTGTTCATCTC AAACTGCTCCCACACTCTAAG	143
rLDHA mRNA NM_017025.1	TGGCTTGTGCCATCAGTATC CCATCATCTCTCCCTTTAGCTTATC	93
rHKII mRNA NM_012735.2	ACTCAGCCCAGAACTCCTTA GCCTTCTCGATTCCATCCTTATC	87
rHKIV mRNA NM_001270849.1	GGAAGACCTGAAGAAGGTGATG TCCCAGGTCTAAGGAGAGAAAG	108
rSlc16a1 mRNA NM_012716.2	GACCAGTGAAGTGTCATGGAT CTGGACGGCTGCCATATTTA	101
rIDH2 mRNA NM_001014161.1	CACTACCGAGAACACCAGAAG AGAGTCTGTGCAAACCTGATG	134
rInsulin mRNA NM_019129.3	CCCTAAGTGACCAGCTACAATC GGACTTGGGTGTGTAGAAGAAA	217

rmitDNA* **KF011917.1**	CCTCCCATTCATTATCGCCGCCCTTGC GTCTGGGTCTCCTAGTAGGTCTGGGAA	211
rNuclear DNA* **NC_005100.4**	TCCTCAAAGCAGCAGTAAGAC AGGGATTAAGGCGACGTTTC	101

*Primer sequence obtained from Rooney et al. (2015)
(r=Rat, h=Human)

Table 2.2: Human and rat DNA methylation analysis primers

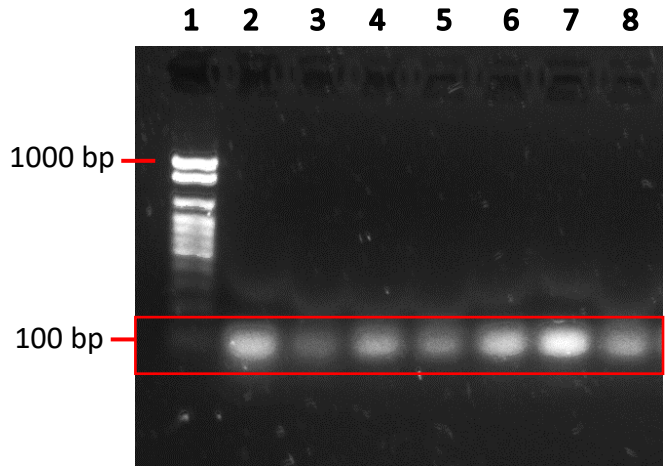
Primer target	Primer sequence	Amplicon
rIdh2 DNA **NC_005100.4**	GCTGTGCGGTGGGGACT TCCCGGGTGAGACCCAATG	92
rInsulin DNA **NC_005100.4**	CCAGAACCTGTTTCCCAAAGT GTGCTTGATCTCTCCTGTTCTC	92
hIdh2 Primer 4 F **NC_000015.10**	CCATGTAAACCTGGATGTCAGT TAATGCGCCTTTGGGTTCA	108
hBnip3CpG nF1 **NC_000010.11**	CCACTAGCAGGATGGAAAGAC GCCGGGTTCTCCTTTGAA	112
hNixV2 CG P1 F **NC_000008.11**	TCTTCTATTTACCAGCACTTGATACTAC CTGAGCTTCAGCTTCCTCATTT	93
BiSulfite-rIDH2 DNA**	GGTTGTTTYGGTTAGTTGGGGATATTTAGTGTAG CCCRAACTCTAAAACAAACCCAAAACTATC	298

**Primer sequence designed using ZymoResearch Primer design seeker
(r=Rat, h=Human)

2.4. Polymerase Chain Reaction (PCR)

2.4.1. PCR, Gradient PCR and gel electrophoresis

Primer efficiency and optimum annealing temperatures were determined using gradient PCR and gel electrophoresis analysis (Figure 2.3). PCR reactions were performed using Taq 2x master mix (NEB) on a StepOnePlus 7500 (Applied Biosystems) with PCR cycling conditions of 30 second at 95°C (Initial denaturation), 30 cycles (denaturation, annealing and extension) of: 20 seconds at 95°C, 20 seconds at 59°C-62°C (depending on primers) and 20 seconds at 68°C. Followed by the final extension for 5 minutes at 68°C. The PCR product was then resolved on 1.5% agarose gel (1.5g of agarose in 100mL of TBE buffer 1x with 10µl of SYBR safe-Invitrogen), and separated DNA bands were observed using Syngene InGenius3 Gel documentation system.



1: Ladder, 2: 55°C, 3: 56°C, 4: 57°C, 5: 58.5°C, 6: 59.5°C, 7: 60.5°C, 8: 62°C

Figure 2.3: Gel electrophoresis analysis of gradient PCR carried out on rat *HMGCR* primers. Lanes 2-8 represent different annealing temperatures analysed for optimum amplification. The data obtained showed highest specificity at temperature 60.5°C for annealing.

2.4.2. Real Time quantitative PCR (RT-qPCR)

Following primer validation by gradient PCR, qPCR analysis was performed using Quantinova SYBR green master mix (Qiagen) on a StepOnePlus 7500 with qPCR cycle design consisting of 2 minutes at 95°C (Initial denaturation), 40 cycles (denaturation and combined annealing/extension) of: 5 seconds at 95°C and 15 seconds at 59°C-62°C (depending on primer). Followed by melting curve analysis consisting of 0.3°C/sec (changed from the default of 1°C/sec) rise in temperature to obtain a higher resolution of specificity and identity of qPCR product. Low concentration of ROX dye (passive reference dye) was used in the master mix (1:10 ratio) and pre-defined in the qPCR protocol in StepOnePlus 7500 for the adjustment of background fluorescence in order to obtain normalisation of fluorescent signal. Expression of target genes was quantified and normalised to the internal housekeeping control GAPDH/18s, using $2^{-\Delta\Delta Ct}$ comparison method, and results were presented as fold change relative to the control condition. Internal qPCR primer specificity was determined using melting curve analysis.

2.4.3. Mitochondrial DNA copy number analysis by qPCR

Optimised primer sequences for mitDNA analysis were obtained from Rooney et al. (2015). human mitDNA primers were normalised to human nuclear DNA primers, and rat mitDNA primers were normalised to rat DNA primers.

2.4.4. Statistical analysis of RT-qPCR

Initially, GAPDH and 18s reference genes were analysed to be used for statistical comparison of gene expression. The housekeeping gene with least standard deviation and closest ct value to target gene were selected. Statistical analysis of $2^{-\Delta\Delta Ct}$ values was performed using GraphPad PRISM 7, where student's t-test was performed on the data obtained from two groups (e.g. control vs acetate treated) and one-way ANOVA analysis for the comparison of data obtained from multiple groups (e.g. control vs galactose treatment post 2 days vs galactose treatment post 5 days). P values obtained from the statistical analysis of <0.05 were considered to be statistically significant.

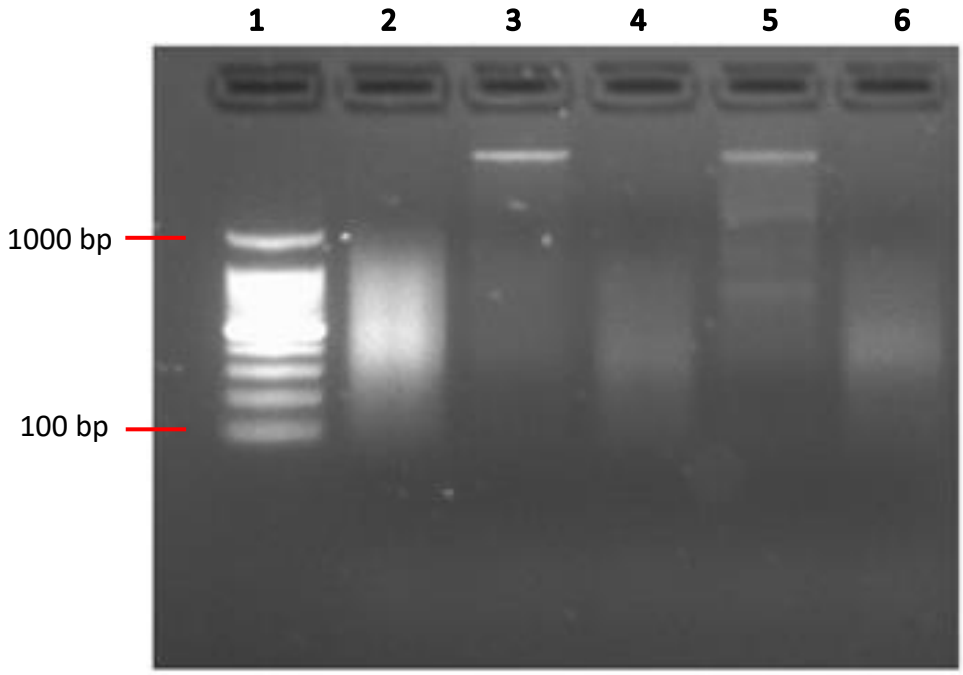
2.5. DNA Methylation analysis

2.5.1. Me-DIP

CpG islands on *IDH2, NIX, BNIP3* and *insulin* promoter regions were identified using Genome Browser (GB) and specific qPCR primers sets were designed based on regions containing high frequency of CpG sites, both embedded or not, in CCGG motifs (Table 2.2). For methylation analysis, DNA was extracted using DNAeasy Kit (Qiagen) from cells under different nutrient regime. Afterwards, DNA was immunoprecipitated using 5-mC rabbit mAb (monoclonal antibody – Cell Signalling Technology) followed by qPCR determination to confirm promoter methylation of *IDH2* and *insulin*.

2.5.1.1. DNA Sonication

The first step of Me-DIP analysis was to sonicate the DNA. 5µg of genomic DNA was eluted in 130µl buffer TE and placed in microtube (Covaris). The microtube was then placed in dH_20 filled S-Series Covaris tank for acoustic shearing. To achieve the target peak of 300 bp, sonication was carried out for different durations (40, 55, 80 seconds) at 140W (Peak incident power) 10% duty factor and 150 – 250 cycles per burst. Subsequently, fragmentations of DNA were analysed by 1.5% agarose gel electrophoresis and visualised using Syngene InGenius 3 gel imager (Figure 2.4). Adequate sonication setting was selected according to gel electrophoresis, and optimised conditions were used in the experimental repeats.



1: Ladder, 2: Sonicated DNA for 80 seconds, 3: Non-sonicated DNA, 4: Sonicated DNA for 55 seconds, 5: Non-sonicated DNA, 6: Sonicated DNA for 40 seconds

Figure 2.4: Gel electrophoresis analysis of sonicated DNA for 40 – 80 seconds. 5µg of DNA was sonicated using Covaris S-series at 140W and 150-250 cycles per burst. ~300ng of DNA was loaded onto the 1.5% agarose gel and analysed alongside non-sonicated DNA. The desired size of sheared DNA (400-700 bp) with the highest intensity was obtained from 80 seconds bursts.

2.5.1.2. DNA Immunoprecipitation and qPCR

For immunoprecipitation, protocol from the manufacturer was followed (NEB – MeDIP Kit). Briefly, DNA was denatured to obtain single stranded product and incubated with mAb against 5-mC for immunoprecipitation (IgG mAb Isotype control was used as a negative control – Cell Signalling Technology). Initially, 5-mC antibody or IgG Isotype control was added to samples and incubated overnight at 4°C. Thereafter, ChIP grade magnetic beads (Cell Signalling Technology) in combination with magnetic separation rack was used to precipitate antibody bound DNA (5-mC rich DNA fragments). The elution of DNA was carried out using Thermomixer at 65°C for 30 minutes. Beads were separated by magnetic rack, and eluted DNA obtained. Once the DNA was purified and isolated from mAb and beads, qPCR quantification was carried out. An 10% DNA input reaction was carried out alongside as a control measure to assess immunoprecipitation efficiency determination, in addition to IgG isotype control and qPCR negative control (without DNA) (Figure 2.5).

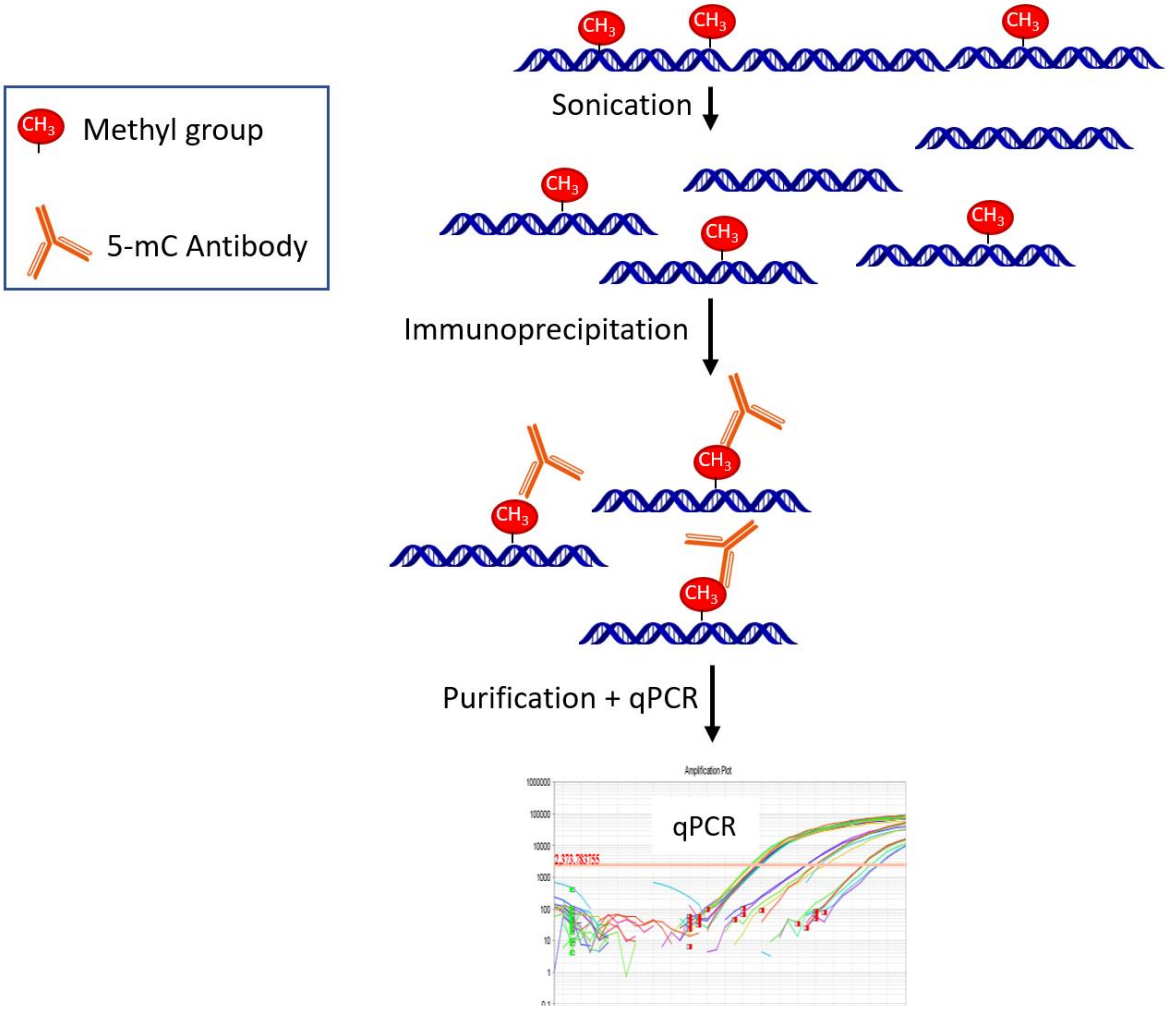


Figure 2.5: Overview of MeDIP combined qPCR methodology for the analysis of DNA methylation (5mC). Initially, genomic DNA is sonicated into fragments of ~300bp and denatured into single strands. Antibody directed against the methylated cytosines is then employed to immunoprecipitate 5mC using magnetic beads. Subsequently, purified DNA is quantified using qPCR, with primers specific for the target genes.

2.5.2. DNA methylation analysis by SREMA

DNA methylation assessed using sensitive restriction enzyme methylation analysis (SREMA) was carried by digesting 1µg of DNA with the methylation-sensitive enzyme HpaII (5 Units) (NEB) (isolated from E. coli strain carrying the gene from Haemophilus parainfluenzae) for 5 hrs at 37°C. HpaII, is a sequence specific restriction enzyme that cleaves the C^CGG sites when the internal cytosine in CCGG motif is unmethylated. Following HpaII digestion, the enzyme was heat-inactivated at 85°C for 20 minutes. Then, the resulting fragments were analysed by qPCR, assessing a subset of gene promoter CCGG sites in the genes (*IDH2, NIX, BNIP3* and *insulin*). Finally, promoter methylation status was normalised to the quantification of a region of DNA lacking the CCGG motifs, and data presented relative to control condition.

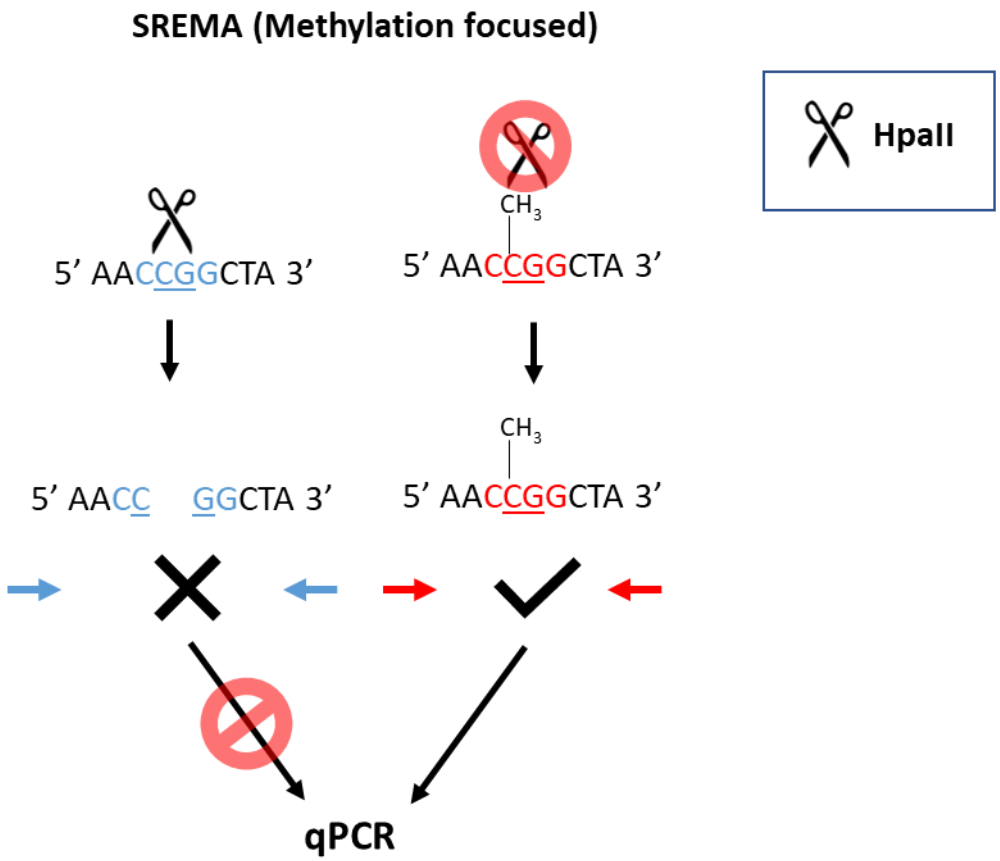


Figure 2.6: Overview of SREMA combined qPCR methodology for the analysis of DNA methylation. HpaII is a methylation-sensitive enzyme that targets CCGG nucleotide sequence; which will cut the CCGG sites only in the absence of DNA methylation, whereas the action of this enzyme is blocked by methylated cytosines. The product from the HpaII restriction digest reaction is then quantified using qPCR, with primers targeting specific gene promoter region.

2.5.3. DNA Hydroxymethylation analysis by SREMA

Hydroxymethylation status of *IDH2* and *NIX* was analysed by modified SREMA analysis, using differential methylation analysis employing isoschizomers MspI and HpaII restriction enzymes. Genomic DNA from HeLa was initially treated with T4-BGT: converting the hydroxymethylated CpG sites to glucosylated CpG sites (5-ghmC) in a sequence independent manner, which in turn prevents cleavage by MspI. MspI acts in a sequence-dependent manner (C^CGG), cleaving 5-mC and 5-hmc, but not 5ghmC. Whereas, HpaII cleaves only unmodified cytosine, but not 5-mC, 5hmC and 5-ghmC. Following MspI and HpaII digestion, the enzymes were treated with proteinase k. Then, the resulting fragments were analysed by qPCR, assessing a subset of gene promoter CCGG sites in the genes (*IDH2* and *NIX*). Finally, promoter hydroxymethylation status was normalised to the quantification of a region of DNA lacking the CCGG motifs, and data presented relative to control condition.

2.5.4. DNA Methylation analysis by Bisulfite combined Sequencing

2.5.4.1 Bisulfite combined sequencing and primer design

Bisulfite treatment of the DNA converts the unmethylated cytosines to uracil and then to thymines in the PCR reaction. Therefore, in order to design the primers according to the converted DNA sequence, Zymoresearch bisulfite primer seeker software was utilised. The first primer in the pair of primers was designed to target the bisulfite converted sequence and the second primer in the pair is designed towards the sequence of the amplicon generated by the first primer. Due to lesser GC content in the converted DNA, the primer Tm was compensated by increasing the length of the primers. Bisulfite conversion of DNA from cells was carried out using EpiMark Bisulfite conversion kit (NEB) following manufacturer's instructions.

2.5.4.1 PCR and purification of amplicon

Following bisulfite treatment, the converted DNA was then used as a template and amplified using PCR with specific bisulfite primers. PCR product was resolved on 1.5% agarose gel using electrophoresis. PCR amplicon was purified from agarose gel using QIprep

miniprep (Qiagen), following manufacturer's instruction. The purified product was analysed using Nanodrop, subsequently sequenced by Sanger sequencing. Data from sequencing was analysed using TIDE software.

2.6. Protein extraction and determination

Proteins were extracted using RIPA lysis buffer (Thermofisher) supplemented with Protease inhibitor (Roche Applied Sciences). Following the appropriate nutrient regime incubation and pelleting (as explained above), the cells were resuspended in PBS and counted using haemocytometer. $5x10^4$ cells were transferred to 1.5mL Eppendorf tube and centrifuged at 200 x g for 5 minutes. Thereafter, cells were resuspended in 400µl of cold RIPA buffer and incubated on ice for 30 minutes. Cells were then centrifuged at 14,000 x g for 30 minutes to pellet the cell debris, and protein containing supernatant was then collected. Protein determination was carried out using BCA Assay Kit (Thermofisher) and Bio-Rad iMark Microplate absorbance reader. BSA standards were used to generate a standard curve and determine protein concertation in a 96-well plate. 25 µl of collected samples were added to each well in triplicates, followed by addition of 200 µl BCA working reagent. Finally, the 96-well plate was incubated for 30 minutes at 37°C and colourimetric analysis carried out at 595nm.

2.7. Western blotting analysis and Densitometry

For western blotting, 20µg of proteins from appropriate samples were subjected to 95°C heating for 5 minutes with laemmli buffer (NEB) and separated by SDS-page on Bio-Rad gradient pre-cast gels. Separation of proteins was achieved by running the gel at 150 volts for 1 hour. After the resolution by SDS-Page, proteins were transferred onto PVDF-membrane (BioRad) (activated in methanol) using Bio-Rad Trans blot. The membrane was then incubated in 10% blocking buffer for 1 hour at room temperature. Thereafter, the membrane was incubated with the appropriate primary antibody overnight at 4°C (Table 2.3). On the subsequent day, the membrane was washed three times in TBST and incubated with appropriate secondary antibody at room temperature for 1 hour, followed by three more washes with TBST (10 minutes each). Lastly, the membrane was visualised using Bio-

Rad ECL detection reagent on LAS-3000 Fuji Imager. Subsequently, the process was repeated for control (e.g. GAPDH-Sigma Aldrich) protein expression analysis.

The image obtained from Fuji Imager was uploaded to ImageJ and converted to 8-bit format. Appropriate sections were selected using 'Rectangular' selection tools and plot profile formed. The background intensity was subtracted from the selection by 'Straight line' selection. Following the selection, intensities of the area were measured with the 'Wand' tool and data obtained analysed as a fold change.

Table 2.3: Antibodies used for Western Blot analysis

Target	Source	Dilution
IDH2	Protein Tech	**1:500**
DNMT3a	Santa Cruz Biotechnology	**1:1000**
GAPDH	Abcam	**1:10,000**
mtCOI	Abcam	**1:1000**
mtCOII	Abcam	**1:1000**
NIX	Santa Cruz Biotechnology	**1:500**
Bnip3	Cell Signalling Technology	**1:500**
β-actin	Abcam	**1:10,000**
Acetylated Histone 3	Active Motif	**1:1000**
Acetylated Histone 4	Active Motif	**1:1000**
Histone 3 Total	Active Motif	**1:500**
Histone 4 Total	Active Motif	**1:500**
Cdh1	Calbiochem	**1:1000**
Acetylated IDH2	GeneTel Laboratories LLC	**1:1000**
5-mC	Cell Signalling Technology	**1:50**
IgG	Cell Signalling Technology	**1:50**

2.8. Cell cycle co-ordination and Flow cytometry analysis

HeLa cells were coordinated with 0.1 µg/mL Nocodazole (NCZ) for 14 hours to achieve cell cycle synchronisation, thereafter, the cells were released in standard DMEM media. In order to assess the effect of acetate on cell cycle, the coordinated HeLa cells were released in either glucose or 5mM acetate media. The cells were then collected at 0, 4, 8 and 12 hours post-release from NCZ. Flowcytometry analysis was carried out by pelleting NCZ coordinated 1×10^6 cells and fixing at 80% methanol at −20 °C for overnight. Thereafter, cells were removed from methanol by centrifugation, and resuspended in 50 µg/mL propidium iodide (PI) containing PBS. 200 µl of HeLa cells (fixed and stained) were diluted as 1:5 in PBS prior to subjection onto flow cell to obtain $1x10^6$ cells/mL concentration. Cell cycle analysis was carried out with Merck MUSE analyser by quantifying the populations in G_1, S and G_2/M, following manufacturer's instructions.

In order to determine the protein content and acetylation during cell cycle. The coordinated HeLa cells were subjected to protein extraction (as describe in section 2.6) at 0, 4, 8 and 12 hours post release into DMEM. The extracted proteins were then analysed by western blotting.

2.9. Metabolite determination

2.9.1. ATP assay

To determine ATP levels of INS-1 cells treated w/wo 5mM acetate, a Firefly luciferase (Photinus pyralis) based assay was employed. Protocol from the manufacturer was followed (PerkinElmer – ATPlite assay). Briefly, 100µl of cells alongside 'Mammalian cell lysis solution' were added to 96 well plate to lyse and stabilise. After adding the substrate solution consisting of Luciferase, the plate was 'dark' adapted for 10 minutes, and finally the luminescence was measured. Data obtained was then normalised to proteins per µg (determined using BCA kit as described above).

2.9.2. Lactate assay

HeLa and INS-1 cells were grown in 6 well plate at $1x10^6$ cells/well in DMEM for 24 hours. Thereafter, cells were subjected to the appropriate nutrient regime. Following treatment of cells with acetate (INS-1 cells) or galactose (HeLa cells), the culture media was collected. Lactate assay (Trinity Biotech) was utilised in the analysis of lactate production by the cells under different nutrient regime. The assay was carried out in 96 well plate, generating a standard curve by lactate reagent and standards provided with the kit; where lactate oxidase converted the lactic acid to pyruvate and hydrogen peroxide, which was then measured. 10µl of the sample was added to plate alongside lactate reagent and incubated for 10 minutes at room temperature, thereby reaction was analysed at 540 nm on Bio-Rad iMark microplate reader. The data collected was 'Blank' adjusted and normalised to protein per µg.

2.9.3. α-Ketoglutarate assay

Intracellular α-KG levels of INS-1 (w/wo acetate) and HeLa (w/wo galactose) were measured using α-ketoglutarate Assay Kit (Abcam). Cells were seeded at a density of $1x10^5$ cells/well in 6 well plate under the nutrient regimes. Cells were then washed with cold PBS (stored at 4°C before use) and resuspended in assay buffer on Ice. After centrifugation supernatant was collected and subjected to deproteination by Perchloric acid (4M and ice cold). Thereafter, samples added to a 96 well plate alongside reaction mix and fluorescence of the cells was examined using BMG Labtech ClarioSTAR at Ex/Em 535/587.

2.10. Apoptosis and Necrosis assay

Apoptosis and Necrosis of HeLa cells was evaluated using Apoptosis/Necrosis kit (Abcam) utilising apoxin green for apoptosis and 7-AAD for necrosis. Cells were seeded at a density of $1x10^5$ cells/well in 96 well w/wo 25mM galactose for a total of 5 days. Culture media decanted and cells were washed with assay buffer. The cells resuspended in 200 µl of assay buffer and subjected to 2 µl Apopxin green, 1 µl of CytoCalcein and 1 µl of 7-AAD. Thereafter, cells were incubated at room temperature for 1 hour and the assay buffer was replaced. Finally, the fluorescence of the cells was examined using BMG Labtech ClarioSTAR at Ex/Em 490/525 (apoptosis) and 550/650 (necrosis).

2.11. Proliferation assay

Proliferation analysis of galactose treated HeLa cells was evaluated using WST-1 assay (Sigma Aldrich), following manufacturer's instructions. Briefly, cells were seeded at a density of $1x10^5$ cells/well in 96 well w/wo 25mM Galactose for a total of 5 days. WST-1 was added and incubated at 37°C. Cell proliferation analysed after 1 hour of incubation with Bio-Rad Microplate reader at 415nm.

2.12. Insulin content analysis by ELISA

INS-1 cells were passaged in 6-well plates at the density of 1×10^6 cells/well and grown for 24 hours prior to the differential nutrient regime (i.e. acetate). After 24 hours of acetate treatment, 2 mL of Krebs-Ringer Modified Buffer (KRB) consisting of 20mM KCl and 400mM NaCl was added for 2 hours directly to the existing 2 mL of RPMI media and incubated at 37°C with 5% CO_2. Following the completion of 2-hour incubation, the media was replaced with standard RPMI or RPMI+5mM acetate. Total insulin content was quantified using an Insulin ELISA kit (Mercodia). Cells were lysed as described before with RIPA, and clarified supernatant was used for insulin content determination using anti-insulin antibody coated ELISA plate. Insulin content was normalised to protein concentration, using BCA kit (Thermofisher).

2.13. Modulation of hydroxymethylcytosine levels in the IDH2 promoter by dCAS9-Tet1CD

Fuw-dCas9-Tet1CD, a gift from Rudolf Jaenisch (plasmid # 84475, (Liu et al. 2016)) and lentiGuide-Puro, a gift from Feng Zhang (plasmid # 52963, (Sanjana et al. 2014)) were obtained from Addgene. Suitable guidance RNA sequences targeting the *IDH2* promoter (Figure 2.7) region were designed using the online tool E-CRISP (E-crisp.org, (Heigwer et al. 2014)). Selected candidate sequences were cloned into the pLentiPuroGuide following instructions described by Sanjana et al. (2014). HEK293 cells were transfected with a combination of both plasmids (or alternatively with an empty pLentiPuro-guide in control cells) using a PEI protocol as outlined in section above. Finally, transfected cells were selected for 48 hours in 1 µg/mL of puromycin and then, used for DNA and proteins

analysis. Hydroxymethylcytosine content was evaluated using an SREMA 5-hmC protocol as described above.

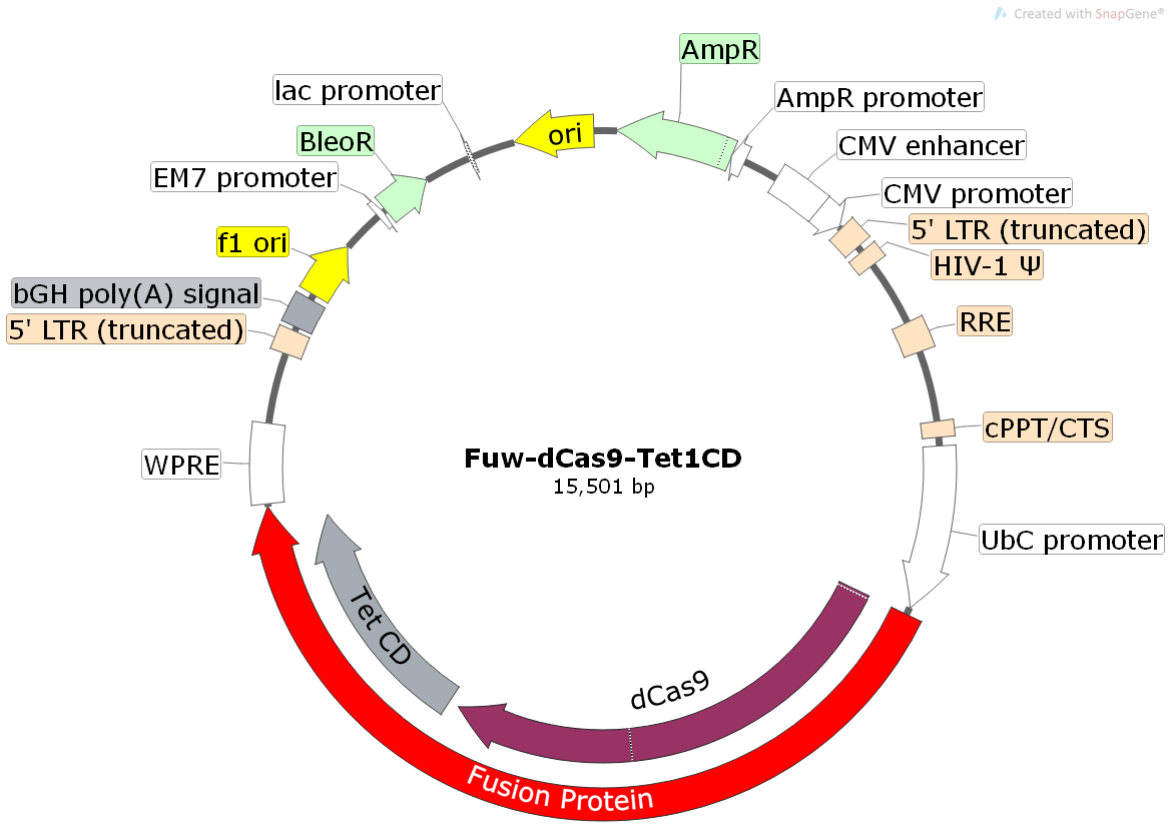


Figure 2.7: Plasmid maps indicating restrictions digest enzyme sites in Fuw-dCas9-Tet1CD.

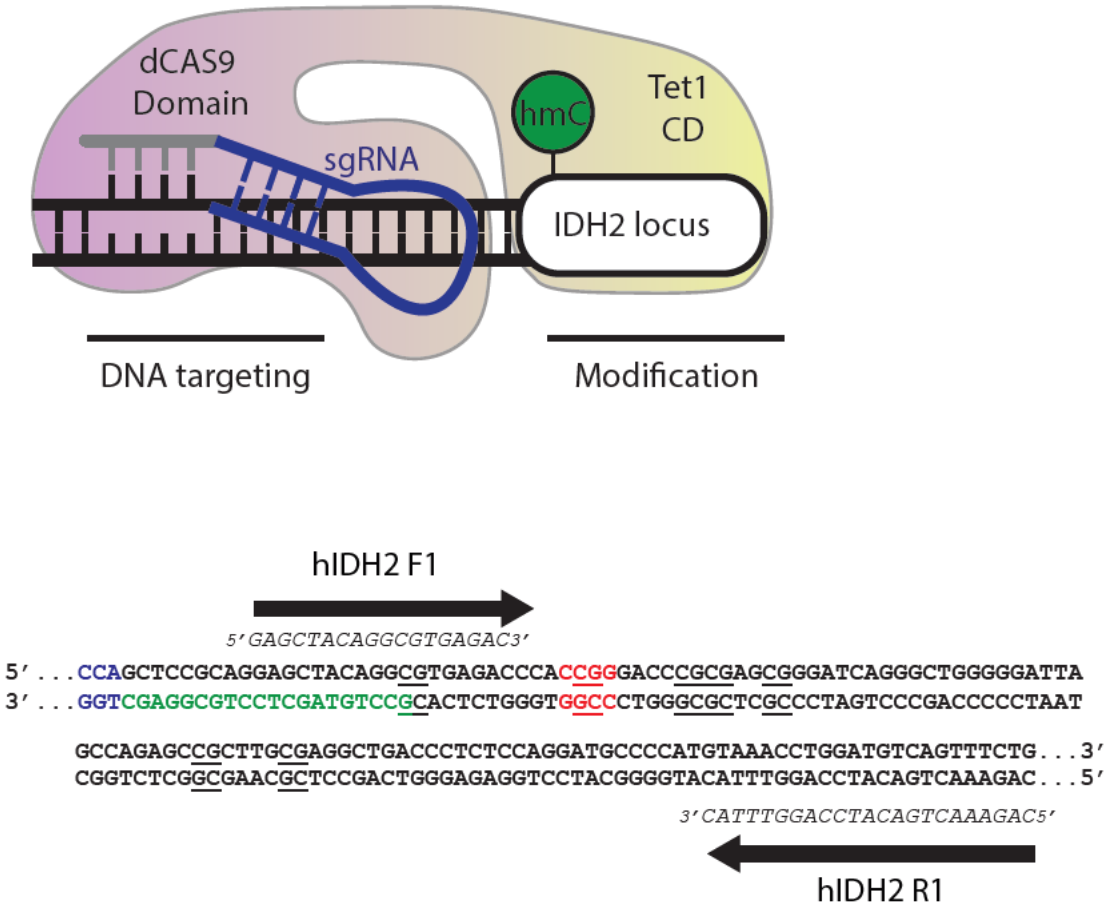


Figure 2.8: Schematic representation of the mechanism of action of dCAS9-Tet1CD and the small guidance RNA. The IDH2 locus is recognized by the specific identification of the hybrid sgRNA + DNA target. The Tet1CD domain modifies all the methylcytosines in the boundaries of the dCAS9 binding site into hydroxymethylcytosine residues. Panel B: Targeted sequence in the IDH2 promoter region for dCAS9-Tet1CD + sgIDH2 RNA. Sequence highlighted in green is the specific guidance RNA sequence recognized in the IDH2 promoter. Underscored nucleotides are the CpG islands in the sequence. Sequence in red indicates the CCGG domain used for SREMA hydroxymethylation analysis. Arrows indicate primer sequences used for qPCR analysis. Blue sequence indicates Protospacer adjacent motif (PAM) sequence.

2.14. Mycoplasma testing

All cells used in the research were regularly tested for Mycoplasma infection using EZ-PCR Mycoplasma Test Kit (BioInd). PCR in combination with 1.5% agarose gel electrophoresis was utilised for this testing methodology. Supernatant from cell culture media was collected after at least 48 hours of incubation of cells at 37°C with 5% CO^2. The supernatant was briefly centrifuged at 250 x g to pellet cell debris, and the supernatant transferred to fresh tube. After sedimentation of potential mycoplasma by centrifugation at 14,000 x g for 10 minutes, the supernatant was decanted, and any remaining pellet resuspended in 50µl of buffer solution provided with the kit. The solution was heated at 95°C for 3 minutes and subjected to PCR with the following cycling conditions: 30 second at 94°C (Initial denaturation), 35 cycles (Denaturation, Annealing and Extension) of: 30 seconds at 94°C, 2 minutes at 60°C and 20 seconds at 72°C. Followed by final denaturation, annealing and extension for 30 seconds at 94°C, 2 minutes at 60°C and 5 minutes at 72°C. The PCR product was then resolved on 1.5% agarose gel (1.5g of agarose in 100mL of TBE buffer 1x with 10µl of SYBR safe-Invitrogen) and separated DNA band with the size of 270bp were observed using Syngene InGenius3 Gel documentation system. This reaction was carried out alongside positive control templates provided by the manufacturer.

2.15. General statistical analysis

All statistical analyses were performed using Microsoft Excel and GraphPad Prism (GraphPad Software Inc.), where student's t-test was performed on the data obtained from two groups (e.g. control vs acetate treated) and one-way ANOVA analysis for the comparison of data obtained from multiple groups (e.g. control vs galactose treatment post 2 days vs galactose treatment post 5 days). Data was expressed as means ± standard error of the mean (SEM). A p value of <0.05 was considered to be statistically significant.

CHAPTER 3: THE EFFECT OF ACETATE ON EPIGENETICS AND METABOLISM IN THE DIABETIC MODEL INS-1

3.1. Introduction

The number of adult patients reported globally with diabetes have increased radically in the last few decades from 108 million in 1980 to 422 million at present, with expected 193 million undiagnosed cases. Although population increase and the rising number of ageing population may account for some of this increase, but it does not explain the near four-fold rise in diabetes prevalence. By 2030, Diabetes is predicted to be one of the top 10 causes of morbidities, with globally rising costs of care (Ang 2018). More than 90% of diabetic cases are T2D. The underlying characteristics of T2D, include insulin deficiency in pancreatic β-cells, insulin resistance in targeted organs, β-cell failures/loss, hyperinsulinemia, hyperglycaemia, increased platelet reactivity and fibrinolysis (Chatterjee et al. 2017). In order to manage these complications, the current first-line therapy for T2D is metformin, which reduces glucose output from the liver, stimulates Glucagon like peptide-1 (GLP-1) secretion and enhances peripheral tissue sensitivity (DeFronzo et al. 2016). However, there are number of complications arising from the use of metformin, including chronic kidney disease, vitamin B12 deficiency and GI difficulties (Rena et al. 2017). Additionally, caloric intake control and bariatric surgery may also be explored for diabetes remission. However, both options are difficult to implement on wide-scales and growing numbers of diabetic patients. Currently emerging T2D treatment strategies, include stem cell therapy aimed at increasing or replacing the lost β-cell volume, and bionic pancreas therapy to regulate the insulin levels (Chatterjee et al. 2017).

Different types of diabetes are differentiated into Type 1 Diabetes, T2D and Monogenic/MODY (Mature onset of diabetes of the young). Recently, this differentiation has been challenged by the increasing number of T2D cases diagnosed at a young age. The changes in lifestyle and diet have been suggested to play an important role in the onset of T2D in younger population, but there is a lack of molecular and biochemical evidence to support this statement (Chatterjee et al. 2017). Additionally, diabetic patients have been shown to be affected by hyperglycaemic associated complications, even when their blood glucose is mostly controlled by therapeutic approaches (Katada et al. 2012). Collectively, these findings suggest certain metabolic reprogramming and/or epigenetics could play a key role in the development of these complications. In some cases, T2D patients have been shown to achieve remission of the disease, which further indicates the role of reversible epigenetic modifications. Remission is defined by the control of blood glucose levels

without pharmacological or surgical interventions; fasting glucose of 5.6 mmol/l for at least 1 year (Buse et al. 2009).

3.1.1. Diabetes and Epigenetics

Environmental and nutritional factors have been suggested to drive epigenetics and therefore alter disease susceptibility. Genome wide association studies analysing T2D heritable genetic markers, such as nucleotide polymorphism, were only able to identify a small number of genetic markers (Prasad, Groop 2015). Dayeh et al. (2014) identified 16 T2D associated CpG Single nucleotide polymorphisms (SNPs), that were targeted DNA methylation sites. SNPs at CpG islands deregulate the methylation at the site it occurred on, and the methylation levels at nearby CpG sites. Davegårdh et al. (2018) correspondingly reported 40 SNPs that were strongly associated with T2D. From these 40 SNPs, 19 CpG sites were associated with aberrant methylation. DNA methylation also plays a significant role in regulating mitochondrial genes. For example, the aberrant DNA methylation of *CIX7A1*, known to be regulated by *PGC1-α* (master regulator of mitochondria), was associated with ageing and diabetes in mouse models (Ling, Groop 2009). These findings indicate that the DNA methylation mediated deregulation of mitochondrial associated genes can be involved in diabetes pathogenesis. Collectively, these findings suggest a potential role for DNA methylation in the pathogenesis of T2D; however, further research is required to understand the underlying mechanism that regulate this epigenetic change in diabetes. Other factors known to regulate epigenetics, including diet (e.g. acetate, glucose and fatty acids), physical inactivity, age and obesity; are emerging as key regulators of diabetes and mitochondrial diseases, emphasising the importance of epigenetic changes in gene regulation of T2D (Davegårdh et al. 2018).

3.1.2. Insulin synthesis

Until the isolation of insulin, the management of young diabetic insulin dependent patients was carried out using Frederick Allen's starvation diet. Following the isolation of insulin (by Banting and colleagues in 1921) for treatment of these patients, the management of insulin dependent diabetes became manageable (De Meyts 2004). This discovery led to improved understanding of insulin and insulin-receptor biochemistry and structure. Monomeric

insulin consists of two chains, Chain A and B consisting of 21 and 30 amino acids, respectively, linked by three disulphide bonds. The secondary structure of the B chain contains β-sheets and α-helices (Fu et al. 2013). In β-cells, insulin (approximately 40mM) is stored in dense and clustered granules, containing insoluble crystalline hexamers (De Meyts 2004). Insulin is a small protein (5.8 kDa) consisting of 51 amino acids that forms a complex secondary and tertiary structures with evolutionarily conserved regions throughout the phylogeny tree among different species (Fu et al. 2013). These conserved regions have been shown to play an important role in insulin-receptor binding affinity. Interestingly, the *insulin* gene codes for 110 amino acids that translates into preproinsulin consisting of hydrophobic N-terminal signal peptide. The role of this peptide is to interact with signal recognition particles from the cytosolic ribonucleoprotein resulting in the translocation of preproinsulin through rough ER to the lumen, subsequently, leading to the cleavage of the signal peptide by signal peptidase. This cleavage results in the formation of proinsulin, which folds and forms three disulphide bonds and is then transported to Golgi apparatus where proinsulin is packed into the immature secretory vesicles and is cleaved to yield C-peptide and insulin. Finally, insulin and C-peptides are stored as granules ready to be secreted (Fu et al. 2013).

3.1.3. Nutrients and Diabetes

Maintaining the insulin storage in pancreatic β-cells depends on the expression of the *insulin* gene. Moreover, metabolism plays an essential role in the biosynthesis of insulin, especially glucose metabolism, a well-known regulator of *insulin* transcription and translation. The promoter region of *insulin* gene has been shown to play a key role in the biosynthesis of insulin. Several transcription factors involved in the gene expression regulatory network interact with the *insulin* gene promoter region and thereby control its expression. In terms of pancreatic β-cell's insulin regulation by metabolism, glucose metabolism controls preRNA splicing and mRNA stability, in addition to regulating histone methylation and acetylation: which has been suggested to play an important role in the regulation of *insulin* gene expression (Poitout et al. 2006). The stabilisation of *insulin* mRNA serves as an crucial step in controlling its gene expression, which has been proposed to be mediated via the action of glucose-regulated pyrimidine-rich sequence binding with polypyrimidine tract-binding protein; the pyrimidine-rich sequence lies in the 3'-UTR of *insulin* mRNA (Tillmar et al. 2002).

The high concentrations of glucose, termed glucotoxicity, can also dysregulate the *insulin* gene expression. Glucotoxicity of pancreatic β-cells has previously been shown to impair *insulin* gene expression. It was also shown that the mechanism involved in glucotoxicity-mediated insulin dysregulation is carried out in a number of ways, including binding impairment of transcription factors, oxidative stress and dedifferentiation of pancreatic β-cells. The glucotoxicity mediated dedifferentiation occur as a result of large number of gene dysregulation, specifically genes involved in defining β-cell and its functionality. Another form of β-cell toxicity is mediated by high concentrations of lipids or fatty acids, termed lipotoxicity, that can also dysregulate the *insulin* gene expression. Lipotoxicity has been suggested to dysregulate *insulin,* at least partly, through a shared mechanism with glucotoxicity; hence the combination of both, i.e. high concentrations of glucose and lipids; glucolipotoxicity (GLT) was shown to hinder the insulin content and secretion by pancreatic β-cells (Poitout et al. 2006).

In addition to glucose and fatty acids, acetate was shown to correlate with the progression of diabetes. The sources of acetate include the breakdown of dietary fibres by gut microbiota either by acetogenesis or saccharolytic fermentation, through lumen and colon bacteria mediated fermentation resulting in short-chain fatty acids acetate, from diary and meat products (Schug et al. 2016; Schug et al. 2015). Akanji et al. (1989) demonstrated an impairment in acetate metabolism of diabetic patients, which exhibited relatively higher fasting plasma acetate levels in comparison to control. In addition, it was also shown by Hameed et al. (2015) that acetate contributes to unfavourable outcome in diabetes, by regulating Free fatty acid receptor 2 (FFAR2), known factor of diabetes pathogenesis. Furthermore, acetate was previously identified as a major carbon source for yeast and bacteria, and recently, was shown to be utilised as an alternative carbon source in humans along with glutamine, glucose and fatty acids (Gao et al. 2016). For example, acetate can be ligated to coenzyme A forming acetyl-CoA, that plays a central role in various intermediary metabolic pathways. This ligation is mediated by three isoforms of mammalian Acyl-CoA synthetase (ACSS) (figure 3.1) located at nuclear/cytoplasm (ACSS2) and mitochondrial (ACSS1/ACSS3) (Lyssiotis, Cantley 2014). The ACSS-mediated generation of acetyl-CoA plays a central role as a metabolite linking glycolysis to TCA cycle. Acetyl-CoA is a also a major component, widely involved in the PTM of proteins and histones, and synthesis of ketones, hexosamines and sterols (Schug et al. 2016).

Previous work from our group (Dr M Turner – Secondary supervisor) showed that *HMGCR* expression was downregulated in INS-1 cells treated with high levels of glucose and lipids (GLT). This *HMGCR* downregulation was associated with a reduction in insulin secretion. GLT and acetate have previously been shown to associate with diabetes. Moreover, glucose, lipids and acetate are known regulators of intracellular acetyl-CoA levels (Bagnati et al. 2016). In addition to being an important metabolic intermediate, acetate, contributes to unfavourable outcome in diabetes (Hameed et al. 2015). Herein, to characterise the role of acetate in the development of diabetes, INS-1 cells in acetate nutrient regime were investigated.

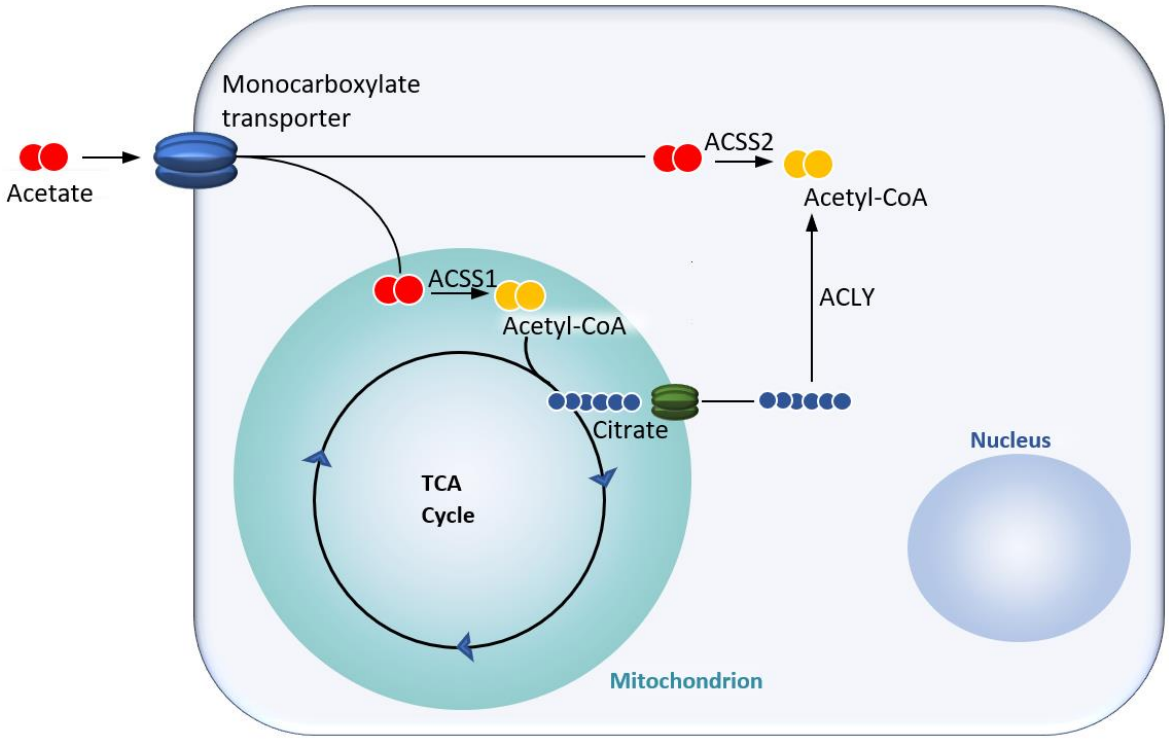


Figure 3.1: Schematic diagram depicting the function and metabolic fate of acetate in mammalian cells. The monocarboxylate transporter mediates the transfer of acetate from extracellular environment into the cell. Acetate can then serve as a metabolic source that can be transported into the mitochondria or directly converted into acetyl-CoA by the cytosolic ACSS2. Mitochondrial acetate can be converted into acetyl-CoA via ACSS1. Acetyl-CoA in the mitochondria can then join the TCA cycle for oxidation, and before getting catalysed by IDHs, can be exported into cytosol by Citrate-Malate shuttle. Exported citrate is then converted into acetyl-CoA by ACLY. (ACSS: Acyl-CoA Synthetase Short Chain Family Member (1 and 2), ACLY: ATP citrate lyase)

3.2. Aims of Chapter 3: Diabetic Model/INS-1 cells

- Evaluating the role of acetate in regulating key metabolic genes involved in mitochondrial function
- Characterising the effect of acetate on promoter DNA methylation of the *IDH2* and *insulin* gene
- Characterising the cellular content of insulin following acetate nutrient regime

3.3. Results

Insulin secreting cells INS-1 β-cells were subjected to acetate nutrient regime for 2 days. RT-qPCR and western blotting were utilised to assess the impact of acetate treatment on the gene expression profile. Initially, cells were grown in 5mM acetate and number of metabolically relevant genes were analysed for mRNA expression. The genes with significant regulation under acetate nutrient regime were selected for further analysis. Subsequently, protein expression, DNA methylation and functional consequence of the modulated genes were investigated. In order to examine the DNA methylation status of acetate treated INS-1 cells, MeDIP, SREMA and Bisulfite combined sequencing were employed.

3.3.1. Assessment of gene expression profiles in acetate treated INS-1 cells

The regulation of *HMGCR* in acetate treated INS-1 cells was investigated using RT-qPCR. After establishing the impact of acetate treatment on the expression of *HMGCR*, a number of other key metabolic genes, including *IDH2, HKII, HKIV, LDHA* and *Slc16a1*, known to associate with diabetes, were also assessed. The primer specificity for the candidate genes were evaluated using gradient PCR, and further confirmed by melting curves. The optimal annealing temperature were then selected and used in downstream mRNA analysis using RT-qPCR. The RT-qPCR data showed a significant regulation in the mRNA expression of *HMGCR* (0.6 fold), *IDH2* (0.6 fold) and *LDHA* (3.7-fold) in acetate treated INS-1 cells relative to control treatment (glucose). The IDH2 protein expression was confirmed using western blotting, demonstrating a significant downregulation by 0.7-fold (Figure 3.2B).

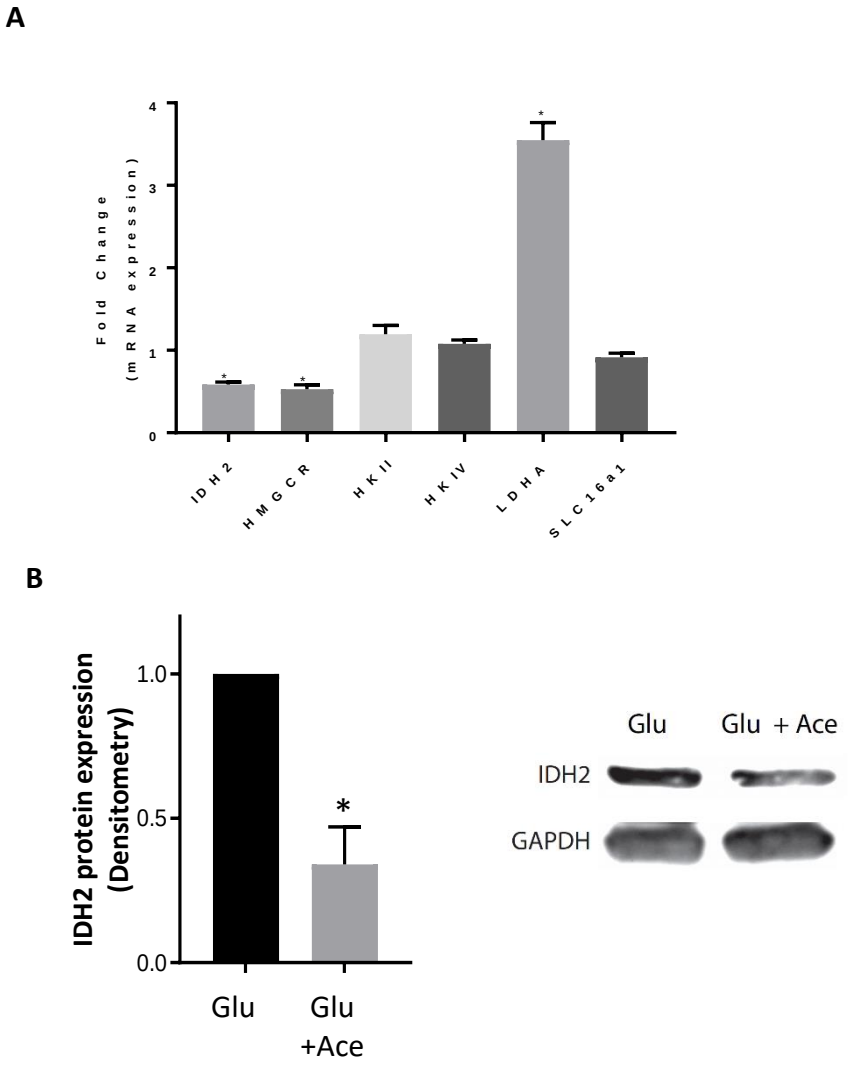


Figure 3.2: The effect of acetate on the expression of *IDH2, HMGCR, HKII, HKIV, LDHA* and *SLC16a1*. (A) mRNA expression levels of *IDH2, HMGCR* and *LDHA* were significantly regulated in INS-1 cells subjected to acetate nutrient regime. (B) IDH2 protein expression was significantly reduced in INS-1 cells subjected to acetate, relative to control (glucose). The average mRNA expression levels were normalised to GAPDH. Western blotting of IDH2 was normalised to GAPDH. Values are mean ±SEM (n=3), *P<0.05 (Glu: Glucose 11mM, Ace: Acetate 5mM).

3.3.2. DNA methylation analysis of the *IDH2* promoter region

In order to examine the relationship between gene expression and promoter DNA methylation status of *IDH2*, the target CpG and CCGG dinucleotide islands were identified using UCSC Genome browser (GB) (March. 2012 assembly genome (NCBI Assembly ID: 382928) (Rnor_5.0)) (Figure 3.3A). Using GB, the range of potential CpG and CCGG islands were mapped on the promoter region within -1200 bp from gene transcription start site (TSS) (according to the default reference genome build). It was also indicated by GB, that the majority of target CpG and CCGG islands were located within -200 bp from the TSS of *IDH2*. Notably, five CCGG islands were identified within the high frequency region (-200 bp from TSS) and were further subjected to DNA methylation analysis (Figure 3.3B).

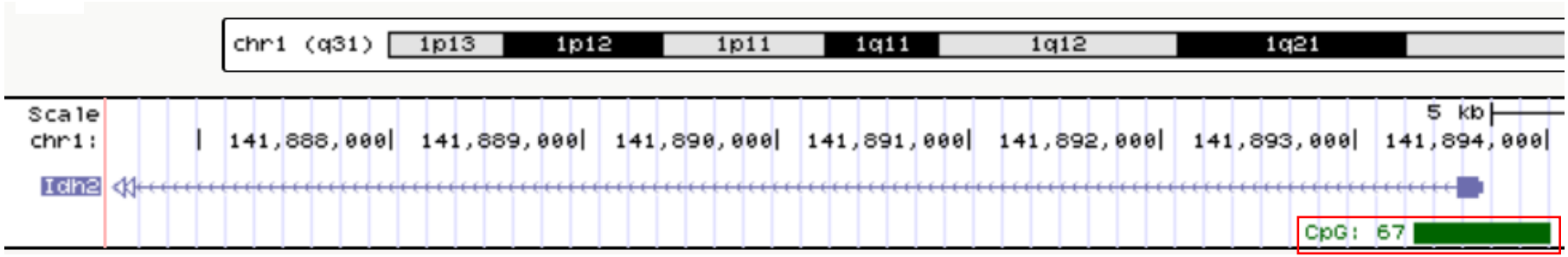
3.3.2.1. DNA methylation analysis of *IDH2* by SREMA

Initially, to examine promoter DNA methylation of *IDH2*, a methodology combining the action of specific restriction enzyme (i.e. HpaII) with qPCR was employed. Briefly, primers designed to target the amplification of different *IDH2* promoter regions containing a CCGG island were utilised in SREMA assay. Specificity of primers used in SREMA were validated by detection of unique amplicons in agarose gel electrophoresis and qPCR melting curves. After selecting the optimal primers for qPCR, the analysis of *IDH2* was carried out alongside a control primer designed to target the genomic region excluding any CpG islands (non-CpG primers). The data obtained from SREMA analysis showed that acetate nutrient regime increases DNA methylation of *IDH2* promoter region. This observation was depicted in acetate treated INS-1 cells, where significantly increased levels of 5-mC content were detected relative to control cells (Figure 3.3C). Also, no change was observed in the region targeted by non-CpG control-primers. The overall promoter methylation status of *IDH2* using SREMA was significantly increased by 6-fold in acetate nutrient regime of INS-1 cells.

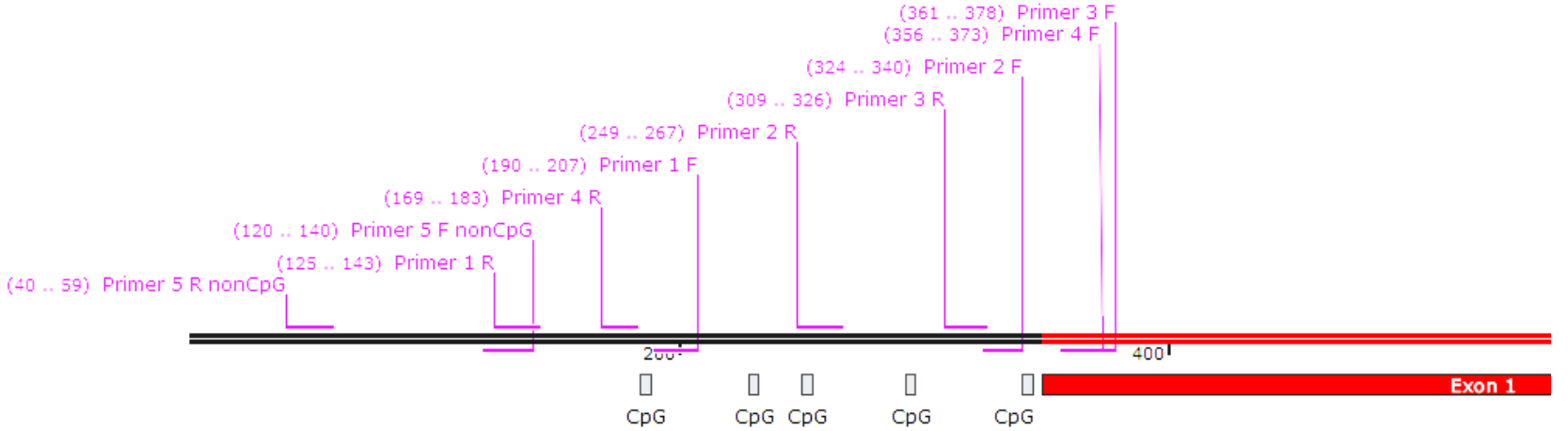
3.3.2.2. DNA methylation analysis of *IDH2* by Me-DIP

The *IDH2* DNA methylation status was further confirmed by an independent method, Me-DIP. This method is based on antibody (5-mC) DNA precipitation. The precipitated methylated DNA was thereafter analysed using qPCR to quantify the methylated CpG islands in acetate treated INS-1 cells. Briefly, total DNA obtained from INS-1 cells was sonicated using Covaris S220 Focused-ultrasonicator, to achieve DNA to fragments of 250-300 bp spanning the IDH2 promoter region and a group of CCGG islands (as indicated by GB) (Figure 3B). Sonication was optimised as stated in method chapter 2 (Figure 2.4). In correspondence to the SREMA results, Me-DIP detected high levels of 5-mC content on *IDH2* promoter region in acetate treated INS-1 cells (Figure 3.3D). The overall promoter methylation status of *IDH2* using MeDIP was significantly increased by 4-fold in acetate nutrient regime of INS-1 cells. The methylation status of these CpG islands was negatively correlated with the gene expression of IDH2.

A



B



Idh2 RAT CpG islands

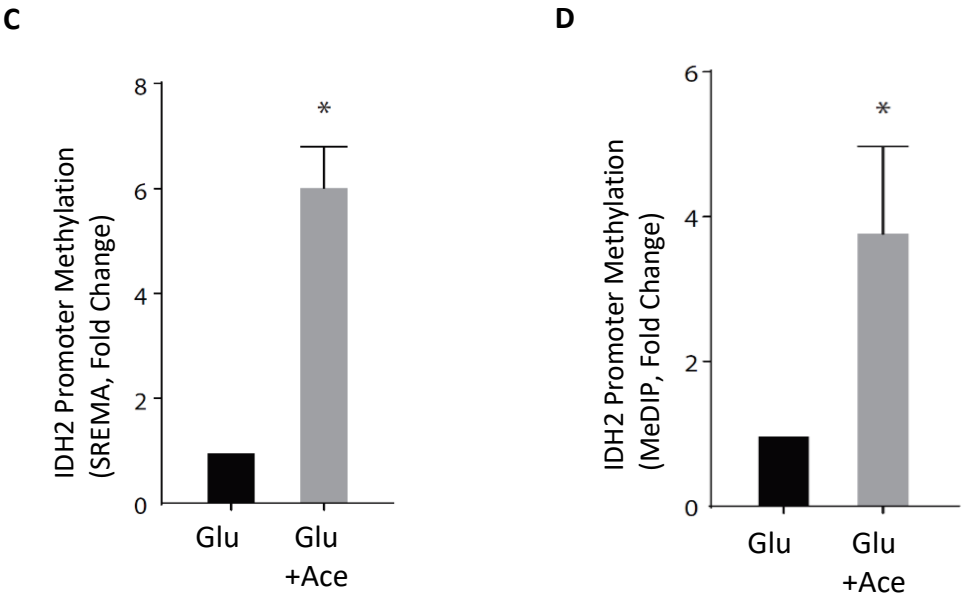


Figure 3.3: SREMA and Me-DIP analysis of the *IDH2* DNA promoter methylation in acetate treated INS-1 cells. (A and B) Distribution of CpG islands in the *IDH2* promoter region. The gene of interest (i.e. *IDH2*) and predicted CpG islands are represented by green strand and rectangle (outlined in red). Each grey box represents a CCGG island within -200bp upstream of TSS (high frequency CpG region identified using GB), and primers spanning these CCGG islands are highlighted above the *IDH2* DNA strand representation. After acetate treatment, the DNA promoter methylation of *IDH2* in INS-1 cells was significantly increased, confirmed using (C) SREMA (D) and MeDIP. Values are mean ±SEM (n=4), *P<0.05. (Glu: Glucose 11mM, Ace: Acetate 5mM)

3.3.3. Bisulfite combined sequencing for *IDH2* DNA methylation analysis

Bisulfite combined sequencing was also used to further confirm the observations from SREMA and Me-DIP. Briefly, DNA was treated with bisulfite to convert non-methylated cytosines to thymines. The treated product was then amplified using PCR with primers designed to target *IDH2* promoter region (Figure 3.4A). The amplified products, including the converted and non-converted DNA (untreated) were then analysed by Sanger sequencing. The TIDE-Sanger sequencing analysis demonstrated trivial increase of DNA methylation status in *IDH2*. The data showed partial methylation of CCGG islands upstream of *IDH2* TSS (Figure 3.4B and C) relative to the control (glucose).

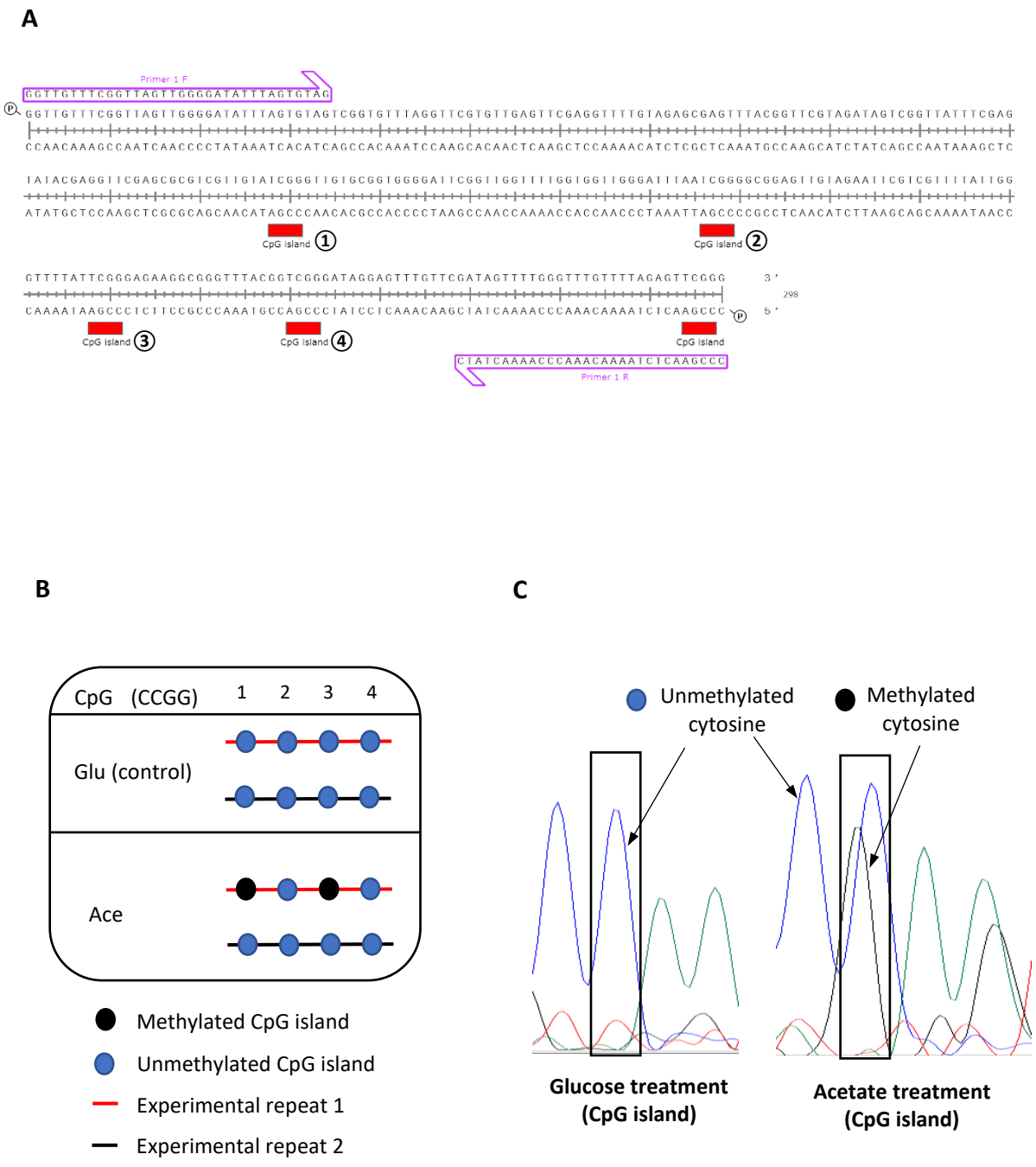


Figure 3.4: Bisulfite combined sequencing analysis of the *IDH2* DNA promoter region in acetate treated INS-1 cells. (A) Predicted bisulfite converted DNA of the *IDH2* promoter region. Primers used to target bisulfite converted DNA are shown in the purple outlined arrows, consisting of a group of CCGG islands (1 to 4). (B) Schematic of CCGG islands obtained from two independent experimental repeats (red line, experimental repeat 1; black line, experimental repeat 2). Circles represent CCGG island (black, methylated; blue, unmethylated). (C) TIDE analysis of bisulfite combined sequencing data represented in DNA chromatograms.

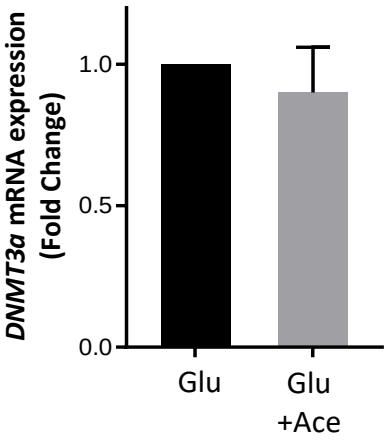
3.3.4. Acetate increases DNMT3a protein abundance in INS-1 cells

Data from SREMA and Me-DIP demonstrated significant changes in DNA methylation of *IDH2* promoter region in acetate treated INS-1 cells. As part of the analysis of epigenetic regulation of IDH2 expression in INS-1, DNMT3a, a key regulator of DNA methylation, mRNA and protein expression were assessed by RT-qPCR and Western blot analysis, respectively. The data obtained showed a substantial increase by 4.9-fold change in DNMT3a protein abundance in cells treated with 5 mM acetate (Figure 3.5B). However, RT-qPCR analysis showed no significant change in *DNMT3a* mRNA levels and remained unchanged in the acetate nutrient regime (Figure 3.5A). Furthermore, we investigated the effect of acetate treatment on DNMT3a protein abundance in IDH2 overexpressing INS-1 cells. Briefly, INS-1 cells were transfected with IDH2 expression plasmid (pcDNA3.1) and enriched by mammalian antibiotic puromycin. The transfection efficiency was assessed by fluorescence microscopy and confirmed by western blotting. DNMT3a protein abundance was evaluated in acetate treated IDH2 overexpressing INS-1 cells. The data obtained showed that the overexpression of IDH2 reverted the acetate mediated increase of DNMT3a protein abundance in INS-1 cells (Figure 3.5B), with a substantial decrease by 2.7-fold, relative to wild type INS-1 cells treated with acetate.

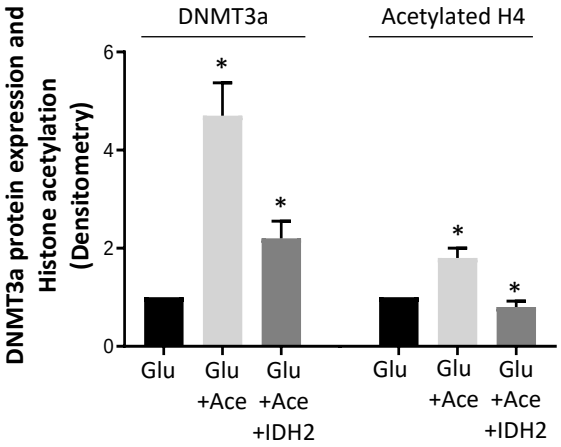
The lack of correlation between mRNA and protein expression, and reversion by IDH2 overexpression, suggested that DNMT3a may be regulated by factors such as PTMs, that may mediate its protein content in INS-1 cells. To address a possible PTM regulatory mechanism in the control of DNMT3a abundance, INS-1 cells were treated with the proteasomal inhibitor MG132 (or DMSO) at concentrations ranging from 5 µM to 20 µM for 2 and 4 hours. MG132, is a potent drug known to induce to cell death. Therefore, it is important to evaluate the concentrations required to inhibit the proteasomal degradation pathway without killing the cells. Thereafter, MG132 treatment was optimised and the cell pellet collected from 0, 2- and 4-hours post-treatment were analysed by western blotting. The optimal concentration of 10 µM MG132 were selected for further analysis. DMSO (MG132-reagent diluent) was used as a control treatment (Figure 3.5C). MG132 treatment increased DNMT3a protein abundance by 1.2-fold and 1.8-fold at 2 and 4 hours, respectively, an effect that was not detected when the proteasomal inhibitor was replaced by DMSO (Figure 3.5C). After characterising the effect of acetate on the PTM-modulated DNMT3a protein abundance. Acetylation status of Histone 4, a surrogate assessment of nutrient treatment efficacy, was investigated. The data obtained showed substantial

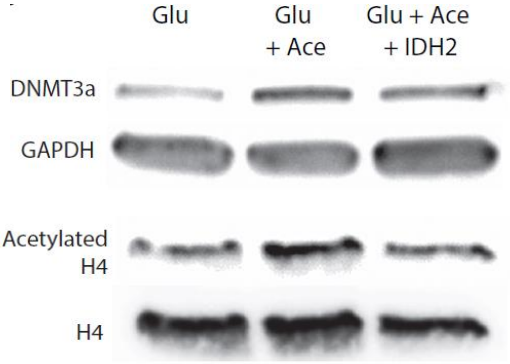
increase when INS-1 cells were grown in the nutrient regime, i.e. acetate. However, the overexpression of IDH2, even under similar treatment conditions, reverted the acetylation status of H4 to a similar extension observed in the DNMT3a protein abundance (Figure 3.5B).

A

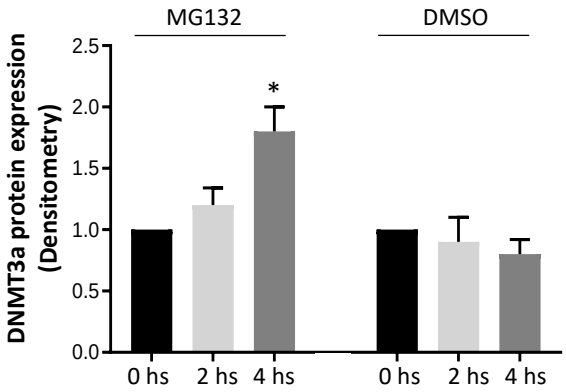

DNMT3a mRNA expression
(Fold Change)
1.0
0.5
0.0
Glu
Glu
+Ace

B


DNMT3a protein expression and
Histone acetylation
(Densitometry)
DNMT3a
Acetylated H4
6
4
2
0
*
Glu
Glu
+Ace
Glu
+Ace
+IDH2
Glu
Glu
+Ace
Glu
+Ace
+IDH2


Glu
Glu
+ Ace
Glu + Ace
+ IDH2
DNMT3a
GAPDH
Acetylated
H4
H4

C


DNMT3a protein expression
(Densitometry)
MG132
DMSO
2.5
2.0
1.5
1.0
0.5
0.0
*
0 hs
2 hs
4 hs
0 hs
2 hs
4 hs

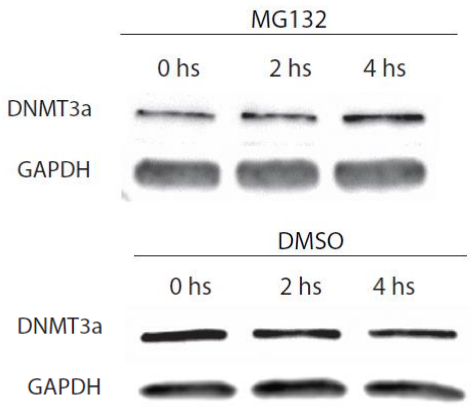

MG132
0 hs
2 hs
4 hs
DNMT3a
GAPDH
DMSO
0 hs
2 hs
4 hs
DNMT3a
GAPDH

Figure 3.5: IDH2 overexpression partly reverts the acetate induced DNMT3a protein abundance and histone acetylation in INS-1 cells. Cells were treated with 5mM acetate for duration of 48 hours and analysed by RT-qPCR and western blotting. (A) *DNMT3a* mRNA expression showed no significant regulation in the presence of acetate. (B) However, acetate increased DNMT3a protein abundance. Cells overexpressing IDH2 were able to partially revert the acetate-induced DNMT3a protein abundance. Changes in histone 4 acetylation were analysed by western blot, where acetate treatment increased the acetylation, and IDH2 overexpression significantly reverted the effect. (C) Cells treated with MG132 (Proteasomal degradation inhibitor) increased the protein abundance of DNMT3a in a time-dependent manner. DMSO, used as a negative control showed no significant changes. Values are mean ±SEM (n=3), *P<0.05. (Glu: Glucose 11mM, Ace: Acetate 5mM, Glu+Ace+IDH2: Overexpressing IDH2 cells in acetate regime).

3.3.5. Effect of Acetate on β-cell mitochondrial function

Following the determination of acetate's role in regulating DNMT3a, DNA methylation and IDH2 expression. The next phase in our research was to investigate the effect of acetate on key mitochondrial functions, such as ATP content, α-KG levels, mtCOI expression and mitDNA copy number. The data obtained showed that acetate led to a decreased ATP content in INS-1 cells (Figure 3.6D). Moreover, overexpression of IDH2 partially recovered acetate-induced ATP reduction in INS-1 cells (Figure 3.6D). In contrast to the previous data of *LDHA* mRNA expression (section 3.3.1), the lactate levels of acetate treated INS-1 cells did not indicate significant changes in the glycolytic activity, i.e. the lactate content of acetate treated wild type INS-1 cells and IDH2 overexpressing INS-1 cells displayed no significant difference, relative to control (Figure 3.6E). The characterisation of mitDNA content and mtCOI expression was assessed under the acetate nutrient regime (Figure 3.6A and B). The data showed a significant reduction in mitDNA content, which was correlated with a reduced expression of the mtCOI protein in acetate treated INS-1 cells. In addition, α-KG levels were also decreased in acetate treated INS-1 cells, relative to control (Figure 3.6C). Overall, α-KG, ATP production, mitDNA and mtCOI expression suggested a decrease of mitochondrial function, whereas, lactate levels did not indicate a change in the glycolytic activity of acetate treated INS-1 cells.

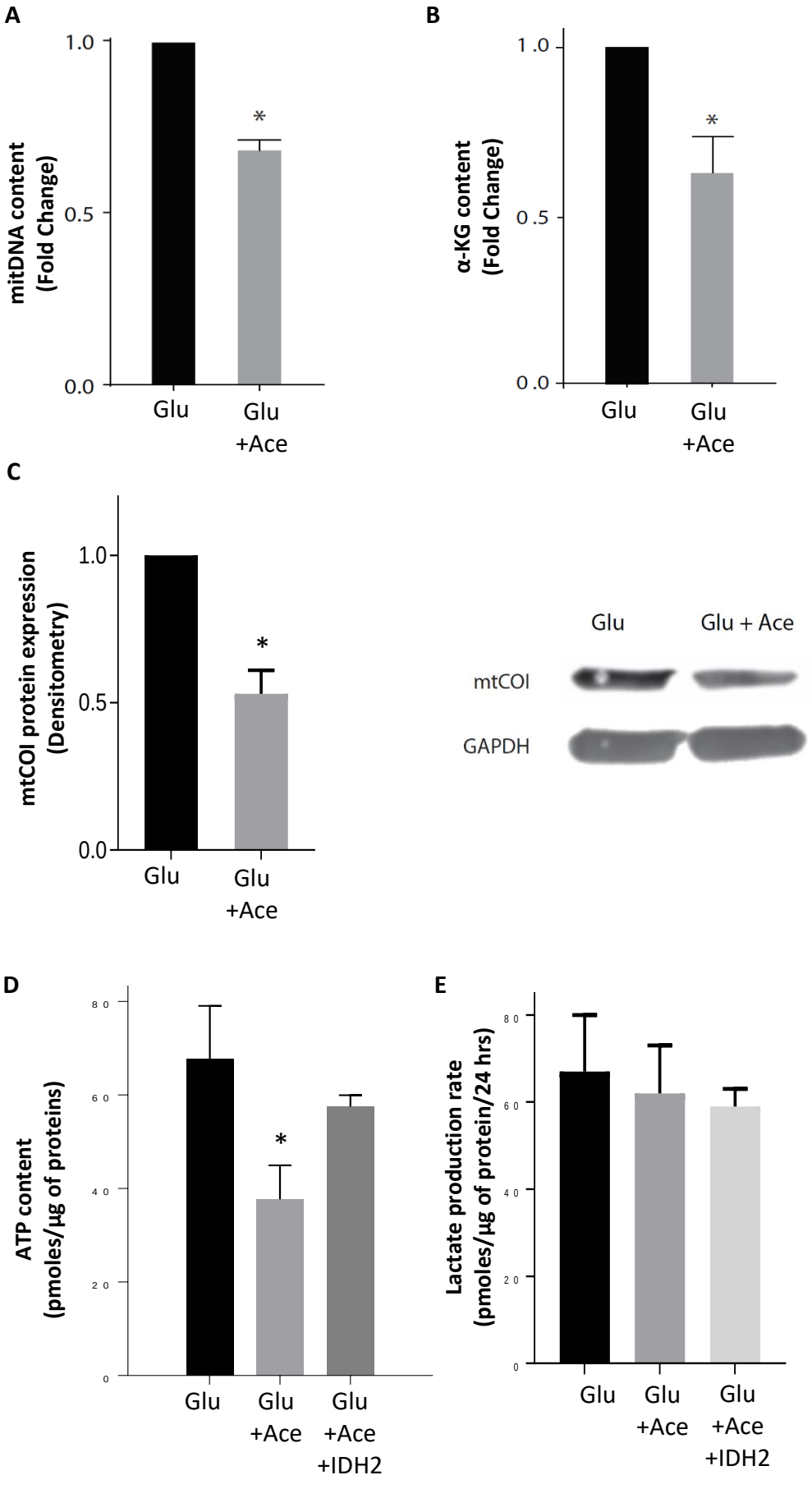

A
mitDNA content
(Fold Change)
1.0
0.5
0.0
*
Glu
Glu
+Ace
B
α-KG content
(Fold Change)
1.0
0.5
0.0
*
Glu
Glu
+Ace
C
mtCOI protein expression
(Densitometry)
1.0
0.5
0.0
*
Glu
Glu
+Ace
Glu
Glu + Ace
mtCOI
GAPDH
D
ATP content
(pmoles/µg of proteins)
80
60
40
20
0
*
Glu
Glu
+Ace
Glu
+Ace
+IDH2
E
Lactate production rate
(pmoles/µg of protein/24 hrs)
80
60
40
20
0
Glu
Glu
+Ace
Glu
+Ace
+IDH2

Figure 3.6: Acetate alters mitochondrial activity in INS-1 cells. The analysis showed acetate-mediated significant decrease in (A) mitDNA copy number, (B) α-KG, (C) mtCOI protein expression and (D) ATP content, analysed by qPCR, western blotting, alpha ketoglutarate fluorometric assay and ATP luminescence assay, respectively. The overexpression of IDH2 in INS-1 cells partially but significantly reverted the acetate-reduced ATP content. (E) The lactate content of acetate treated wild type INS-1 cells and IDH2 overexpressing INS-1 cells displayed no significant difference, relative to control (Glu). mitDNA copy number was normalised to nuclear DNA. mtCOII expression was normalised to GAPDH. Values are mean ±SEM (n=3), *P<0.05. (Glu: Glucose 11mM, Ace: Acetate 5mM, Glu+Ace+IDH2: Overexpressing IDH2 cells in acetate regime)

3.3.6. Acetate induces *insulin* gene DNA promoter methylation and downregulates its expression

Acetate treated INS-1 cells exhibited significant downregulation of *insulin* gene, confirmed by RT-qPCR (0.45-fold, Figure 3.7A), and this has a correlative effect on the abundance of the insulin, confirmed by ELISA (0.4-fold, Figure 3.7B). To evaluate the contribution of epigenetic regulation in the *insulin* gene, the changes in DNA methylation of the promoter region were assessed using previously described methodologies (e.g. SREMA and MeDIP). The data showed that acetate led to a significant increase in the 5-mC content of the *insulin* DNA promoter region (Figure 3.7C and D), ranging from 1.5 to 2-fold changes, when compared to control media conditions.

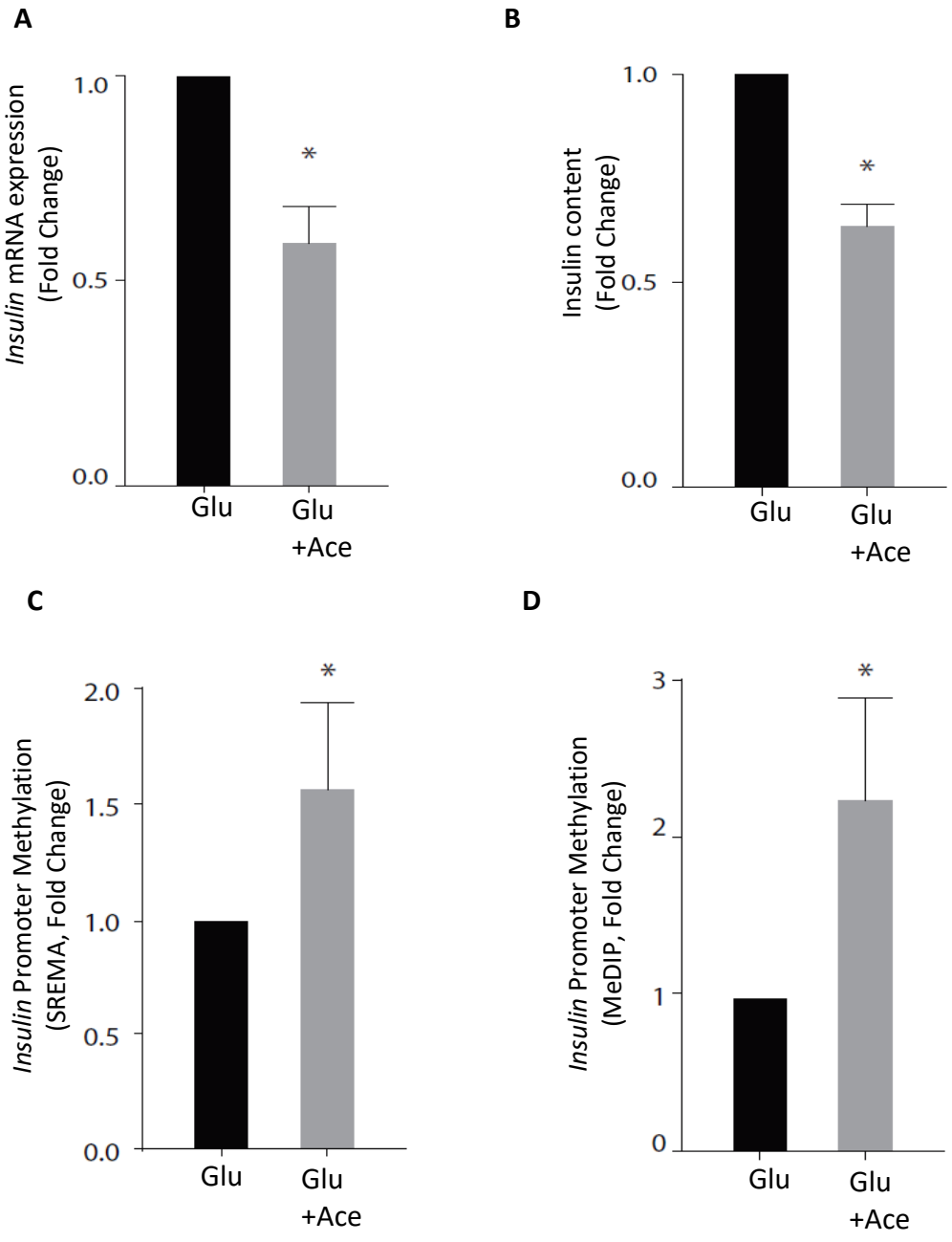


Figure 3.7: Effect of acetate on insulin mRNA expression, content and DNA promoter methylation. (A) *Insulin* mRNA expression from INS-1 cells treated with acetate was assessed by RT-qPCR. Data obtained showed that *insulin* expression was significantly reduced by acetate nutrient regime, normalised to GAPDH. (B) Insulin protein content, quantified by ELISA, showed a significant reduction, comparable to that observed in mRNA expression, when cells were subjected to acetate nutrient regime. (C and D) Levels of *insulin* promoter methylation in both media conditions (i.e. control and acetate) were assessed using MeDIP and SREMA methodologies. Acetate nutrient regime stimulated a significant increase in *insulin* promoter methylation, ranging from 1.5- to 2.2-fold. Quantitative analysis of 5mC content showed a negative correlation between *insulin* mRNA expression and DNA methylation status. Values are mean ±SEM (n=3), *P<0.05. (Glu: Glucose 11mM, Ace: Acetate 5mM)

3.4. Discussion

Diabetes is a metabolic disorder intrinsically linked with lifestyle changes in addition to genetic factors. Although population increase and the rising number of ageing population may account for some of this increase, but it does not explain the near four-fold rise in diabetes prevalence (Zhao et al. 2012; Ang 2018). The nutrient sensing β-cells that secrete insulin in response to circulating nutrients (e.g. glucose), play an important role in the pathogenesis of T2D (Wollheim, Maechler 2002). These nutrient sensing features suggest that the pancreatic β-cell metabolism is more sensitive to nutrients than other cells types. Therefore, understanding the effect of acetate nutrient regime on these cells is necessary for our quest to understand the pathogenesis of diabetes. Acetate is a potent substrate for the intracellular acetyl-CoA level (DeBerardinis, Chandel 2016). The accumulation of acetyl-CoA is a common metabolic feature in diabetic models (Layden et al. 2012). The main aim of this chapter was to characterise the effect of acetate on promoter DNA methylation of the selected genes in INS-1 cells, i.e. IDH2 and insulin. To achieve this aim, we analysed the role of acetate in the regulation of epigenetics, mitochondrial function and insulin content in INS-1 cells. Our data demonstrate the involvement of acetate nutrient in regulating metabolism and consequently affecting insulin content by modulating the epigenetic profile of INS-1 cells. This data adds to our understanding of the role of DNA methylation in regulating key mitochondrial components, such as, mtCOI, mitDNA, ATP content and α-KG. Additionally, we suggest the role of DNA methylation in the regulation of *insulin* gene expression leading to the reduction in total insulin content of cells, which correlates with DNMT3a protein abundance.

3.4.1. Acetate induces *IDH2* DNA promoter methylation and downregulates its expression

Mitochondrial dysfunction is shown to largely contribute to the development of T2D (Lu et al. 2010). Multiple factors are involved in the regulation of mitochondrial mediated pathogenesis of diabetes, including nutrients, epigenetics, SNPs and mutations. These factors contribute to the complexity of the underlying mechanisms regulating metabolic changes associated with diabetes pathology (Yaribeygi et al. 2018). For example, citrate, a key nutrient, known to regulate energy storage and transformation, also serves as a crucial

component in cellular metabolism and insulin regulation in pancreatic β-cells (Iacobazzi, Infantino 2014). IDH2 is the key player that connects mitochondria and important metabolites, such as citrate, acetyl-CoA and α-KG, to diabetes. IDH2 catalyses the reaction of isocitrate's conversion into α-KG. Previous studies have shown that IDH2 knockdown inhibits insulin secretion under high glucose conditions (Macdonald et al. 2013). Therefore, understanding the role IDH2 in insulin secreting pancreatic β-cells is crucial to identify the underlying mechanisms regulating metabolic changes in diabetes. In our study, INS-1 pancreatic β-cells treated with 5mM acetate showed downregulation of IDH2 mRNA and protein expression. The implications of IDH2 downregulation can include mitochondrial redox imbalance, ROS induced damage (YI et al. 2016), fat/cholesterol biosynthesis and glutathione metabolism irregularity in the cell (Koh et al. 2004). Pancreatic β-cell are exceptionally susceptible to high intracellular ROS content, and its accumulation induces apoptotic pathways leading to cell death (Lenzen et al. 1996). IDH2-generated NADPH reduces ROS-mediated cell damage by modulating antioxidation (Jo et al. 2001). Therefore, the downregulation of IDH2 by acetate can lead to β-cell death. In addition, previous studies carried out in *IDH2*$^{-/-}$ *m*ouse models, showed a significant reduction in mitochondrial oxygen consumption and cell viability (White et al. 2018), further elucidating the importance of IDH2 in cellular respiration and function. Diabetes progression is a multi-step process that involves mitochondrial oxygen consumption downregulation (Mulder, et al. 2017). Michael et al. (2013) demonstrated that knockdown of *IDH1* and *IDH2* leads to the inhibition of insulin secretion. In correspondence with these findings, our data showed significant decrease in IDH2 expression in acetate treated INS-1 cells, and the supporting data from Michael et al. (2013) indicates that this decrease could be substantial to hinder the normal physiological function of pancreatic β-cells. Like acetate, oxysterol treatment, commonly found in western diet, has also been shown to downregulate IDH2 in human aortic endothelial cells (Fu et al. 2014). Overall, our findings and others suggest that IDH2 can be highly regulated by differential nutrient regimes which is crucial for the normal function of pancreatic β-cell.

Chemical epigenetic modifications, such as acetylation of histones and DNA methylation are linked with changes in intracellular metabolic activity. This is particularly evident in the epigenetics of cells harbouring mutations in components of the TCA cycle, such as fumarate hydratase and succinate dehydrogenase (D'Urso, Brickner 2014). Additionally, it is well established that increment in CpG islands methylation leads to transcription repression. Thus, we hypothesised that the observed reduction of *IDH2* expression could be, to some

extent, mediated by CpG island methylation. Based on this, our aim to characterise the effect on promoter DNA methylation of *IDH2* was executed by three complementary methodologies. Because the regions rich in CpG islands has been shown to attract *de novo* methylation, the CpG rich regions of *IDH2* promoter were targeted for DNA methylation analysis (Edwards et al. 2017). Initially, the promoter methylation changes were detected by the combination of a methylation-sensitive restriction enzyme analysis (SREMA) followed by qPCR quantification. Our results indicated a significant increase in *IDH2* promoter methylation, supporting the changes observed in *IDH2* expression. Furthermore, we validated these results by Me-DIP. Once again, the data revealed a significant increment in the methylation of the *IDH2* promoter region, supporting the findings from SREMA. Studies conducted in cancer models has previously revealed that *IDH2* can be regulated by DNA methylation of the promoter region (Chou et al. 2016). Although our study is carried out in rodent β-cells, the functional CpG islands that are regulated by methylation have previously been shown to be conserved amongst various species, including human (Illingworth et al. 2010). Therefore, these results may be replicated in human primary or immortal cell lines.

The methylation status of *IDH2* confirmed using SREMA, MeDIP was also analysed by Bisulfite combined sequencing. However, the data obtained from bisulfite combined sequencing displayed a lower confidence level in the determination of methylated cytosines. Bisulfite treatment converts the unmethylated cytosines to uracils, and the methylated cytosines are conserved in the treatment. During PCR amplification step, these uracils are converted to thymines by DNA polymerase (Frommer et al. 1992). The limitation of bisulfite treated DNA sequencing includes the inability to differentiate between 5-mC and 5-hmC (Huang et al. 2010), which can cause variability in the data. However, this limitation does not apply to Me-DIP. Moreover, a single cytosine can have different methylation profile in different cells of the same treatment which can interfere with the confidence level of the methylation determination by sequencing (Song et al. 2005). These limitations could explain the low confidence level of the results obtained using Bisulfite combined sequencing data.

3.4.2. DNMT3a and histone acetylation regulation in INS-1 cells

CpG islands are generally unmethylated, which allows gene transcription maintenance (Jones, Takai 2001). However, the active regulation of DNA methylation in metabolic conditions such as T2D is not fully elucidated. DNA methylation is carried out by DNMT1, DNMT3a and DNMT3b (Robertson et al. 1999). DNMT1 is involved in the maintenance of DNA methylation status in the cell (Du et al. 2010), whereas, DNMT3a participates in the *de novo* promoter methylation of genes (Robertson et al. 1999). In the current study, the analysis of *DNMT3a* mRNA expression under acetate nutrient regime showed no significant changes. However, DNMT3a protein abundance was significantly increased in the presence of acetate. Similarly, acetylation of histone 4, a surrogate indicator of treatment efficacy, was also significantly increased in acetate nutrient regime. It was previously reported that histone acetylation correlates with insulin gene expression. In the same study, p300, a histone acetyltransferase was found to be modulated by pancreatic and duodenal homeobox 1 (Pdx1) that regulate insulin gene transcription (Bernstein et al. 2017). In terms of DNA methyltransferase, Park et al. (2008) studied the implications of DNA methylation during intrauterine growth in mouse models. Their findings suggested that the development of T2D was associated with epigenetic changes, including DNMT3a-initiated *de novo* DNA methylation resulting in the locking of a silenced state of *Pdx1* in the intrauterine growth, subsequently restricting pancreatic function, ultimately resulting in diabetes. Dhawan et al. (2015) also demonstrated the importance of DNA methylation in β-cell and insulin secretion. The transition from neonatal to adult β-cell involves a specific DNA methylation profile. Neonatal β-cell secretes insulin irrespective of blood glucose levels (Blum et al. 2012); however, this mechanism changes during cellular maturity. DNMT3a was shown to contribute to neonatal β-cell cellular maturity by initiating glucose-stimulated insulin secretion, suggesting suitable levels of DNMT3a are required for β-cell function. The balance in DNA methylation is an important factor for maintaining the identity of pancreatic β-cells. Offsetting the balance of DNMT3a could lead to changes in DNA methylation of pancreatic β-cells resulting in loss of identity and functionality. Specifically, offsetting the balance of DNMT1 has previously been shown to change the identity of β-cells into α-cells after injury induced proliferation (Dhawan et al. 2011). According to these findings, we further evaluated DNMT3a and the functionality of acetate treated INS-1 cells.

Interestingly, in our study, the overexpression of IDH2 in INS-1 cells reverted acetate-induced DNMT3a abundance and Histone 4 acetylation significantly. The metabolic-epigenetic connection has been the focus of research over the past few years. The role of DNA methylation in mitochondrial proteins and diabetes has been previously described (Maghbooli et al. 2015; Reichetzeder et al. 2016; Zhao et al. 2012), however, the association of IDH2 with DNA methylation in diabetes is not yet elucidated. Mutations of *IDH2* in cancers have been associated with increased global DNA methylation, that also correlates with the production of 2-hydroxyglutarate (2-HG) (Reitman et al. 2010; Yang et al. 2012; Fu et al. 2010). 2-HG is suggested to be an oncometabolite that promotes tumour growth by modulating cell epigenetics (Janke et al. 2017). However, in normal cells Malate dehydrogenase, 3-phosphoglycerate and LDHA are also shown to promiscuously produce 2-HG (Ye et al. 2018); likely serving a normal biological function. 2-HG is also the prominent hallmark of 2-hydroxyglutaric aciduria, a rare neurometabolic disease where a mutation in SLC25A1 (mitochondrial citrate carrier) leads to accumulation of 2-HG (Nota et al. 2013). There has been inconsistence observations regarding to the role of 2-HG in cancer (Li et al. 2018; Fu et al. 2010). Additionally, our data also shows that IDH2 downregulation can likely increase DNA methylation, in ways other than producing the oncometabolite, 2-HG.

IDH2 downregulation can lead to the accumulation of acetyl-CoA. Increase in acetyl-CoA levels has been associated with PTMs of intracellular proteins, such as acetylation of lysines (Gao et al. 2016). In addition, it was previously found that the catalytic activity of DNMTs can be modulated by various PTMs, such as acetylation and phosphorylation (Jeltsch, Jurkowska 2016). One study by Cheng et al. (2015) established the role of acetylation in mediating DNMT1. Another study by Deplus et al. (2014) demonstrated the role of Casein Kinase 2-mediated phosphorylation of DNMT3a in DNA methylation of cells. In addition, DNMT3a mediated DNA methylation can also be regulated by ROS (Miozzo et al. 2018) and domain rearrangement (Jeltsch, Jurkowska 2016). These findings suggest that acetate nutrient regime can induce PTMs and subsequently regulate DNMT3a. In our study, we investigated whether the stabilisation or degradation of DNMT3a is regulated by PTMs. INS-1 cells were incubated with proteasomal degradation inhibitor MG132. The inhibition of proteasomal degradation led to a significant increase in DNMT3a abundance in cells. In addition, the mRNA analysis of *DNMT3a* confirmed the above finding, that there was no significant change in *DNMT3a* transcripts levels after acetate treatment of INS-1 cells, despite the significant upregulation observed in protein level. Both combined, the MG123-mediated significant increase of DNMT3a protein, and acetate-induced DNMT3a

abundance while maintaining constant levels of mRNA in acetate, are suggestive of PTMs role in regulating DNMT3a protein abundance. Based on these observations, we speculate that DNMT3a can be stabilised by acetylation leading to evasion from proteasomal degradation pathway.

In addition to evaluating the role of PTM in DNMT3a regulation, we also evaluated the role of acetate in histone acetylation. The data obtained showed that acetate induced hyperacetylation of histones in INS-1 cells. Acetylation/deacetylation of histones is carried out by HATs and HDACs using acetyl-CoA as a substrate (Pons et al. 2009). Emerging insights suggest that the modulation of acetyl-CoA can metabolically regulate the function of other epigenetic changes in addition to histone acetylation (Su et al. 2016; Lempradl et al. 2015; Tran et al. 2017). Radiolabelled acetate has previously been shown to provide acetyl group that binds to histones causing acetylation (Allfrey et al. 1964). Gao et al. (2016) correspondingly reported that acetate can induce hyperacetylation of histone 3 in hypoxic cells by increasing ACSS1 and ACSS2 expression, leading to the production of acetyl-CoA. Moreover, Schug et al. (2015), using $^{13}C_2$-acetate demonstrated that acetate conversion to citrate is mediated by ACSS1 in mitochondria. Both studies combined with our data provide evidence that acetate induces hyperacetylation of histones by mediating the expression of IDH2, ACSS1 and ACSS2, thereby increasing intracellular acetyl-CoA levels. The downregulation of IDH2 mediated by DNA promoter methylation can lead to the accumulation of citrate, which can then be transported into cytosol and further increase acetyl-CoA concentration. Modifications of histones have been previously shown to play an important role in the metabolic memory of diabetic patients (Miao et al. 2014). The increase in acetyl-CoA levels has been linked with numerous cellular process simultaneously including histone and protein acetylation (Pietrocola et al. 2015). Therefore, the increase in histone acetylation in acetate treated INS-1 cells could also indicate the increased acetylation of other cellular proteins including DNMT3a, resulting in modulated protein abundance.

The general concept of histone acetylation leading to gene expression came from studies conducted in isolated calf thymus nuclei (Allfrey et al. 1964). This was further elucidated by cloning HATs and HDACs chromatinised templates that resulted in gene deregulation (Brownell et al. 1996; Taunton et al. 1996). Histone acetylation is mainly associated with increased gene expression, but our data shows increased histone acetylation can also correlate with decreased expression, possibly mediated by DNA methylation. This finding

suggests that a relationship between histone acetylation and gene deregulation needs to be further elucidated.

In order to establish a cause-consequence relationship between the observed INS-1 functional features and the reduced IDH2 content, an ectopic overexpression of *IDH2* was utilised. INS-1 cells overexpressing IDH2, showed a substantial reversion of DNMT3a protein abundance and histone acetylation, suggesting increased IDH2 levels can decrease intracellular acetyl-CoA levels resulting in the observed reversion. Supporting evidence from Kobayashi et al. (2014) and Pietrocola et al. (2015) demonstrated that IDH2 overexpression leads to increased TCA cycle and reduces intracellular acetyl-CoA levels.

3.4.3. Acetate decreases mitochondrial activity in INS-1 cells

The availability and composition of nutrients available to the cell *in vivo* has been shown to dictate the metabolism. Cells can modulate their metabolism according to the predominant metabolite from the available nutrients in order to meet the structural and functional need (Khurshed et al. 2017). The amounts and availability of metabolites can also be controlled by metabolic changes mediated by key metabolic enzymes. For example, the knockdown of IDH1 and IDH2 was shown to associate with intracellular decreased α-KG and increased citrate levels, indicating a role of deregulating key metabolic genes in controlling the composition and availability of metabolites (Macdonald et al. 2013). IDH2 plays a key role in TCA cycle by mediating irreversible oxidation and decarboxylation of isocitrate to α-KG (Zou et al. 2017). Therefore, following acetate-induced IDH2 downregulation, we measured the level of α-KG in INS-1 cells. The data obtained showed decreased α-KG levels in acetate treated INS-1 cells. α-KG serves as a signal for insulin secretion in β-cells (Iacobazzi, Infantino 2014), and the level of α-KG can largely depend on IDHs (Park et al. 2016). IDH2 overexpression leading to increased α-KG have previously been shown to be associated with increased mitochondrial function (Vohwinkel et al. 2011), suggesting that IDH2 can regulate α-KG levels and mitochondrial activity, simultaneously. Thereafter, we investigated the function of mitochondria by evaluating mtCOI expression, mitDNA content, ATP content and α-KG levels in acetate treated INS-1 cells. The results obtained indicated a decrease in mitochondrial activity.

Mitochondria are the central site for energy metabolism and cellular respiration. Moreover, mitochondrial dysfunction correlates with insulin secretion and sensitivity (Song

et al. 2001). Gong et al. (1998) showed a rapid (<1hr) increase in mitochondrial activity after irradiation of cells, which correlated with increased mtCOI expression and ATP production. Irradiation of cells displayed the observed changes within a short period, thus suggesting that mtCOI expression and ATP production are direct representatives of mitochondrial activity rather than an indirect effect from mitochondrial stimulation. In our study, mtCOI expression and ATP production were significantly reduced in acetate treated INS-1 cells. Complex IV regulates the mitochondrial function and activity. Moreover, impaired complex IV gene expression, including mtCOI impairment has been linked with T2D pathology (Holvoet et al. 2016). A single missense mutation of *mtCOI* was associated with a significant loss of complex IV activity (Acín-Pérez et al. 2003). Additionally, several cases of diabetes were shown to arise from familial mitochondrial related pathologies (Mkaouar-Rebai et al. 2013). *mtCOI* is the first gene encoded in mitDNA (Acín-Pérez et al. 2003), and variations in mitDNA have been suggested to be a metabolic risk factor for diabetes (Pravenec et al. 2007). mitDNA and encoded genes are essential components of β-cells function, i.e. insulin secretion (Nicholas et al. 2017). Therefore, we assessed the content of mitDNA in acetate treated INS-1 cells. The data obtained showed a significant reduction in the mitDNA content. Pravence et al. (2007) showed that conplastic strains of rats consisting of the same nuclear but different variations of mitDNA, displayed different metabolic risk factors towards T2D, suggesting the important role of mitDNA in the pathogenesis of the disease.

The underlying mechanisms of mitDNA regulation in T2D are yet to elucidate, but insulin-dependent cellular stress was suggested to damage mitDNA, leading to the loss of mitDNA content. The extranuclear location and large copy number (each mitochondrion contains several nucleoids consisting of mitDNA) of mitDNA, increases its susceptibility to damage (Gilkerson 2016). The acquired decrease in mitDNA copy number of T2D patients was also observed in their offspring, indicating the copy number variation of mitDNA can be inherited (Song et al. 2001). While a single mutation in nuclear DNA is sufficient for a profound effect, a heteroplasmy in mitDNA is required to alter phenotype, due to its large copy number in a cell (Mulder 2017). The results from our study also support the previous statement that mitDNA is regulated by mechanisms other than mutations in INS-1 cells. TCA cycle plays an important role not only in the generation of ATP but also in generating metabolic intermediates including α-KG, citrate and etc. Citrate was shown to inhibit the TCA cycle, subsequently reducing ATP production (Iacobazzi, Infantino 2014). Acetate treated INS-1 cells in our study also demonstrated a reduction in α-KG and ATP content of the cells, indicative of a decrease in the TCA cycle activity. The TCA cycle and ATP

production was previously shown to play a central role in insulin gene transcription and translation (Maechler 2013; Poitout et al. 2006). Additionally, T2D patient-derived islets cells showed morphologically altered mitochondria and thereby mitochondrial function (Wiederkher, Wollheim 2008), further supporting our findings.

Barres et al. (2009) indicated that PGC-1α expression correlates with mitDNA copy number and mitochondrial content of the cell. This regulation in T2D patients was shown to be negatively correlated with DNA methylation status of PGC-1α promoter region. Moreover, in the same study, PGC-1α mRNA expression was shown to be regulated by the nutritional status of the cells, exhibiting a long-term consequence in T2D patients. (Rodgers et al. 2005). The rate of lysine acetylation mediated PGC-1α downregulation and consequent mitochondrial deregulation is limited by the availability of acetyl-CoA (Fernandez-Marcos, Auwerx 2011). Acetylation of PGC-1α can be regulated by GCN5, a protein acetyltransferase (Kelly et al. 2009). In addition, ACLY generates intracellular acetyl-CoA, that is required for acetylation of proteins and histones. Silencing of GCN5 and ACLY result in highly similar cellular changes, suggesting common pathways of action between the two (Wellen et al. 2009). Therefore, it is possible that the changes observed in the acetylation of histones following the treatment of acetate (in our study) and the reversion of histone acetylation by the overexpression of IDH2 can also be observed in PGC-1α.

3.4.4. DNA methylation of *insulin* gene and its correlation with insulin content

The transcription of *Insulin* gene was previously shown to be regulated by active DNA promoter methylation (Kuroda et al. 2009). Following these findings, we investigated the regulation and DNA methylation status of the *insulin* gene in acetate treated INS-1 cells. The data obtained showed increased DNA methylation of *insulin* DNA promoter region, that inversely correlated with *insulin* mRNA and protein content. Kuroda et al. (2009) correspondingly reported that DNA methylation alterations affecting *insulin* transcription is a long-term effect, which can result in the development of diabetes. Moreover, in the same study, *in vitro* methylation of *insulin* gene resulted in 90% transcription reduction by hindering the transcription factor binding to cAMP responsive element (CRE) site of *insulin* promoter region. Insulin secretion is generally higher in female islets in comparison to males; however, the DNA sequence of the gene is identical or similar in both. Hall et al. (2018) demonstrated that these differences arise from sex-specific DNA methylation

associated with a number of genes known to affect *insulin* transcription. In our study, we observed 40% reduction in insulin mRNA and protein levels that inversely correlated with increase in DNA methylation. Combination of findings from others and ours, can concluded that the acetate-induced reduction in *insulin* gene can be a result of direct interference from increased promoter DNA methylation. Moreover, DNA methylation is heritable; when an individual is exposed to nutritional changes, the epigenetic changes can be intergenerationally or transgenerationally inherited (Heard, Martienssen 2014).

Glucotoxicity and lipotoxicity causes a decrease in mitochondrial activity and increased ROS production leading to apoptosis in β-cells (Maedler et al. 2003; Fu et al. 2013). Our study also demonstrated a reduction of IDH2 and mitochondrial activity, these changes have previously been shown to be associated with increased ROS production. Collectively, these findings suggest that long term exposure of acetate from the diet can lead to pancreatic β-cell dysfunction and therefore, diabetes.

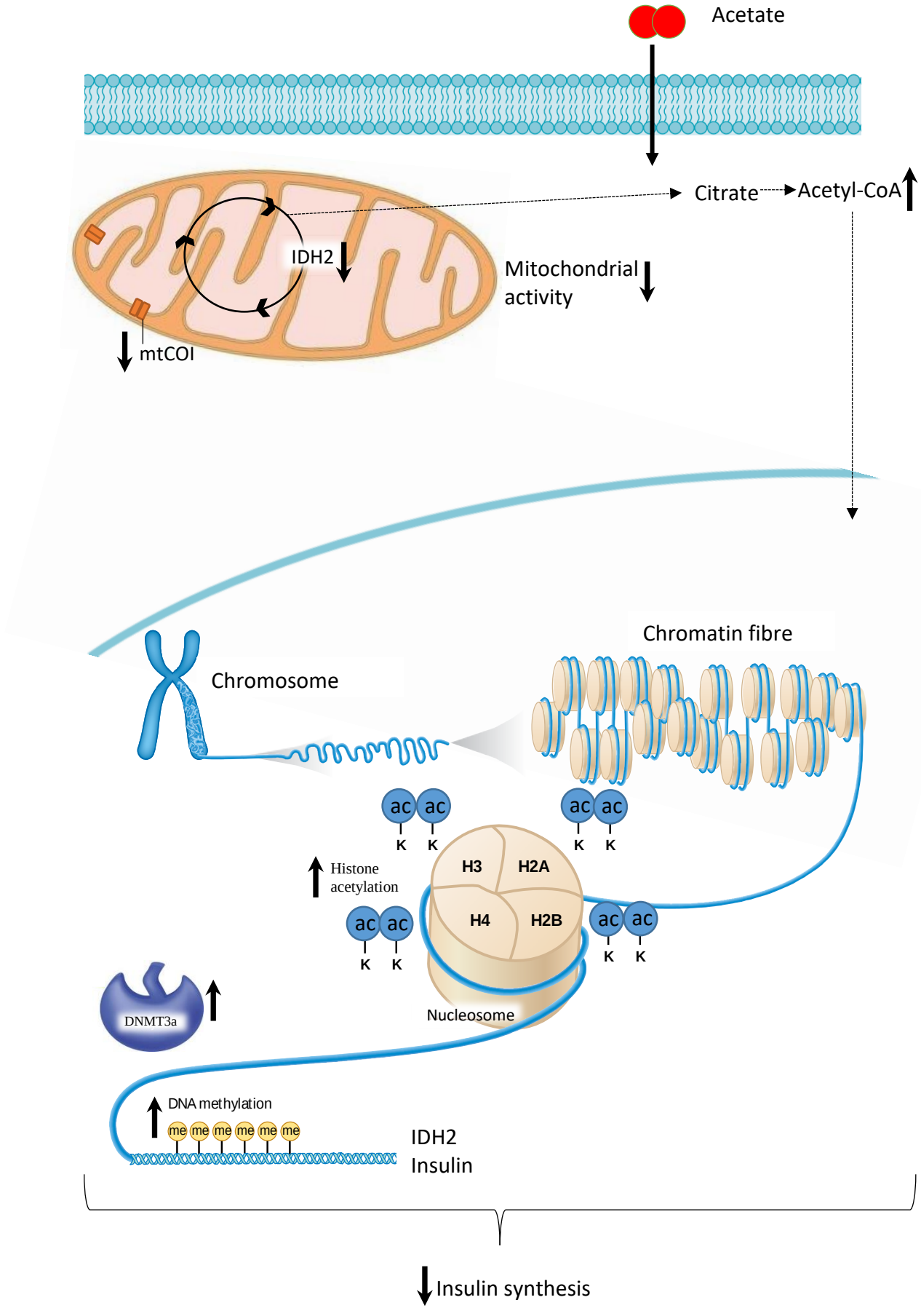


Figure 3.8: Schematic figure illustrating the conclusion of chapter 3 (for further explanation see section 3.5).

3.5. Conclusion

In conclusion, our data shows that in the β-cell model INS-1, increased acetate levels lead to a significant decrease in *IDH2* and *insulin* expression by an active epigenetic control. These changes were accompanied by reduction in key mitochondrial components including mtCOI and mitDNA copy number, α-KG levels and ATP content. Additionally, acetylation of histone and stabilisation of DNMT3a was observed, thereby, explaining the increased rate of DNA methylation on *IDH2* and *insulin* promoter region. Overexpression of an ectopic IDH2 led to functional recovery of the INS-1 cells bioenergetics, with both a decreased histone acetylation and DNMT3a protein abundance. Finally, acetate was also found to reduce the insulin content of INS-1 cells (Figure 3.8). Overall, our study provided a rationale for the design of therapeutic strategies aiming to modulate the epigenetic mechanism of β-cells in metabolic disorders such as T2D.

CHAPTER 4: THE EFFECT OF GALACTOSE ON EPIGENETICS AND METABOLISM IN THE CANCER MODEL HeLa

4.1. Introduction

Otto Warburg and colleagues suggested the concept of 'The Warburg effect' in 1920s. The Warburg effect states that the cancer cells utilise large amounts of glucose in comparison to the surrounding tissues and produce an extensive amount of lactate, even in the presence of abundant oxygen (Warburg et al. 1927). Herbert Crabtree confirmed the findings of Otto Warburg and further elucidated the heterogenicity of aerobic glycolysis in tumour cells. Crabtree and colleagues suggested that tumour cells exhibited a substantial amount of respiration with a variable degree, therefore, require functional mitochondria (Crabtree 1929). However, Warburg and colleagues later suggested in the 1950s that dysfunctional mitochondria are the causative for cancer cell's metabolic profiles (Warburg 1956). Subsequent findings showed that the mitochondria is not defective in tumours, and even cancer cells require functional mitochondria to survive (Moreno-Sánchez et al. 2007; Weinhouse 1976). These findings suggested the existence of an alternative method of mitochondrial and OXPHOS regulation in cancer cells. Cancer was originally thought to be regulated only by changes in the genetic sequence of key tumour suppressors and proliferation regulators. But, recent findings established the role of epigenetics leading to the alterations observed in cancer cells (Berdasco, Esteller 2010). Normal differentiated cells primarily rely on OXPHOS for energy expenditure; however, cancer cells instead rely on the inefficient method of energy generation called aerobic glycolysis (Figure 4.1). Whether the aerobic glycolysis/Warburg effect is a consequence of cancer or inversely a pathogenic factor remains to be clarified. Studies also suggest that 'The Warburg effect' is required for tumour growth, and the necessity of aerobic glycolysis in cancer cells extends beyond the generation of new cellular components for proliferation (Shim et al. 1998; Fantin et al. 2006).

Cancer cells are observed to proliferate increasingly and have reduced apoptosis. In addition to the metabolism, mitochondria also plays a central role in proliferation and apoptosis, (Arciuch et al. 2012). Pro-apoptotic proteins such as Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 Like (NIX) and BCL2 interacting protein 3 (BNIP3), either form an integral part of mitochondrial membrane (NIX) or localise to the mitochondrial outer membrane (BNIP3), that mediate apoptosis (Chourasia et al. 2015). NIX is a mitochondrial protein forming heterodimers with Bcl-XL and Bcl-2 via its transmembrane domain (Abe et al. 2005). NIX stimulates mitophagy by the disrupting Beclin-1 and Bcl-2 complex. NIX also

stimulates autophagy in malignant cardiomyocytes and glioma cells and trigger apoptosis by promoting Bax/Bad-dependent release of pro-apoptotic mediators from the mitochondria and by seizing Bcl-2 family proteins (Radogna et al. 2015). Moreover, metabolic reprogramming in cancer was shown to be accompanied with downregulation of NIX (Drake et al. 2017).

4.1.1. Metabolic changes in cancer

The role of metabolism in cancer has been the focus of research for decades, however, there has always been a discrepancy between studies. It is widely accepted that aerobic glycolysis is the main source of energy production, but OXPHOS still contributes to the energy production in cancer cells. Tumour cells reprogram nutrient utilisation, and therefore the metabolism in order to increase survival and proliferate. Number of therapies targeting metabolism are regularly being used in clinical settings, such as asparaginase, used for the treatment of acute lymphoblastic leukaemia, by converting the asparagine amino acid into ammonia and aspartic acid. Asparagine is usually not synthesised in the leukemic cells due to their high protein synthesis and demand, therefore these cancerous cells rely solely on the uptake of asparagine from the bloodstream (DeBerardinis et al. 2007). Similar to glycolysis, the TCA cycle also plays a vital role in providing intermediates for macromolecule synthesis in cancer cells; hence the TCA cycle is required even at low rate for survival in tumours (Ahn, Metallo 2015). In addition to pyruvate production by glycolysis, the TCA cycle can also generate metabolic intermediates and ATP from amino acids and fatty acids. The breakdown of fatty acids is known as β-oxidation that generates acetyl-CoA, an important intermediate for epigenetic and PTMs. Amino acids including valine, leucine and isoleucine are generally elevated in cancer patients, and can also be utilised by TCA cycle for acetyl-CoA generation. These factors suggest the adaptability of TCA cycle, and therefore an important therapeutic target in cancer research (DeBerardinis, Chandel 2016). In order to prohibit the growth of cancerous cells, Israelsen et al. (2013) targeted pyruvate kinase to limit glycolysis-mediated ATP production; however, this effort failed to prevent tumorigenesis, suggesting an alternative role of glycolysis in cancer. Additionally, cancer cells have been shown to reprogram the surrounding tissue and induce the Warburg effect (Koit et al. 2017). Whether cancer cells induce the Warburg effect in surrounding tissue by secreting metabolic intermediates and consequently causing epigenetic modifications, is yet to be fully elucidated.

4.1.2. Cancer and epigenetics

Cancer stem cells (CSC) can arise from normal cells that have undergone epigenetic reprogramming to display stem cell-like features. Different CSCs display heterogenic features representing different disease entities, and are often resistant to many cancer treatments (Poli et al. 2018). Conventionally, genetic factors were thought to be the leading cause for cancer plasticity, metastasis, high proliferation rate and apoptotic evasion. Genetic mutations rely mainly on the gain or loss of function of the targeted proteins in pathogenesis of cancer; however, epigenetic factors dictate the rate of reaction rather the complete loss or gain of functionality (Di Cerbo, Schneider 2013). Recently, epigenetics has been recognised as a major contributing factor in prompting the distinct features in cancer (Sandoval, Esteller 2012). Besides genetic factors, epigenetics play an important role in the differences that exist between cancers of the same types and therefore leading to the variations in treatment responses. DNA methylation patterns is one of the key players that dictate the metabolic differences in different organs (De Sousa E Melo et al. 2013).

Genome wide hypermethylation of the promoter regions is characterised as a key component of many cancers (Miremadi et al. 2007). DNA methylation plays an important role in regulating gene expression. Moreover, number of genes involved in cell cycle have been shown to be regulated by DNA methylation at the promoter region: including PTEN and BRCA1/2 (Minami et al. 2014). In gliomas, MGMT (DNA mismatch repair protein) is commonly downregulated but is not mutated; current findings suggest a role of DNA methylation in the regulation of this protein in cancer instead of mutations (Jones 2007; Baylin, Jones 2011). Furthermore, several genes associated with different cancers have been found to be hypermethylated and were suggested as potential biomarkers (Sandoval, Esteller 2012). Interestingly, Fernandez et al. (2012) conducted DNA methylation analysis on 1628 samples from patients with various cancers, it was found that 1505 CpG sites at promoter regions of various genes were hypermethylated during tumorigenesis. In addition to DNA methylation, the second main epigenetic modification is histone acetylation. The increase in acetylation of histones due to mutations in HDACs was associated with higher risk of breast and lung cancer. The acetylation levels also depend on HATs and its substrate acetyl-CoA, that can dictate the fate of the cell (Miremadi et al. 2007). Acetylation of histones should be maintained for gene expression controls and genomic stability (Di-Cerbo, Schneider 2013).

4.1.3. Nutrients and cancer

Nutrients play an important role in the prevention of cancer and recovery/treatment of patients. The Gerson therapy, a nutrition-based therapy for melanoma, was evaluated in a five-year interventional study. This study observed an increased survival rate in comparison to other conventional therapies (Hildenbrand et al. 1995; Donaldson 2004). However, the cellular mechanisms involved in this nutrition-based diet was not identified, but the percentage of patients survived on the Gerson therapy was significantly larger than reported by some of the other clinical trials of cancer therapy (Donaldson 2004). Sodeberg et al. (1980) and Petrova-benedict et al. (1992) demonstrated that galactose is lethal to cells harbouring defective mitochondrial respiratory chain; however, normal cells were capable of surviving galactose treatment in cultures. The biological importance of galactose extends beyond metabolic intermediate. Galactose is an important constituent of the nutrition composition of milk, which forms the disaccharide with glucose to generate lactose. Therefore, galactose plays an important role in fetal and neonatal development (Coelho et al. 2015). Lactose (glucose and galactose) is known to play an important role in health and disease as it is an integral part of almost all species producing milk, however marsupials and sea lions do not contain lactose in their milk but instead contain a tri-saccharide of galactose (Urashima et al. 2014). The cellular mechanism involved in galactose utilisation relies on mitochondrial oxidation almost solely for the generation of ATP. Galactose in the cells is converted to UDP-glucose mainly by the Leloir pathway through subsequent action of multiple enzymes using 2 ATPs as cofactors. Therefore, cells relying on glycolysis (generating 2 net ATPs) are forced to utilise OXPHOS to generate additional ATP (Coelho et al. 2015). All these findings suggest that galactose may serve as an important factor in the treatment of cancers, especially by modulating 'The Warburg effect'.

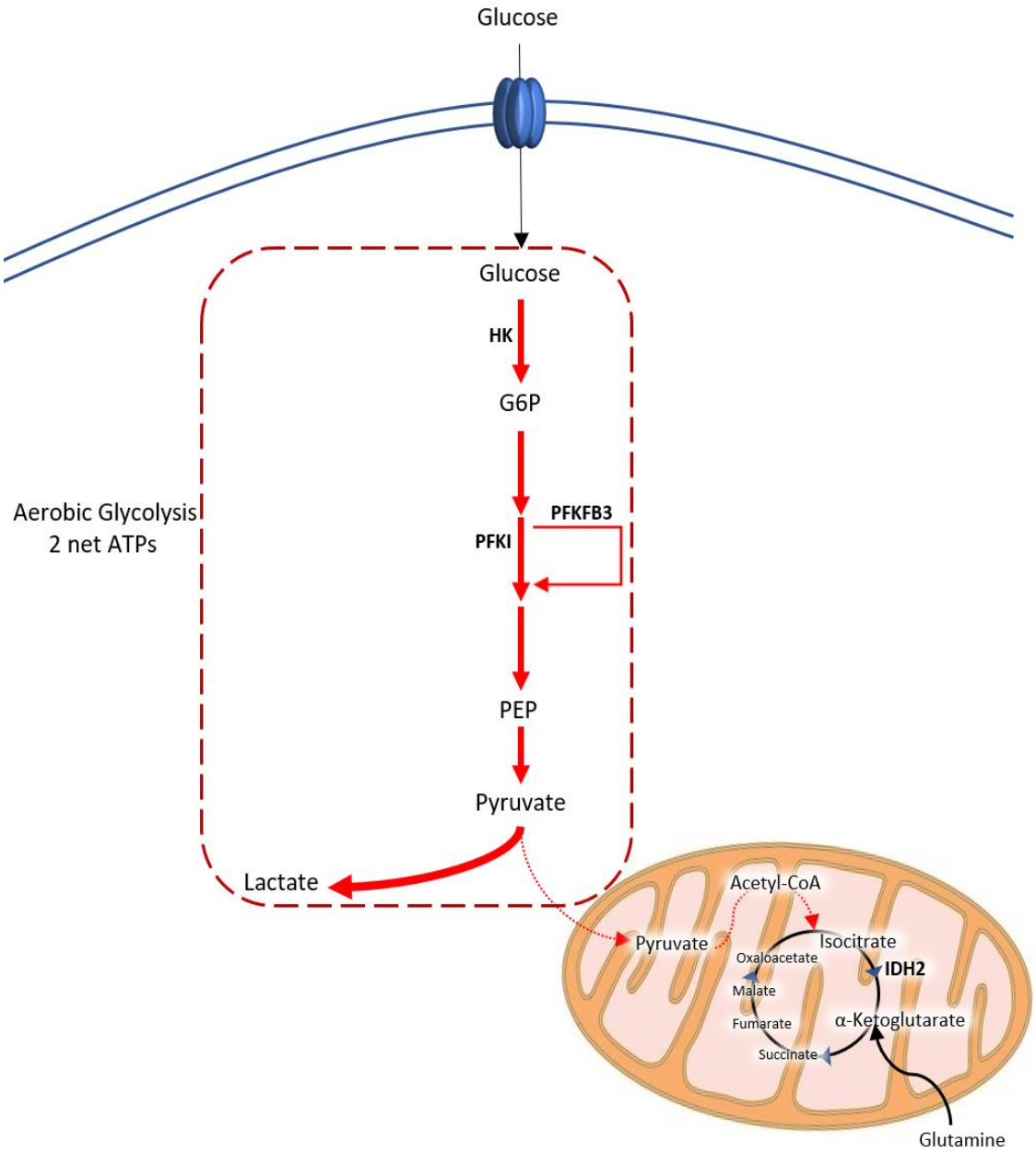


Figure 4.1: Schematic representation of the metabolic changes in cancer cells. Glucose metabolism is modified in cancer cells. Instead of OXPHOS, the cells 'switches' to a phenomena known as aerobic glycolysis (Fu et al. 2017). Initially, glucose is taken up by the cells through one of the 13 glucose transporters (GLUTs); i.e. GLUT 1-12 and H+/Myoinositol cotransporter. The transported glucose is then phosphorylated to produce glucose-6-phosphate by hexokinases (HK). Thereafter, phosphofructokinase-1 (PFK1) can catalyse the conversion to fructose-1,6-bisphospate (Hamanaka, Chandel 2012), or fructose-2,6-biphosphatase 3 (PFKFB3) can degrade fructose-2,6-biphosphate into fructose-6-phosphate by hydrolysis (Shi et al. 2017). Further down the glycolytic pathway, pyruvate can enter the mitochondria to be utilised in OXPHOS or get converted to lactate by lactate dehydrogenase. In cancer cells, the majority of the glucose transported into the cell is metabolised through less efficient (generating 2 ATPs-in comparison to OXPHOS, generating 38 net ATPs) aerobic glycolysis pathway. Collectively, these changes in metabolism contribute to reduced apoptosis, increased macromolecule biosynthesis and balancing of intracellular redox in cancer cells (Fu et al. 2017).

4.2. Aims of Chapter 4: Cancer Model/HeLa cells

- Evaluating the role of galactose in regulating IDH2, NIX and BNIP3
- Characterising the effect of galactose on promoter DNA methylation of the *IDH2, NIX* and *BNIP3*
- Characterising apoptosis and proliferation status of the HeLa cells in galactose nutrient regime

4.3. Results

The effect of galactose on cancer cell metabolism (*in vitro*) was studied using HeLa cell line. Briefly, cells were grown in 25mM Galactose, and consequently, the effect on mitochondrial and apoptotic related genes were investigated. The regulation of *IDH2, BNIP3* and *NIX* transcript levels by galactose nutrient regime and subsequent effect on protein expression, DNA methylation, DNA hydroxymethylation and functional consequence (i.e. cell proliferation and apoptosis) was evaluated.

4.3.1. Galactose upregulates mitochondrial function and reduces lactate in HeLa cells

Initially, the effect of galactose on lactate production and mitochondrial function was investigated. mitDNA is representative of mitochondrial health and function. Therefore, qPCR analysis was used to determine the mitDNA content of HeLa cells and normalised to the nuclear DNA. The data obtained showed a significant increase in the mitDNA content of HeLa cells cultured in galactose nutrient regime for 5 days, exhibiting a 2.7-fold increase (Figure 4.2A). Further characterisation of mitochondrial function was carried out by investigating mtCOII protein expression under galactose nutrient regime. The data showed an upregulation of mtCOII protein, as evaluated by western blotting, demonstrating a positive correlation of mtCOII protein expression with mitDNA content of HeLa cells in galactose nutrient regime (Figure 4.2B).

The α-KG levels in HeLa cells were quantified using alpha-ketoglutarate assay kit. The data obtained showed an increased level of α-KG in galactose treated HeLa cells, exhibiting a 1.75-fold increase (Figure 4.2D). Acquired data from α-KG analysis correlated with other mitochondrial related analysis, i.e. mitDNA content and mtCOII expression. Additionally, in order to assess the effect of galactose on glycolytic activity of HeLa cells, the lactate production was analysed using a Lactate Trininty-bitotech assay. HeLa cells were incubated in galactose nutrient regime for the duration of 5 days. The supernatant was then collected and analysed, demonstrating a significant reduction in lactate production by 52%, relative to control (normal DMEM media). The lactate production negatively correlated with mitDNA, mtCOII and α-KG levels. Collectively, these results indicate a reduction of glycolysis and upregulation of mitochondrial function.

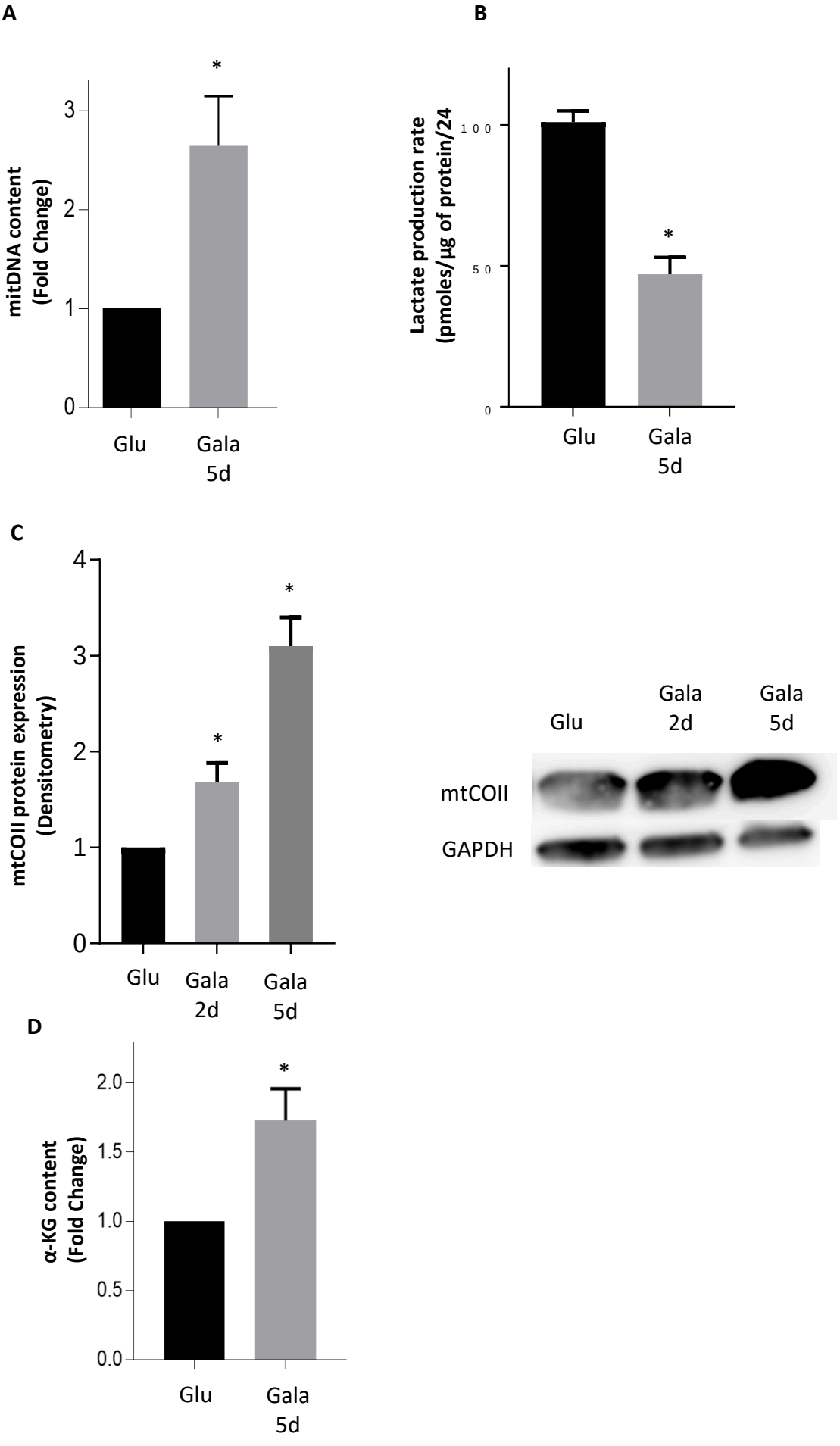

A
mitDNA content
(Fold Change)
3
2
1
0
*
Glu
Gala
5d
B
Lactate production rate
(pmoles/µg of protein/24
100
50
0
*
Glu
Gala
5d
C
mtCOII protein expression
(Densitometry)
4
3
2
1
0
*
*
Glu
Gala
2d
Gala
5d
Glu
Gala
2d
Gala
5d
mtCOII
GAPDH
D
α-KG content
(Fold Change)
2.0
1.5
1.0
0.5
0.0
*
Glu
Gala
5d

Figure 4.2: Galactose alters mitochondrial function and lactate production in HeLa cells. (A) mitDNA copy number was analysed using qPCR (normalised to nuclear DNA), the data obtained showed a significant increase in mitDNA copy number. (B) Lactate production was measured in galactose nutrient regime using colorimetric Lactate Trinity-biotech assay. The data obtained showed a significant decrease in lactate content in cells. (B) mtCOII protein expression was analysed using western blotting (normalised to GAPDH), the data obtained showed a significant increase in mtCOII protein expression in galactose nutrient regime of HeLa cells. (D) α-KG metabolite levels were measured using fluorometric alpha-ketoglutarate kit (Abcam), the data obtained showed a significant increase of α-KG levels in galactose treated HeLa cells after 5 days, relative to control. Values are mean ±SEM (n=3), *P<0.05. (Glu: Glucose 25mM, Gala 2d: Galactose 25mM for 2 days, Gala 5d: Galactose 25mM for 5 days)

4.3.2. Expression analysis in galactose treated HeLa cells

Following the determination of the role of galactose in regulating mitochondrial function and lactate production, we investigated the effect of galactose on key mitochondrial related genes. HeLa cells were subjected to galactose nutrient regime for a duration of 5 days, thereafter DNA, RNA and proteins were extracted. Primer annealing temperatures and specificity were optimised by gradient PCR and melt curve analysis. The RNA obtained from HeLa cells was subjected to reverse transcriptase reaction using Thermofisher 2 step RT kit, followed by mRNA expression analysis by qPCR (AB Biosystem StepOne plus).

Previous chapter (chapter 3) of this thesis, demonstrated that the downregulation of IDH2 correlated with a decrease in mitochondrial function. Moreover, in the present chapter, mitochondrial function increase was indicated in galactose nutrient regime according to the data obtained from lactate, α-KG, mitDNA and mtCOII analysis. Following these findings, *IDH2* expression was assessed in galactose nutrient regime in HeLa cells, by RT-qPCR. The data obtained showed a significant increase by 2-fold change in *IDH2* expression after 5 days of galactose nutrient regime (Figure 4.3A). Following the initial findings of *IDH2* expression, we further investigated mRNA expression of a number of other candidate genes related to mitochondrial function (i.e. *LDHA* and *TET2*) or forming an integral part of mitochondrial structure that can induce apoptosis or reduce proliferation in cancer cells (i.e. *NIX* and *BNIP3*).

4.3.3. Regulation of key metabolic genes

In order to determine the regulation of mitochondrial associated genes, HeLa cells were incubated in galactose nutrient regime for the duration of 5 days, RT-qPCR based relative quantitation approach was then employed. Initially, *IDH2, NIX, TET2, BNIP3* and *LDHA* mRNA were analysed after day 5 of galactose treatment. The data obtained showed significant changes in *IDH2* (2-fold), *NIX* (1.6-fold) *TET2* (1.5-fold) and *BNIP3* (0.72-fold), whereas, *LDHA* mRNA expression showed no significant difference (Figure 4.3 A). It was interesting to evaluate the time course of expression changes in the highest regulated genes by means of RT-qPCR and western blotting. Therefore, we analysed IDH2 and NIX, at day 2 of galactose treatment in HeLa cells. The data obtained showed a time dependent trend in the upregulation of *IDH2*: 1.35-fold increase at day 2, and 2-fold increase at day 5. *NIX* also demonstrated a significant increase by 1.5-fold at day 2 and 1.6-fold increase at

day 5 of galactose nutrient regime (Figure 4.3B). Subsequent analysis by western blotting confirmed previous findings from RT-qPCR analysis, displaying an upregulation of IDH2 and NIX expression in galactose treated HeLa cells. IDH2 was found to be upregulated by 1.8-fold at day 2 and 3.9-fold at day 5. This trend in protein expression change was also evident in NIX, displaying a 2-fold increase at day 2 and 5.3-fold at day 5. This response was a time-dependent increase as previously observed by the mRNA expression analysis.

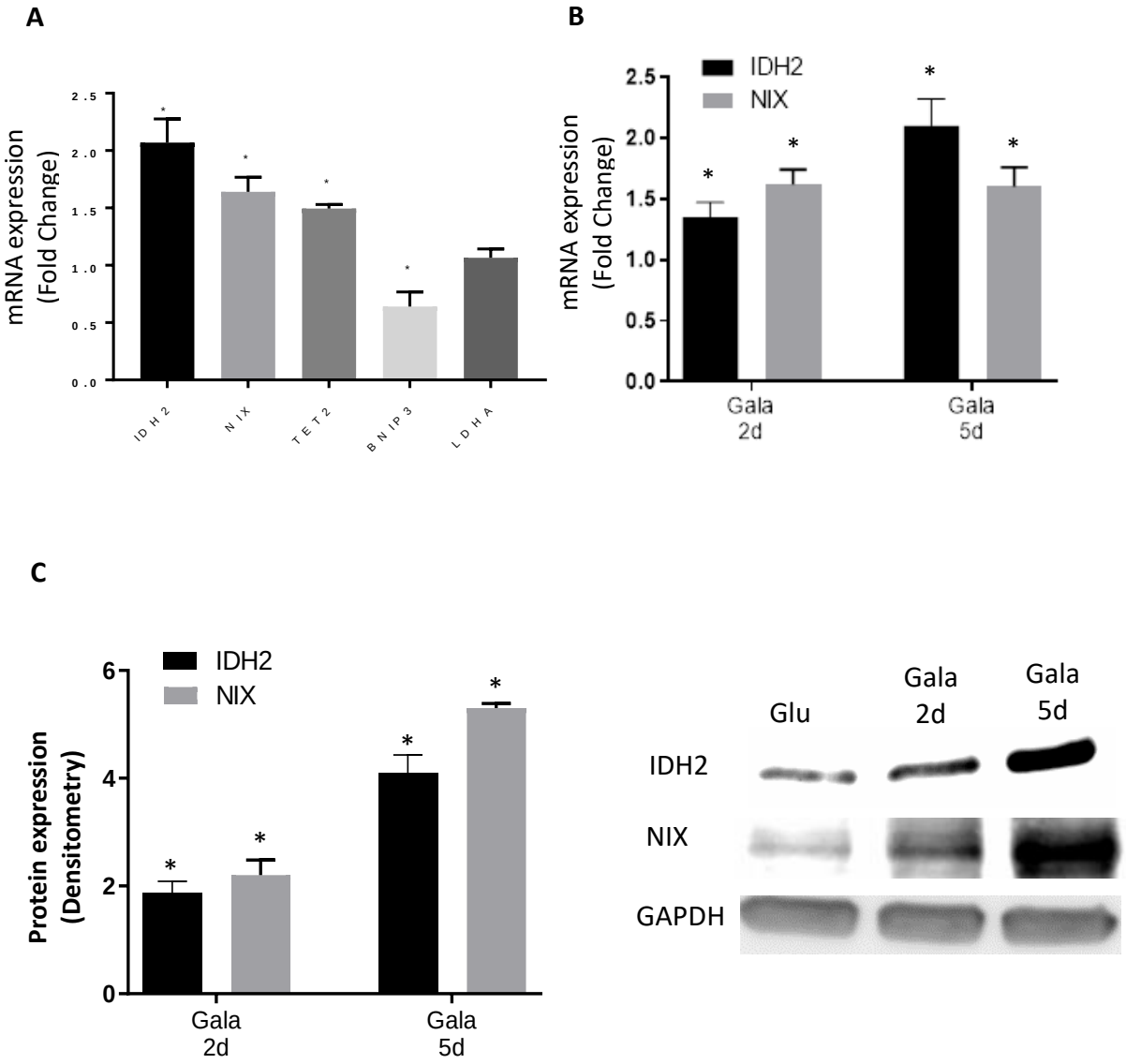


Figure 4.3: Galactose modulates the expression of IDH2, NIX, *TET2* and *BNIP3*. (A) mRNA expressions of *IDH2, NIX, TET2* were significantly increased, whereas, *BNIP3* was significantly decreased in galactose treated HeLa cells at 5 days of treatment. *LDHA* mRNA expression analysis showed no significant changes. (B) Highest regulated genes, *IDH2* and *NIX* in galactose treated HeLa cells were also analysed at day 2 and 5 of treatment. The RT-qPCR analysis showed a significant upregulation of *IDH2* and *NIX* mRNA and (C) western blotting analysis showed upregulation of IDH2 and NIX in a time-dependent manner, confirming previous findings from RT-qPCR analysis. Values are mean ±SEM (n=3), *p<0.05. (Glu: Glucose 25mM, Gala 2d: Galactose 25mM for 2 days, Gala 5d: Galactose 25mM for 5 days)

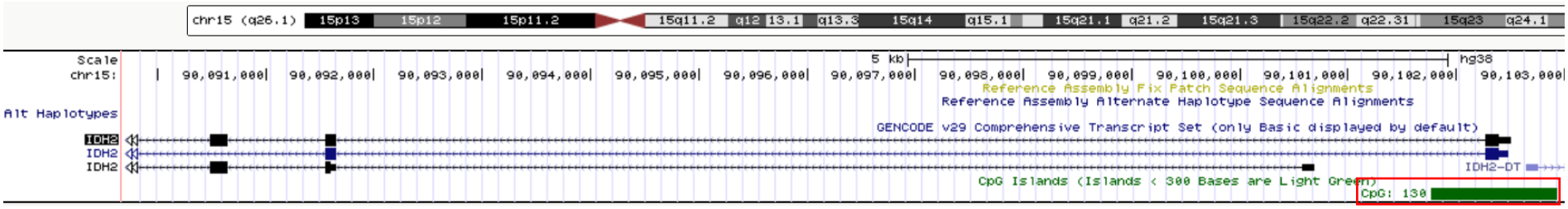
4.3.4. DNA methylation analysis of the *IDH2*, *NIX* and *BNIP3* promoter region

4.3.4.1 Galactose decreases methylation and increase hydroxymethylation of DNA promoter region of *IDH2* and *NIX*

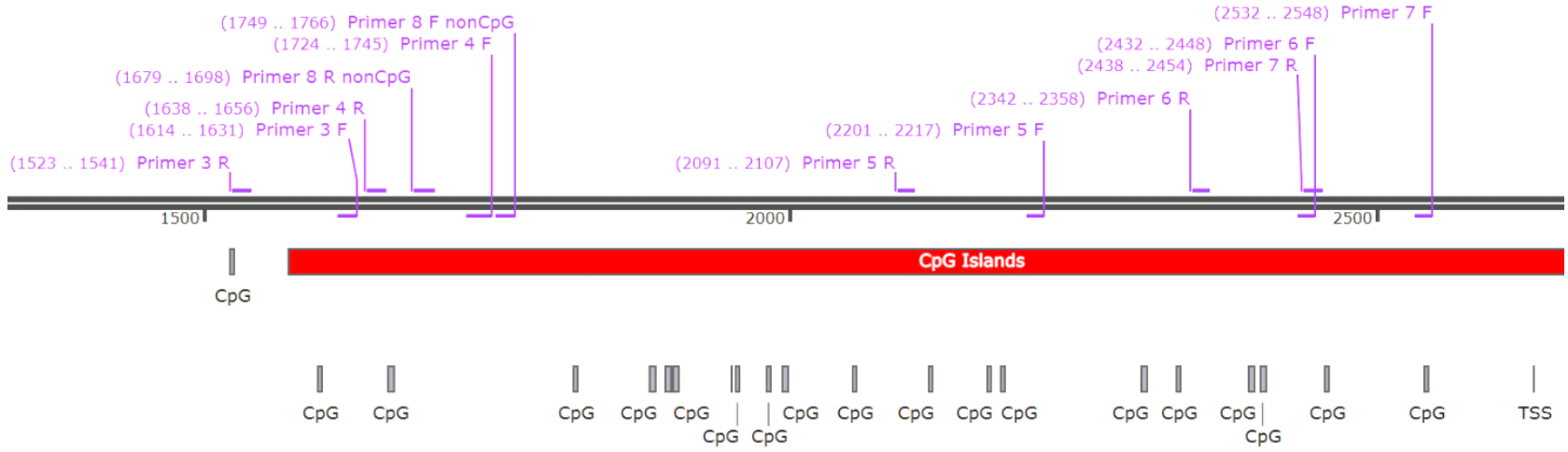
In order to examine the relationship between gene expression and promoter DNA methylation status of *IDH2 and NIX*, the target CpG and CCGG islands were identified using UCSC Genome browser (GB) (Dec. 2013 assembly genome (NCBI Assembly ID: 5800238) (GRCh38.p12)) (IDH2 promoter-Figure 4.4 A). Using GB, the range of potential CpG and CCGG islands were mapped on the promoter region within -1200 bp TSS (according to the default reference genome build). It was also indicated by GB, that the majority of target CpG and CCGG islands were located within -1000 bp from the TSS of *IDH2* and within -400 bp from the TSS of *NIX*. The identified CpG islands within the high frequency region were further subjected to DNA methylation analysis (IDH2-Figure 4.4B, NIX-Figure 4.5).

Initially, to examine promoter DNA methylation and hydroxymethylation of *IDH2* and *NIX*, a methodology combining the action of specific restriction enzyme (i.e. HpaII and MspI) and cytosine glycosylation with qPCR, was employed. Briefly, primers designed to target the amplification of *IDH2* and *NIX* promoter regions containing a CCGG island were utilised in SREMA assay. Specificity of primers used in SREMA were validated by detection of unique amplicons in agarose gel electrophoresis and qPCR melting curves. After selecting the optimal primers for qPCR, the analysis of *IDH2* and *NIX* was carried out alongside a control primer designed to target the genomic region excluding any CpG islands (non-CpG primers). The data obtained from SREMA analysis showed that galactose nutrient regime decreased DNA methylation and increased hydroxymethylation of both, *IDH2* and *NIX* promoter region. This observation was depicted in galactose treated HeLa cells, where significantly decreased levels of 5-mC and increased levels of 5-hmC content on *IDH2* (Figure 4.4 C and D) and *NIX* (Figure 4.5 A and B) promoter region were detected relative to control cells. Also, no change was observed in the region targeted by non-CpG control-primers. The overall promoter methylation status of *IDH2* using SREMA was significantly decreased by 0.25-fold and hydroxymethylation status increased by 1.35-fold, and *NIX* methylation decreased by 0.2-fold and hydroxymethylation increased by 1.5-fold in galactose nutrient regime of HeLa cells.

A



B



***IDH2* DNA promoter region**

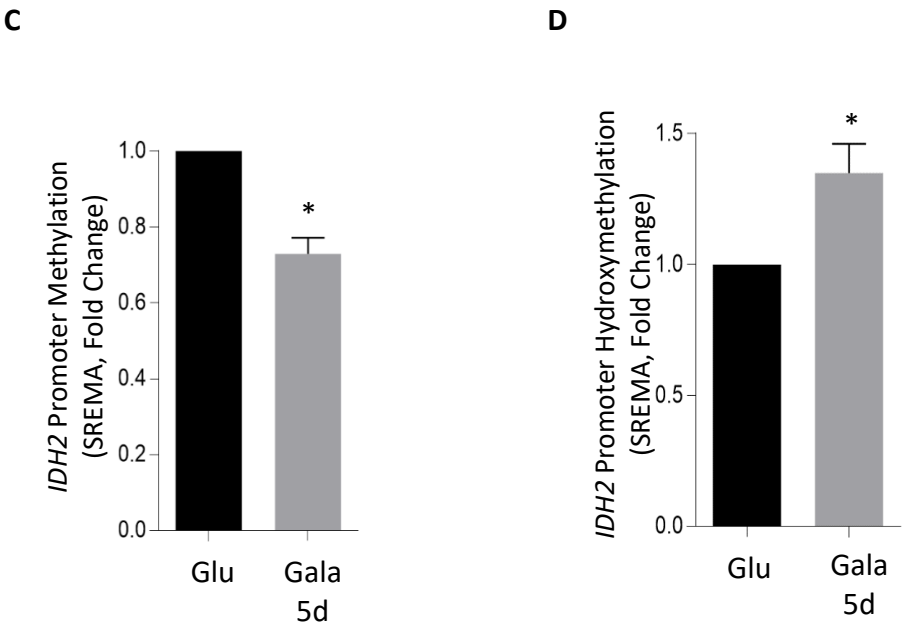


Figure 4.4: SREMA analysis of the *IDH2* DNA promoter methylation and hydroxymethylation in galactose treated HeLa cells. (A and B) Distribution of CpG islands in the *IDH2* promoter region. The gene of interest (i.e. *IDH2*) and predicted CpG islands are represented by green strand and rectangle (outlined in red). Each grey box represents a CCGG island within -1000bp upstream of TSS (high frequency CpG region identified using GB), and primers spanning these CCGG islands are highlighted above the *IDH2* DNA strand representation. After galactose treatment, the DNA promoter methylation and hydroxymethylation of *IDH2* in HeLa cells was significantly decreased and increased, respectively. This was confirmed using (C and D) SREMA. Values are mean ±SEM (n=4), *p<0.05. (Glu: Glucose 11mM, Gala 5d: Galactose 25mM for 5 days, TSS: Transcription start site)

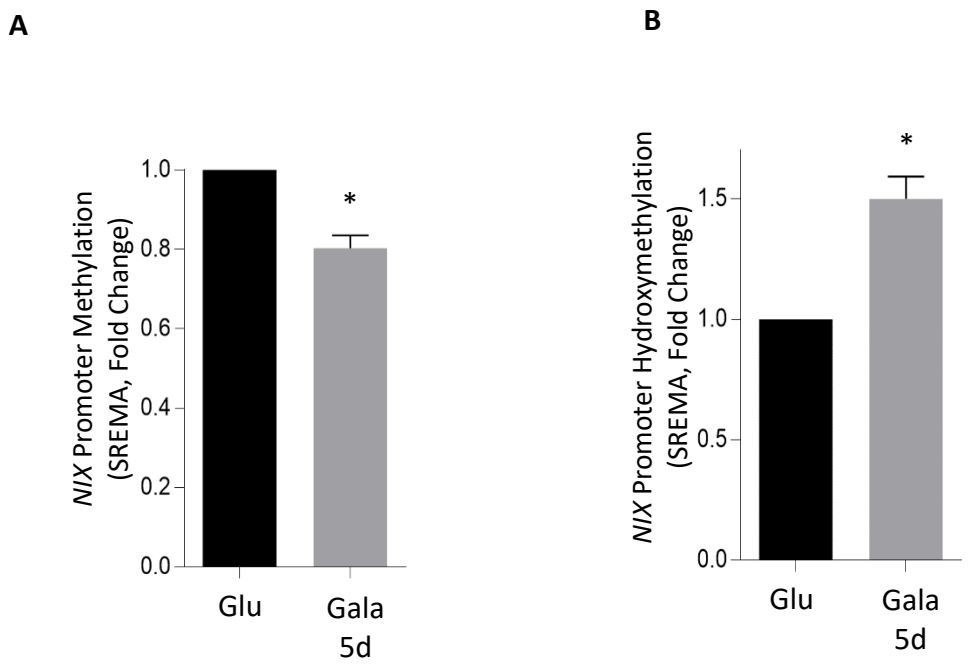


Figure 4.5: SREMA analysis of the *NIX* DNA promoter methylation and hydroxymethylation in galactose treated HeLa cells. Cells were subjected to galactose nutrient regime for 5 days. (A) DNA promoter methylation status of *NIX* was analysed using SREMA, the results obtained showed a significant decrease in the methylation status of *NIX* promoter region. (B) DNA promoter hydroxymethylation was analysed using SREMA, the results obtained showed increase in hydroxymethylation status of *NIX* promoter region in galactose nutrient regime. Values are mean ±SEM (n=3), *p<0.05. (Glu: Glucose 25mM, Gala 5d: Galactose 25mM for 5 days)

4.3.4.1. Galactose downregulates BNIP3 without significant changes in DNA methylation

In contrast to *IDH2* and *NIX*, *BNIP3* mRNA expression was downregulated in galactose treated HeLa cells. To further characterise the changes observed in RT-qPCR data, the protein expression of BNIP3 was assessed by western blotting. The data obtained showed significant downregulation of BNIP3 by 0.7-fold in galactose nutrient regime (Figure 4.6A), which is in agreement with mRNA expression analysis. Furthermore, to assess the DNA methylation status of *BNIP3*, the identical SREMA approach as described in chapter 3 (section 3.3.2.1) was employed. The data obtained showed no significant methylation changes at the promoter region (Figure 4.6B).

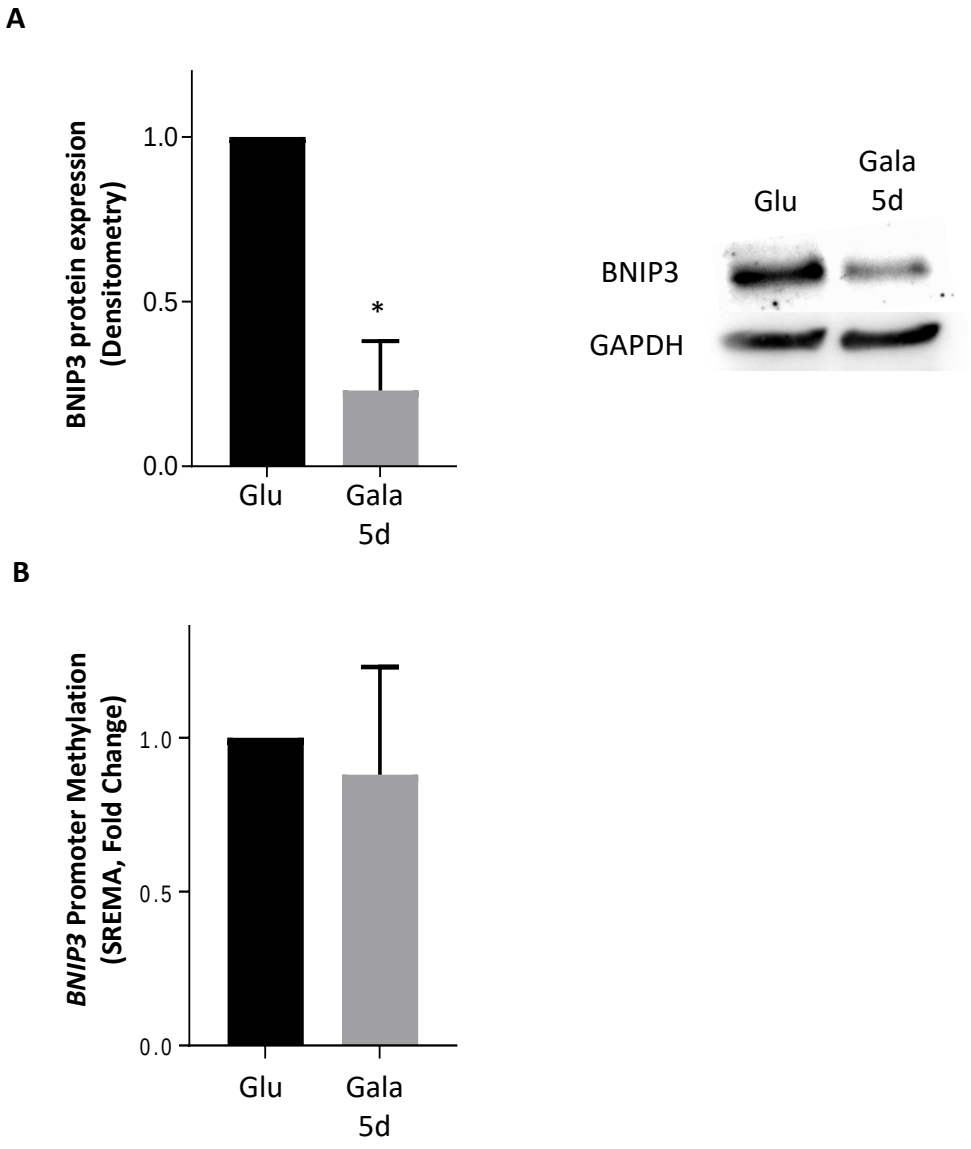


Figure 4.6: Galactose downregulates BNIP3 expression but displays no significant changes in methylation levels at the DNA promoter region. HeLa cells were subjected to galactose nutrient regime for a duration of 5 days. (A) The data obtained showed significant downregulation of BNIP3 protein expression, analysed by western blotting (normalised to GAPDH). (B) DNA promoter methylation status of *BNIP3* was analysed using SREMA. The data obtained showed no significant changes in the methylation level of the promoter region. (Glu: Glucose 25mM, Gala 5d: Galactose 25mM for 5 days)

4.3.5. Galactose negatively regulates DNMT3a at the post-transcriptional level

Previous data from this chapter demonstrated significant changes in DNA methylation status of *IDH2* and *NIX* promoter region in galactose treated HeLa cells. In order to extend these findings, the modulation of DNMT3a in galactose treated HeLa cells was assessed by RT-qPCR and western blotting. The data obtained showed a significant decrease in DNMT3a protein abundance in cells treated with 25mM galactose (Figure 4.7A), displaying a 0.6-and 0.8-fold downregulation after day 2 and day 5, respectively. However, RT-qPCR analysis showed no significant changes in *DNMT3a* mRNA levels in galactose treated HeLa cells at day 5 of treatment (Figure 4.7D). The lack of correlation between mRNA and protein expression, and previous data (Chapter 3) in rat INS-1 cells suggested that DNMT3a may be regulated by other factors, such as PTMs, that may mediate its protein content in HeLa cells. To address a possible PTM regulatory mechanism in the modulation of DNMT3a abundance, HeLa cells were treated with the proteasomal inhibitor MG132 at concentrations of 10 μM. MG132 treatment increased DNMT3a protein abundance by 1.5-fold and 1.4-fold at 2 and 4 hours, respectively (Figure 4.7B).

4.3.5.1. Galactose decreases histone acetylation in HeLa cells

After characterising the effect of galactose on the PTM-modulated DNMT3a protein abundance, the acetylation status of Histone 3, a surrogate assessment of nutrient treatment efficacy, was investigated. The data obtained showed substantial decrease of histone 3 acetylation by 0.6-fold and 0.95-fold at day 2 and 5 of galactose treatment, respectively in HeLa cells (Figure 4.7C). The acetylation changes observed in histones also revealed a time-dependent alteration, similar to DNMT3a.

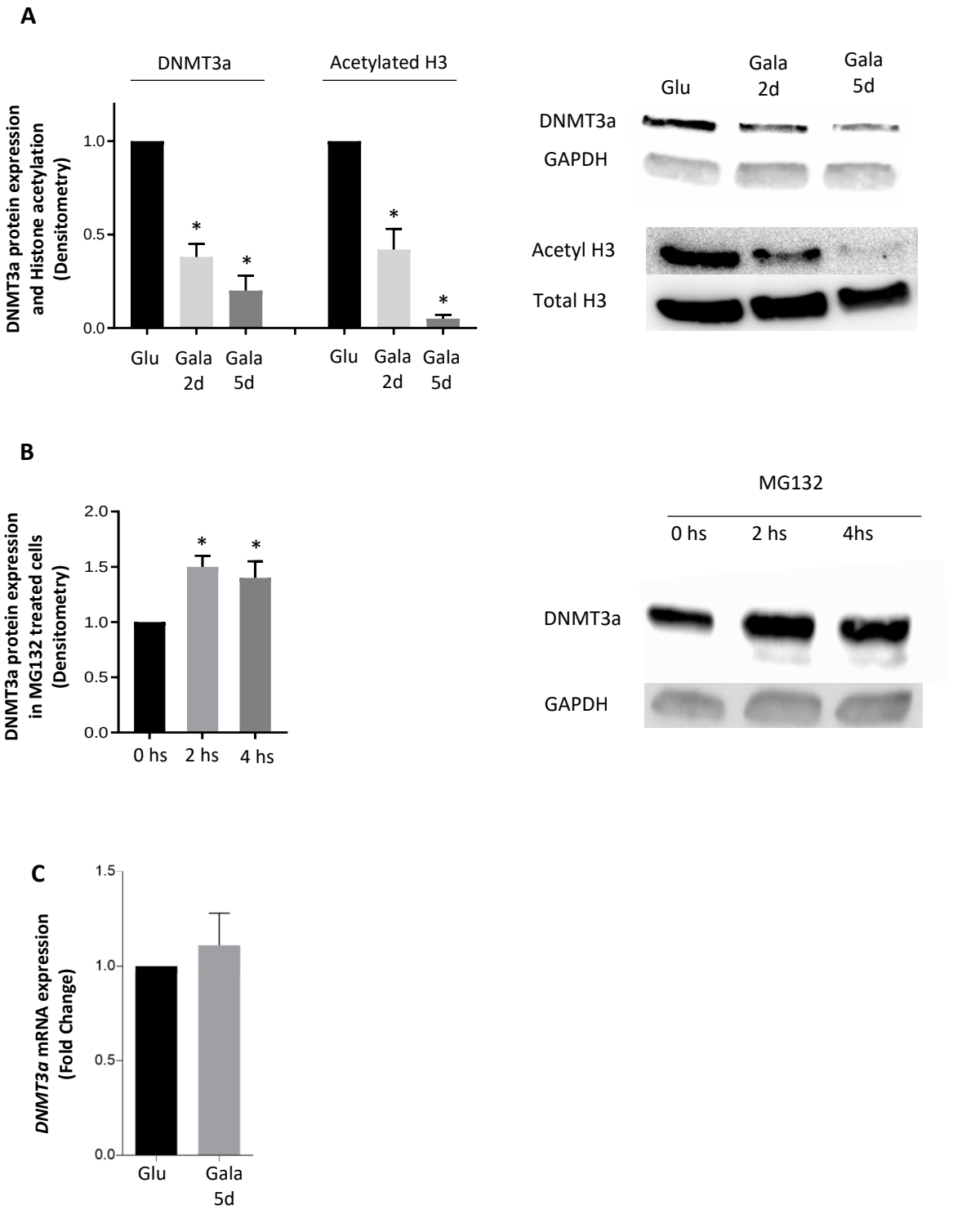

A
DNMT3a
Acetylated H3
DNMT3a protein expression and Histone acetylation (Densitometry)
1.0
0.5
0.0
*
*
*
Glu
Gala 2d
Gala 5d
Glu
Gala 2d
Gala 5d
Glu
Gala 2d
Gala 5d
DNMT3a
GAPDH
Acetyl H3
Total H3
B
DNMT3a protein expression in MG132 treated cells (Densitometry)
2.0
1.5
1.0
0.5
0.0
*
*
0 hs
2 hs
4 hs
MG132
0 hs
2 hs
4hs
DNMT3a
GAPDH
C
DNMT3a mRNA expression (Fold Change)
1.5
1.0
0.5
0.0
Glu
Gala 5d

Figure 4.7: Galactose decreases DNMT3a protein abundance and histone 3 acetylation. HeLa cells were treated with galactose nutrient regime for 2 and 5 days. (A) DNMT3a protein abundance showed significant downregulation in the presence of galactose, in time a dependant manner, assessed by western blotting. Changes in histone 3 acetylation were analysed by western blotting and demonstrated significant decrease in acetylation of galactose treated HeLa cells. (B) Cells treated with MG132 (proteasomal degradation inhibitor) increased the protein abundance of DNMT3a in a time dependant manner. (C) RT-qPCR analysis of *DNMT3a* showed no significant changes in galactose treated HeLa cells. (Glu: Glucose 25mM, Gala 2d: Galactose 25mM for 2 days, Gala 5d: Galactose 25mM for 5 days)

4.3.6. Galactose reduces proliferation and induces apoptosis

In order to investigate the functional consequence of galactose on HeLa cells, the rate of apoptosis and proliferation were assessed. This was achieved by subjecting HeLa cells to 25mM galactose for a duration of 5 days. Cisplatin was used as an experimental positive control for apoptosis/necrosis assay. The rate of apoptosis and necrosis were then assessed using Apoptosis/Necrosis Abcam kit. The data obtained showed a significant increase of apoptosis in galactose treated HeLa cells by 92%, relative to control. Whereas, necrosis analysis displayed no significant difference between control or galactose nutrient regime. In addition to the findings obtained from apoptosis assay, the proliferation in galactose treated HeLa cells showed significant decrease in proliferation by 87%, relative to control. The data collected from digital microscope also confirms the findings of proliferation assay, displaying a substantial decrease in the cells at 5 day of galactose nutrient regime.

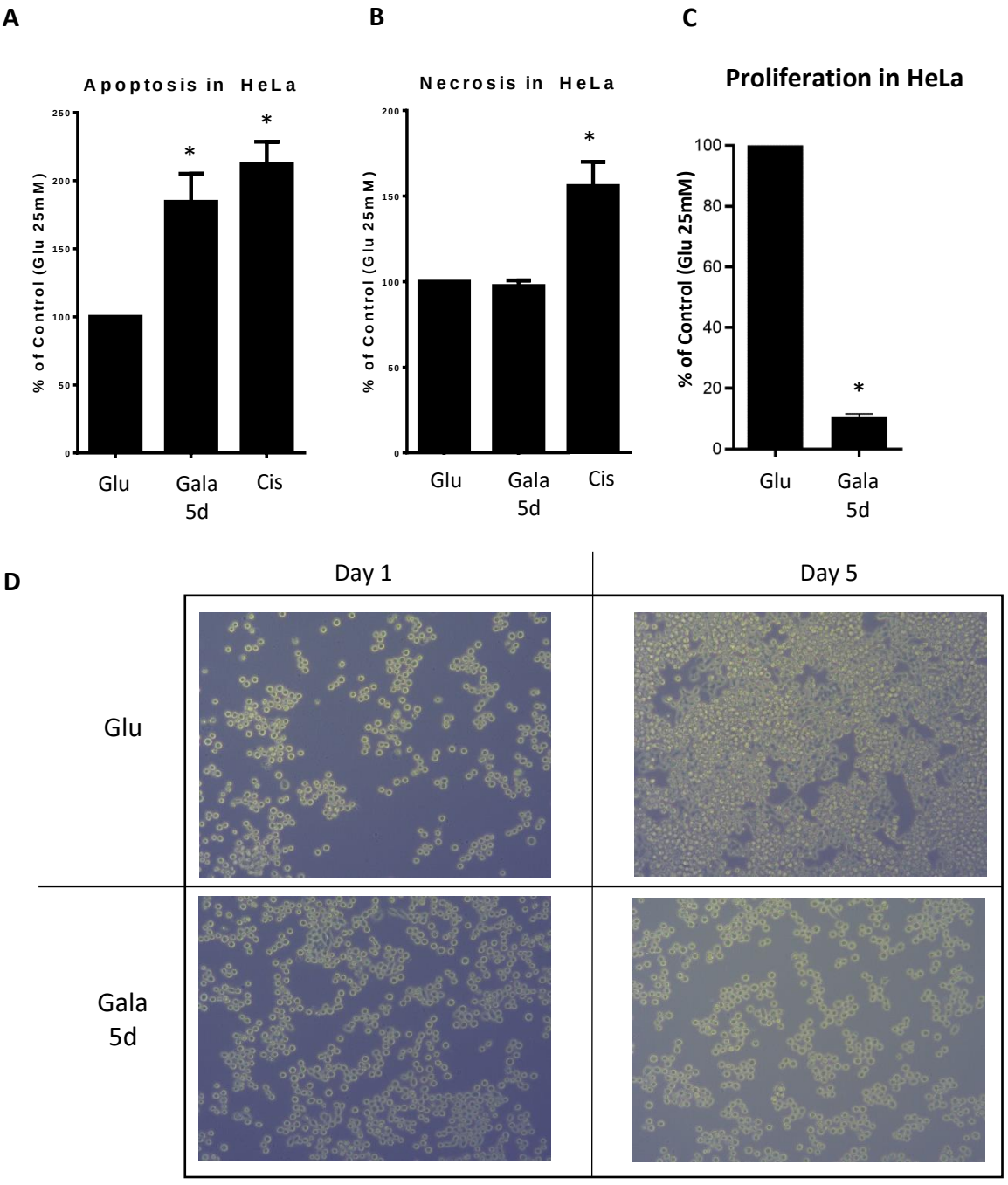


Figure 4.8: Galactose increases apoptosis and decreases proliferation in HeLa cells. Cells were treated with galactose (25mM) for a duration of 5 days. (A) The results obtained showed significant increase in cell apoptosis, as analysed by fluorescence based apoxin dye. (B) Moreover, no significant changes were observed in necrosis analysis of galactose treated HeLa cells. The positive control in apoptosis and necrosis assay was Cisplatin (Cis). (C, D) Cell proliferation was significantly decreased as analysed using WST-1 colorimetric assay and as demonstrated by digital microscopy. Values are mean ±SEM (n=3), *p<0.05. (Glu: Glucose 25mM, Gala 5d: Galactose 25mM for 5 days, Cis: Cisplatin)

4.3.7. IDH2 expression can be modulated by inducing hydroxymethylation at the promoter region

To validate the role of epigenetics in the regulation of IDH2 expression, a specific dCAS9-Tet1CD system was targeted to the DNA promoter region of the *IDH2* to induce hydroxymethylation. HEK293 cells were transfected with a combination of a specific guidance RNA and a construct encoding the fusion of a defective CAS9 and the catalytic domain of Tet1 and were used to induce IDH2 promoter hydroxymethylation and protein content. Figure 4.9 shows the hydroxymethylcytosine enrichment achieved in the *IDH2* promoter region by the dCAS9-Tet1CD system (over a 9-fold change), when compared to control cells. Similarly, the treatment resulted in a significant increase in IDH2 protein abundance (Figure 4.9B, over 2.8-fold change increment).

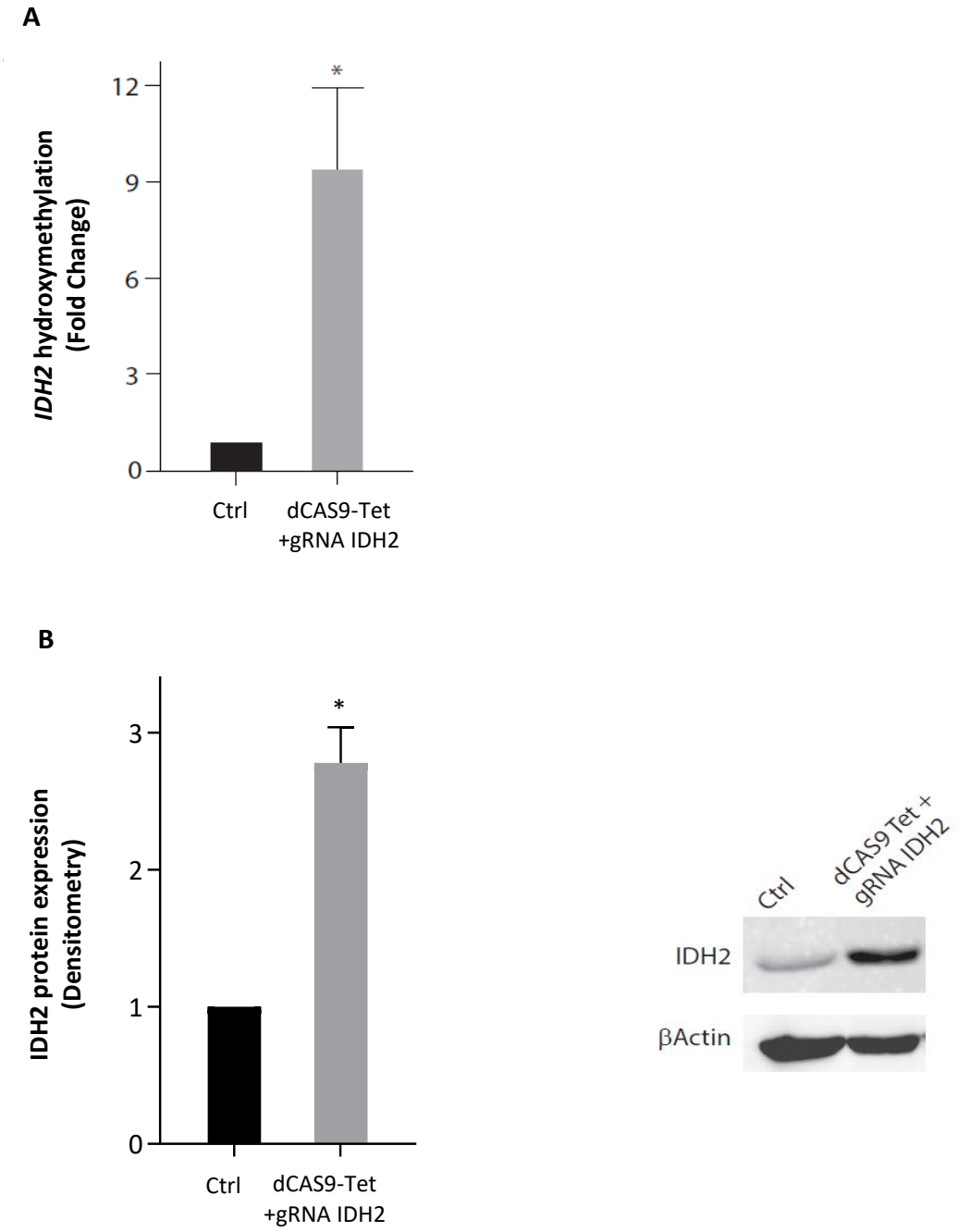


Figure 4.9: Changes in IDH2 expression by induction of DNA promoter hydroxymethylation. (A) The status of *IDH2* promoter DNA hydroxymethylation, induced by a specific dCAS9-Tet1CD + IDH2 gRNA system was assessed as described previously (section 2.5.3). HEK293 cells transfected with this system (and selected under puromycin for 2 days), showed an 9-fold increase in *IDH2* promoter hydroxymethylation, when compared to mock transfected cells. (B) Representative western blotting analysis from these cells showed a 2.8-fold increase in protein abundance, judged by densitometry. Values were normalised to internal housekeeping expression. Values are mean ±SEM (n=3), *p<0.05. (Ctrl: Untreated/Wild-type HeLa cells, dCAS-TET+gRNA IDH2: HeLa cells targeted by dCAS9 methodology)

4.4. Discussion

The incompetence of normal proliferating cell to generate sufficient ATP from glucose reactivates catabolism and induces cell cycle arrest (Heiden et al. 2009). However, cancer cells evade this mechanism and perform aerobic glycolysis in a highly proliferative state; this phenomenon is also known as 'The Warburg effect'. Nevertheless, 'The Warburg effect' in cancer cells remains to be fully understood. An important factor also noted by Warburg and colleagues was that depriving the cancer cells of glucose and oxygen did not affect their viability. To our understanding of cancer cell's metabolic changes, this chapter confirms that galactose is a key regulator of glycolysis and deregulates key mitochondrial-related gene expression by inducing epigenetic changes in HeLa cells. Our data demonstrates the involvement of galactose nutrient regime in regulating metabolism and epigenetic profile of cancer cells. We also found that galactose induces apoptosis and reduces proliferation in HeLa cells.

4.4.1. Lactate production and Mitochondrial activity

Aerobic glycolysis and high levels of lactate production are considered the fundamental metabolic changes frequently detected in cancer cells. To assess this metabolic shift, HeLa cells were subjected to galactose treatment for a duration of 5 day, followed by subsequent metabolic and epigenetic investigations. Cells treated with galactose exhibited a substantial reduction in lactate production. Consistently, the generation of lactate has been linked with aerobic glycolysis (Fantin et al. 2006), a process often observed in cancer. Jimenez et al. (2011) correspondingly reported the reduction in lactate production in Chinese Hamster Ovaries cells treated with galactose. Lane et al. (2015) also reported similar findings in Chromocytes (primary cells). During the presence of glucose, highly proliferative cells produce biomass and lactate along with other metabolic components, whereas, in the depletion of glucose, cells tend to the consume galactose and lactate (Jiménez et al. 2011). These findings suggest that the reduction in lactate observed in our study, could be accounted not only by downregulation in lactate production but also by the uptake of lactate into the cells.

Rogatazki et al. (2015) suggested that lactate production is a result of a near equilibrium reaction, this equilibrium invariably favours the production of lactate from pyruvate, and support the cytosol-to-mitochondria lactate shuttle, during this process the activity of LDH defines the flow of lactate. In order to extend on the findings from lactate assay, *LDHA* mRNA was assessed by RT-qPCR analysis. The *LDHA* mRNA expression displayed no significant regulation in galactose treated HeLa cells. The reduction in lactate production and *LDHA* mRNA expression do not correlate in our study. Similar findings were observed in the cerebral cortex of rat, demonstrating the lack of correlation between LDHA activity and expression (Nunes et al. 2015). LDHA activity has previously been found to be regulated by PTMs, such as acetylation (Zhao et al. 2013), which could suggest the differences between mRNA expression and lactate production observed in our study.

mitDNA copy number and mtCOII are representatives of mitochondrial health and function. Defects in mtCOII have been previously linked with reduced Complex IV activity in mitochondrial diseases (Clark et al. 1999); complex IV, is a major regulator of OXPHOS (Signes, Fernandez-Vizarra 2018). Therefore, studying the regulation of mitDNA copy number and mtCOII in galactose treated HeLa cells was an important step in understanding the underlying mechanisms involved in galactose-mediated metabolic changes. The data obtained from mtCOII expression and mitDNA copy number suggested that galactose upregulates mitochondrial activity. mitDNA is a hallmark of mitochondrial health (Rooney et al. 2015), proliferation (Baldelli et al. 2014) and is associated with OXPHOS (Deus et al. 2015). Moreover, mtCOII is a mitDNA-encoded structural subunit that forms the catalytic core of Complex IV (Fernández-Vizarra et al. 2009), hence the importance of assessing mitDNA and mtCOII together. The upregulation of mtCOII was observed after 2 days of galactose nutrient regime, and this change was significantly increased after 5 days. Li et al. (2006) reported that complex IV suppression dictates a reduction in total cellular respiration. Aguer et al. (2011) correspondingly reported that galactose increased complex IV activity in myotubes leading to enhanced aerobic metabolism. Additionally, Deus et al. (2015) showed that galactose upregulated mitDNA content in H9c2 cardiomyoblast cells. Collectively, these findings and our data suggests that galactose upregulates mitDNA copy number and mtCOII protein expression, that may lead to increased OXPHOS.

Moreover, mitDNA-depleted cells (rho(0)) demonstrated low mitochondrial membrane potential loss-mediated apoptosis (Ferraresi et al. 2008), indicating that mitDNA can play an important role in cancer's metabolic switch in order to avoid apoptosis. This data in

combination with ours suggest that mitDNA downregulation may be an important factor for cancer cell's survival, and galactose nutrient regime can revert this effect by restoring mitDNA content. Moreover, mitDNA copy number correlates with the expression of respiratory genes (van Gisbergen et al. 2015). Seidel-Rogol et al. (2002) showed the effect of depleting and repleting the mitDNA content of HeLa cells on mitochondrial transcripts and transcription. This ethidium bromide-based regulation demonstrated that the mitochondrial proteins are highly coordinated with the mitDNA content. Our data also supports these findings, where we found that galactose increases mitDNA copy number and mtCOII expression simultaneously.

In our study, we aimed to assess the role of galactose in regulating key mitochondrial proteins including IDH2, and metabolites including α-KG. IDH2 is dysregulated in various cancers, and increased IDH2 expression correlates with good patient prognosis (Lv et al. 2012). In addition, α-KG derived from IDH2/3, is an important intermediate in the TCA cycle (Rojanarata et al. 2018). IDH2 and α-KG levels are important aspects of the mitochondrial activity and both play a significant role in OXPHOS and tumour suppression (YI et al. 2016; Lv et al. 2012). Therefore, we assessed the intracellular levels of α-KG in galactose treated HeLa cells. The data obtained showed significant increase in α-KG levels at 5 days of galactose nutrient regime. This data also correlates with the regulation of mtCOII and mitDNA content of HeLa cells.

Previously, IDH2 expression abnormalities were reported in multiple cancers: presenting as downregulation and upregulation, and in some cancers downregulation at early stages followed by upregulation in advanced stages (Lv et al. 2012; Green, Beer 2010; Fu et al. 2010). These findings require further investigations to elaborate on the role of IDH2 in cancer. In our study, IDH2 protein expression analysis by western blotting showed a significant upregulation in galactose treated HeLa cells. Increased cellular levels of IDH2 expression and α-KG content have been previously associated with increased mitochondrial function (Vohwinkel et al. 2011), which supports our findings of mitochondrial function in galactose treated cells. Moreover, IDH2 mutations in cancer have been suggested to incur with simultaneous decrease in the production of α-KG, and gain of a new function leading to the production of 2-HG (Reitman et al. 2010; Yang et al. 2012; Fu et al. 2010). 2-HG, an oncometabolite known to promote tumour growth by inhibiting TET family (Janke et al. 2017). 2-HG mediated TET inhibition hinder 5-mC conversion to 5-hmC, resulting in global DNA hypermethylation at promoter regions (Carbonneau et al. 2016).

However, in normal cells, Malate dehydrogenase, 3-phosphoglycerate and LDHA have been shown to promiscuously produce 2-HG, that likely serves a normal biological function (Ye et al. 2018). 2-HG is also a prominent hallmark of 2-hydroxyglutaric aciduria, a rare neurometabolic disease caused by mutations in *SLC25A1* (mitochondrial citrate carrier) leading to the accumulation of 2-HG (Nota et al. 2013). The role of 2-HG in cancer has contradictory evidence, where the decreased levels of α-KG have been strongly associated with the metabolic shift commonly observed in cancer cells (Li et al. 2018; Fu et al. 2010).

In our study, we focused on the changes in α-KG levels and the metabolic changes caused by galactose, including the reduction in lactate production, increase in mitochondrial function and IDH2 expression. Mitochondrial fusion also plays an important role in cellular metabolism, however previous studies have found fusion index of cells was not regulated by galactose (Aguer et al. 2011), hence it was not explored in our study.

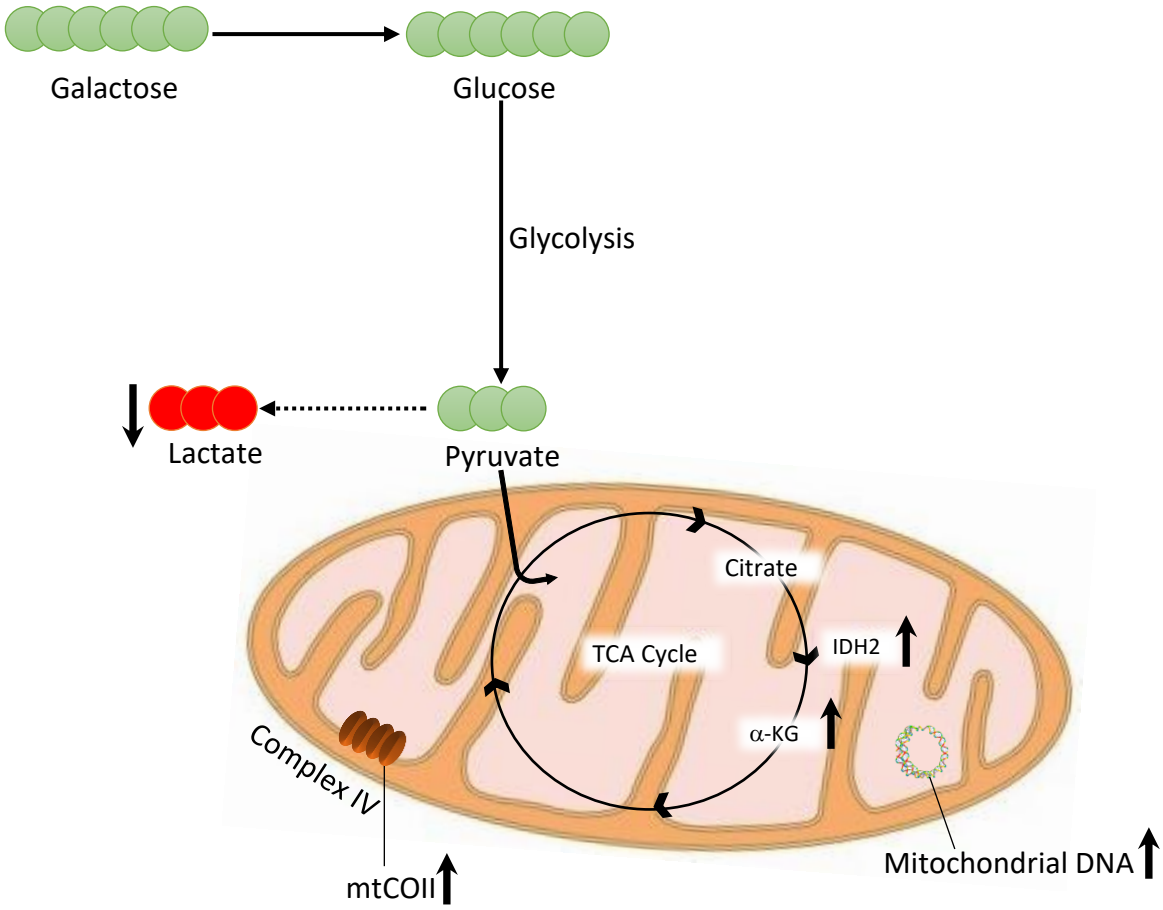


Figure 4.10: Schematic diagram depicting the effect of galactose on lactate production and mitochondrial function. Galactose nutrient regime was able to reduce the production of lactate in HeLa cell. Additionally, upregulation of mitDNA copy number, mtCOII protein expression, IDH2 expression and α-KG levels was observed. Collectively, indicating the reversion of 'The Warburg effect' in cancer cells.

4.4.2. Galactose induces epigenetic changes in *IDH2* and *NIX*

One of the main aims of this chapter was to characterise the effect of galactose on promoter DNA methylation of *IDH2*, *NIX* and *BNIP3.* In order to achieve this aim, we assessed DNA methylation status of *IDH2*, *NIX* and *BNIP3* by SREMA combined qPCR methodology. The data obtained revealed the dual role of galactose in simultaneously regulating DNA methylation and hydroxymethylation of *IDH2* and *NIX* promoter regions. Moreover, the data collected demonstrated a positive correlation between gene expression and 5hmC levels, and a negative correlation between gene expression and 5mC levels of *IDH2* and *NIX*.

Rheb promotes mitophagy via interactions with NIX and LC3-II. Melser et al. (2013) suggested that stimulation of mitochondrial OXPHOS enhances mitophagy by recruiting GTPase Rheb to the mitochondrial membrane. Other studies have demonstrated, that NIX overexpression can promote mitophagy without substantial loss in mitochondrial content and thereby resulting in improved bioenergetics of the cell (Koentjoro et al. 2017; Zhang, Ney 2009). IDH2 and NIX form an integral part of mitochondria, either structurally or functionally, and the expression of both genes is substantially reduced in cancer, correlating with poor patient prognosis (Zhang, Ney 2009; YI et al. 2016). In our study, RT-qPCR and western blotting analysis showed a significant upregulation of NIX expression in galactose treated HeLa cells. The importance of NIX in cancer was previously explored by Du et al. (2017), which revealed that the platinum-based cisplatin drug mechanism of action involves NIX pathway mediated by mir-30c leading to apoptosis. Moreover, Wu et al. (2016) demonstrated that platinum conjugated to galactose had significantly higher toxicity to cancer in comparison to other commonly used drugs, including cisplatin and oxaliplatin. This increase in cytotoxicity was hypothesised to be associated with galactose motif led advantages associated with 'The Warburg effect'. Our data in conjunction with these studies indicates that both galactose and platinum may include NIX mediated apoptosis pathways, therefore the observed effect of increased toxicity of platinum-conjugated-galactose compared to cisplatin or oxaliplatin could be a result from the combined action of galactose and platinum through NIX mediated pathway.

Other mitochondrial adapter proteins including BNIP3 are also shown to regulate mitophagy (Chang et al. 2017). However, in our study we found galactose downregulated BNIP3 at mRNA and protein level at 5 days of treatment. NIX and BNIP3 are proteins with homology to BCL2 in the BH3 domain, and both proteins induce cytochrome c release

leading to apoptosis (Zhang, Ney 2009). Both (NIX and BNIP3) are directly activated by hypoxia induced factor (HIF) (Bellot et al. 2009). Despite these similarities, our data demonstrated a substantial difference in the expression of NIX and BNIP3 in galactose nutrient regime, therefore, suggesting galactose mediated NIX regulation can be independent of HIF. NIX, but not BNIP3, has been suggested to be involved in mitophagy during development, suggesting a distinct role of the two proteins in certain cellular processes (Ney 2015). Furthermore, unlike BNIP3, NIX expression is regulated by p53 binding to its motif site on *NIX*, thereby inducing its expression. Moreover, NIX also mediates red blood cell differentiation by mitophagy (Fei et al. 2004; Sandoval et al. 2008). Collectively, these findings and our data suggests that NIX and BNIP3 can be regulated inversely, and this differential regulation correlates with DNA methylation and hydroxymethylation status of *NIX* promoter region. Interestingly, Schweers et al. (2007) suggested that cells are unable to carry out selective mitochondrial clearance at the autophagosome in the absence of NIX, therefore, suggesting that NIX is essential for selective mitochondrial elimination. However, this autophagosome mediated process doesn't require BAK, BAX or BLC-X_L. In contrast, BNIP3 mediated proapoptotic activity requires BAX or BAK downstream functions (Zhang, Ney 2009; Wei et al. 2001); these findings demonstrate the functional differences between NIX and BNIP3, suggesting the two proteins may involve distinct upstream and downstream pathways.

In addition to mitochondrial activity, IDH2 is directly associated with global DNA increase of 5hmC levels, and mutations in IDH2 genes leads to impaired 5hmC levels in cancer (Rampal et al. 2014). For example IDH2 dysfunction in gastric cancer results in decreased 5hmC and increased 5mC levels, leading to cancer progression (Chou et al. 2016). Increased 5hmC levels have previously been shown to be associated with decreased 5mC levels, therefore demonstrating an inverse correlation between the two DNA modifications (Vető et al. 2018). IDH1, a structurally and functionally similar enzyme to IDH2, has been shown to directly influence the 5hmC levels in tumour, which also correlated with α-KG levels (Ge et al. 2018; Al-Khallaf 2017). These findings indicate that IDH2 is an important regulator of cellular epigenetics in addition to its role in mitochondrial function. Furthermore, α-KG is an essential co-factor that facilitates the conversion of 5mC to 5hmC by TET family of proteins. The association between the IDH2 expression, α-KG and 5hmC levels have been previously described (Mahajan et al. 2017). Interestingly, changes in 5hmC levels in IDH mutant cancers, is usually denoted to the modulation of TET proteins (Pelosi et al. 2016). However, IDH and TET mutant cancers express significant clinical differences; suggesting

distinct differences between the two proteins (Inoue et al. 2016). These findings also indicate that the deregulation of IDH2 and α-KG levels can exhibit a global effect on DNA methylation status in cancer cell. Collectively, these findings suggest, the observed 5hmC increase and 5mC decrease of *NIX* promoter region can be mediated by IDH2. *NIX* has previously been shown to be downregulated by hypermethylation of DNA promoter region in hepatocellular carcinoma (Calvisi et al. 2007), suggesting DNA methylation plays an important regulatory role for this protein.

Additionally, our data showed no significant DNA methylation changes on *BNIP3* promoter region in galactose treated HeLa cells. *BNIP3* has previously been shown to be regulated by DNA methylation in other studies (Murai et al. 2005), but our data doesn't correlate with these findings. However, Diest et al. (2010) demonstrated that *BNIP3*, in invasive breast cancer, is not regulated by DNA promoter methylation, suggesting an alternative regulatory pathway may mediate BNIP3 expression in galactose treated HeLa cells. Our combined findings of *NIX* and *BNIP3* expression and epigenetics contributes to our understanding of how multiple pathways may regulate mitochondrial turnover and therefore can be explored for cancer treatment.

The mechanisms involved in DNA methylation alterations in cancer, remain largely not understood. In order to advance this understanding, in our study, DNMT3a, a *de novo* methyltransferase, that plays an important role in gene regulation by facilitating active promoter methylation (Jia et al. 2016) was examined in galactose treated HeLa cells. Notably, western blotting analysis showed downregulation of DNMT3a in a time-dependant manner. However, RT-qPCR analysis showed no regulation of *DNMT3a* mRNA level. It was previously shown that DNMT3a downregulation correlates with improved patient survival and lower recurrence in lung cancer patients, which also correlated with reduced global DNA methylation (Liu et al. 2018). In terms of DNMT3a regulation, Gu et al. (2017) analysed chorionic villi of early embryos that reached an growth arrest, this study found that DNMT3a was significantly downregulated in these villi. However, this downregulation was only evident at the protein level, but not at the mRNA level. These findings relate to our data in terms of discrepancies observed between DNMT3a mRNA and protein levels.

Furthermore, to demonstrate the reversibility of epigenetic modification in cancer cell by genetic interventions, we utilised a targeted dCAS9-Tet1CD system to modulate the IDH2 promoter hydroxymethylation. The Tet1 enzyme belongs to a family of proteins, catalysing

the conversion of 5-mC in 5-hmC, an epigenetic change associated with the induction of gene expression (Kroeze et al. 2015). The fused protein between a nuclease defective CAS9 (dCAS9) and the catalytic domain of Tet1 allowed a targeted action of this enzyme on a methylated promoter. HEK293 cells, transfected with a combination of a specific IDH2 promoter guidance RNA and dCAS9-Tet1CD, showed a significant increase in hydroxymethylation of *IDH2* promoter region leading to increased protein expression.

4.4.3. Modulation of DNMT3a and Histone acetylation

DNA methylation is carried out by DNMT1, DNMT3a and DNMT3b (Robertson et al. 1999); DNMT1 is widely studied because of its involvement in cell cycle and maintenance of DNA methylation during cell division (Du et al. 2010). DNMT3a and its function in somatic cells remains vastly unexplained. A number of studies have suggested that DNMT1 is regulated by protein acetylation (Cheng et al. 2015; Peng et al. 2011), and depending on the targeted Lysine, the PTM can increase or decrease the protein activity. These findings in combination with our previous data in this chapter led us to speculate the role of PTM in DNMT3a. Therefore, HeLa cells were treated with MG132, a proteasomal degradation inhibitor. The data obtained showed that DNMT3a protein abundance increases in a time-dependant manner. Therefore, suggesting that DNMT3a degradation can be mediated by ubiquitin proteasome pathway and modulated by PTM. Another aspect of the investigation was to address the histone acetylation status in galactose treated HeLa cells. The data obtained showed a significant and time-dependant decrease in acetylation of histone in galactose nutrient regime, demonstrating a similar trend to DNMT3a protein abundance. Histone acetylation is associated with transcriptional upregulation leading to cell growth and proliferation (Cai et al. 2011). Combined with our findings, these results add to the understanding of the epigenetic changes that can be reverted by nutrients in cancer. Epigenetic modification also depend on specific metabolites (Tran et al. 2017). For example, acetylation of histones is carried out by HATs that require acetyl-CoA to carry out their function (Pons et al. 2009). Emerging insights suggests that modulation of these specific metabolites can regulate the function of the associated enzymes leading to epigenetic modifications (Su et al. 2016; Lempradl et al. 2015; Tran et al. 2017). Collectively, these findings suggest that changes in metabolism are reflected in epigenetics of the cell, which supports our findings of the changes mediated by galactose in mitochondrial activity and IDH2 expression that inversely correlate with DNA methylation and histone acetylation.

The impact of metabolic changes on histones is already established, multiple metabolic pathways including glycolysis and β-oxidation can lead to changes in acetyl-CoA and therefore regulate the acetylation of histones (Etchegaray, Mostoslavsky 2016). In support of this, Peleg at al. (2016) also suggested that the level of histone acetylation is susceptible to metabolites such as acetyl-CoA. Additionally, Acetyl-CoA has been shown to be tightly regulated in yeast and controls growth and survival (Cai et al. 2011; Cai, Tu 2011). In cancer cells, the pyruvate derived from glycolysis enters the truncated TCA cycle, and instead of being converted into α-KG by IDH2, it gets exported into cytosol as citrate by the tricarboxylic transporter, followed by ACLY mediated conversion into acetyl-CoA (Hatzivassiliou et al. 2005). These findings combined with our data suggests that galactose leading to increased IDH2 expression, mtCOII expression and mitDNA content may subsequently result in decreasing the pool of intracellular acetyl-CoA by modulating the TCA cycle; this can be further supported by the increase in intracellular α-KG levels. Speculatively, the combined data of histone acetylation, DNMT3a protein abundance and PTM susceptibility of DNMT3a, suggests that DNMT3a might therefore be regulated by acetylation.

DNA methylation and histone acetylation are conserved between different species and serve as major gene regulatory mechanisms. For example, yeast cells have been shown to adapt their epigenetic profile and consequently the gene expression according to nutrient availability. Histone acetylation and deacetylation is usually associated with increase and decrease in transcription, respectively (Etchegaray, Mostoslavsky 2016). However, our data shows the complexity of the interplay between histone acetylation and DNA methylation. In our study, galactose nutrient regime reduced histone acetylation and DNA methylation, and increased hydroxymethylation that correlated with increased mRNA and protein expression of IDH2 and NIX. Moreover, DNMT3a was previously shown to bind with the histone 3 tails, thereby, increasing the DNA methylation by 8-fold globally. Therefore, the IDH2 and NIX upregulation observed in our study could have resulted from the dual action of DNMT3a. Firstly, because of decreased protein abundance of DNMT3a, and secondly, because of its decreased interactions with histone tails (Ooi et al. 2007; Li et al. 2011). This connection between DNMT3a and histone tails was suggested to be a DNA methylation checkpoint, suggesting that the deacetylation of histone 3 observed in our study can result in lower accessibility of histone tails, leading to reduced DNMT3a mediated DNA methylation.

4.4.4. Cancer cell proliferation and apoptosis modulation by galactose

The changes in metabolism, including 'The Warburg effect' has been extensively studied and linked to cancer proliferation and progression. IDH2, an important part of central metabolism, generates α-KG from the oxidation of isocitrate. Moreover, the downregulation of IDH2 has been shown to increase cancer proliferation (Zou et al. 2017; Lv et al. 2012). Therefore, we assessed the proliferation of HeLa cells following our findings of IDH2 in galactose nutrient regime. The data obtained showed that galactose significantly reduced the proliferation of HeLa cells at 5 days of treatment. The connection between metabolism and cell proliferation was previously demonstrated and is extensively discussed by Colombo et al. (The principal investigator of our group) (2012).

Understanding the mechanism of galactose is not only important for therapeutics in cancer but also for non-cancerous pathologies (e.g. diabetes) and normal cells (e.g. T lymphocytes) (Moncada et al. 2012; Swisa et al. 2017). Galactose has been shown to prevent cell death by reducing Endoplasmic reticulum (ER) stress. Moreover, radiolabelled tracer studies demonstrated that the effect of galactose on cells can last even after the cells have been transferred from galactose to glucose (Kase et al. 2013). These findings combined with ours suggest that the lasting effect of galactose on cellular metabolism can be due to the epigenetic modification (i.e. DNA methylation and histone acetylation) introduced during this nutrient regime. Epigenetic modifications are commonly described as epigenetic memory. In terms of epigenetics, a stable gene expression profile can be adapted due to environmental changes and maintained in the cells for the duration of life cycle (D'Urso, Brickner 2014). Cancer cells and fibroblasts cultured in galactose instead of glucose has been shown to shift their metabolism towards OXPHOS instead of glycolysis (Marroquin et al. 2007; Robinson et al. 1992). The mechanism of this shift can provide essential insights into the 'The Warburg effect'. In our study, we show that the shift in metabolism in galactose treated HeLa cells led to changes in DNA methylation, hydroxymethylation, histone acetylation and possibly DNMT3a PTM. Moreover, IDH2 and NIX, known to be associated with cell proliferation and apoptosis, were upregulated in galactose treated HeLa cells. Subsequently, leading to reduced proliferation and increased apoptosis. These findings, at least in part suggest mechanisms or purpose for 'The Warburg effect'.

Otto Warburg's observations link the impairment of respiration in cells to mitochondria, resulting in a switch to aerobic glycolysis. This modulation of metabolism has been shown to assist cell proliferation by facilitating the uptake of nutrients and biosynthesis of amino

acids, lipids and nucleotides (Heiden et al. 2009). Sasaoka et al. (2018) correspondingly to our data also demonstrated reduced proliferation in galactose treated HEK293 cells. Yi et al. (2016) demonstrated that downregulation (RNAi mediated) of *IDH2* led to a pro-proliferative effect in MG-63 and Saos-2 osteosarcoma cells, and it was also noted that IDH2 was inversely correlated with cancer metastasis. AML, Glioma, chondrosarcoma and cholangiocarcinoma are commonly associated with *IDH2* mutations. In these cancers, IDH2 wild-type activity and α-KG production are marginally decreased, corelating with increased proliferation (Fujii et al. 2016). Overall, these findings suggest that IDH2 and α-KG are associated with cellular proliferation. Notably, our data shows galactose increases the expression of IDH2 and production of α-KG: these two changes are interconnected and likely contributed to the observed reduced proliferation in HeLa cells treated with galactose.

As discussed previously in this chapter, *NIX,* a pro-apoptotic gene, has been shown to be frequently downregulated in cancer, and correlating with poor patient prognosis (Zhang, Ney 2009; Du et al. 2017). Our findings from galactose treated HeLa cells showed upregulation of NIX, therefore we investigated the effect of galactose on apoptosis in HeLa cells. The data obtained showed that galactose induced apoptosis in HeLa cells. Although necrosis has been considered an unregulated and passive form of cell death, recent findings suggest that under extreme conditions the cells induce necrosis in a regulated manner (Proskuryakov, Gabai 2010). Peng et al. (2018) demonstrated, in pancreatic acinar cells, that galactose reduced the rate of necrosis. Conversely, in our study, we examined the effect of galactose on necrosis in HeLa cells. However, we found no significant changes in cells treated with galactose.

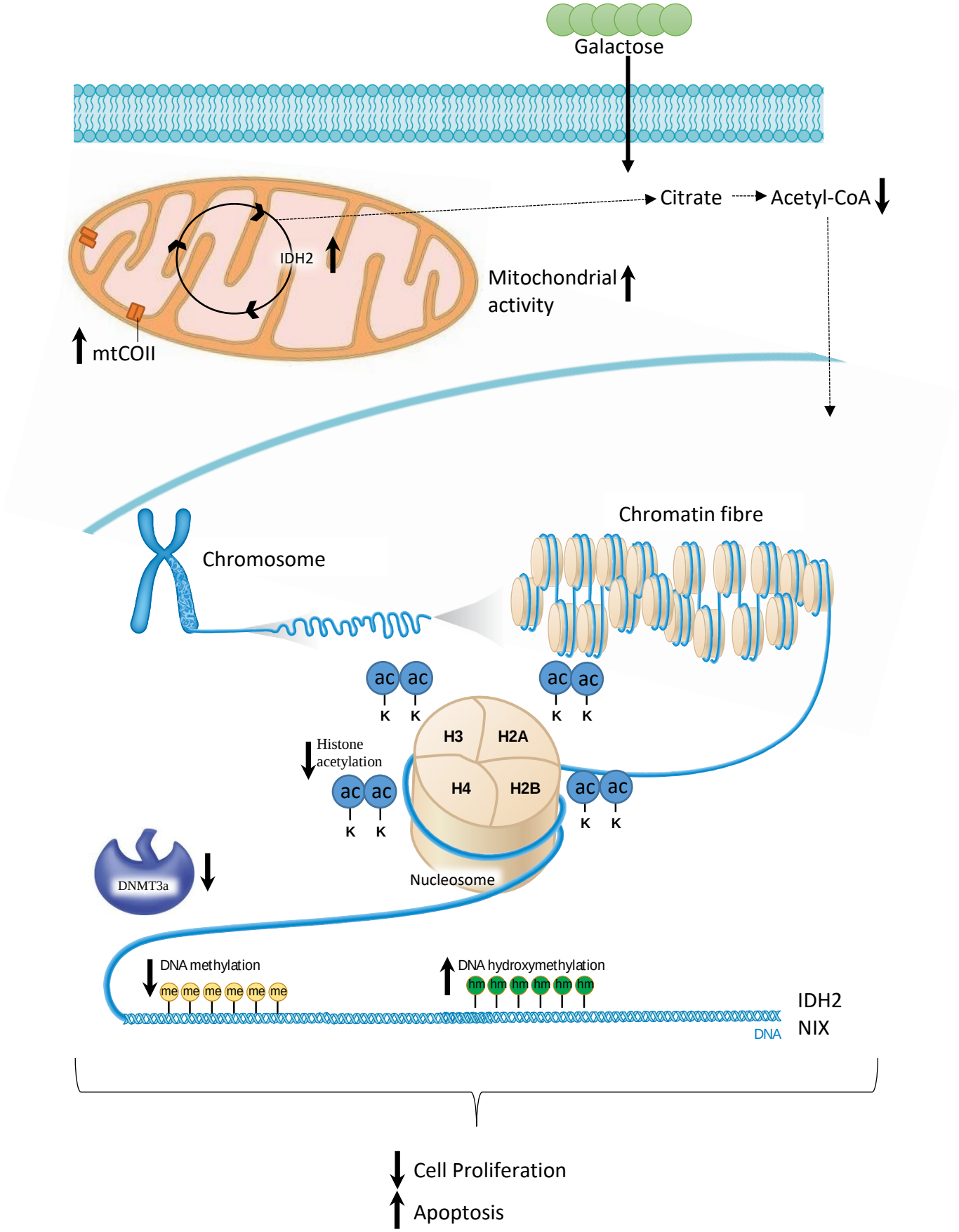


Figure 4.11: Schematic figure illustrating the conclusion of chapter 4 (for further explanation see section 4.5).

4.5. Conclusion

In conclusion, our data shows that in the cancer model HeLa, galactose nutrient regime led to a significant increase in *IDH2* and *NIX* expression by an active epigenetic modulation of the genes (methylation and hydroxymethylation). These changes were accompanied by the upregulation of key mitochondrial components, including mtCOII, α-KG levels and mitDNA copy number, and reduction of lactate production. Additionally, deacetylation of histone and destabilisation of DNMT3a was observed, thereby, explaining the reduced rate of DNA methylation observed in *IDH2* and *NIX* promoter region. Finally, cell proliferation and apoptosis were found to be reduced and increased, respectively in galactose treated HeLa cells. Overall, our study provides a rationale for the design of therapeutic strategies aiming to modulate the epigenetic mechanism of a cancer cells in order to reduce cancer progression and induce cell death.

CHAPTER 5: REGULATION OF CELL CYCLE AND THE ROLE OF ACETATE IN RESTRICTION POINT

5.1. Introduction

The reproduction and growth of eukaryotes depends on the regulation of cell cycle. The multicellular organisms rely on cell cycle for chromosomal replication and subsequent distribution of the biological information into the daughter cells (Kheir, Lund 2010). The typical cell cycle is divided into four different stages: G_1 (Gap 1), S (Synthesis), G_2 (Gap 2) and M (Mitosis); these are in addition to a quiescent stage called G_0 phase (Figure 5.1). Each cell cycle stage performs a specific function (Harashima et al. 2013). The current view of the cell cycle in eukaryotes is dominated by G_1 phase. During the G_1 period of the cell cycle, differentiated cells carry out their physiological function, and usually do not activate S phase from G_1 (Cooper 2000). The formation of cyclin D complex with cyclin dependant kinase 4/6 (Cdk4/6) is a critical step in G_1 phase that performs phosphorylation on retinoblastoma (pRB) protein and induces E2f transcription factor, consequently, facilitating the expression of various genes required for the cell cycle progression (Farioli-Vecchioli, Tirone 2015). In proliferating cells, G_1 phase is followed by the S phase, also known as the DNA replication phase. In G_2 phase cells grow and synthesise proteins for mitosis preparation (Cooper 2000). The cell cycle, in particular S phase, was previously shown to be shortened in proliferating cells. For example Fariolo-Vecchioli et al. (2015) demonstrated that proliferating adult progenitor cells shortened their S phase by 60% in comparison to the surrounding tissue. The nutrient sensitive restriction point (R-point) in G_1 was originally suggested by Arthur Pardee (1974). In nutrient depleted environments, cell remain in quiescent state and do not activate the R-point in order to transition into S phase. However, cells that pass the R-point will continue to progress in cell cycle and divide regardless of nutrient availability after the R-point (before entering quiescent state on the next R-point). The role of R-point is to ensure that prerequisites requirement for cellular division are fulfilled, therefore, allowing the completion of proliferation once this restriction point is passed. R-point is an important factor involved in various biological process in several species, including regulation of mitosis, maturation of oocytes, bacterial nutrient utilisation and yeast mating response, due to its low maintenance and high threshold nature (Yao et al. 2008).

Cyclins are modulated by the availability of specific nutrients and regulatory metabolic enzymes in cell cycle. Cyclins are the regulatory subunits of CDK holoenzyme complexes regulating the cell cycle progression through checkpoints by phosphorylating and

modulating the function of target substrates (Du et al. 2010). Cyclin-dependant degradation plays a key role in the progression of the different cell cycle phases. For example, APC/C mediates the progression from mitosis into G_1 phase (Pesin, Orr-Weaver 2008). Moreover, in addition to controlling motifs in the cell cycle, ubiquitin ligase, such as anaphase-promoting complex/cyclosome-CDH1 (APC/C-CHD1), also controls glycolysis and glutaminolysis by the degrading 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase isoform 3 (PFKFB3) and Glutaminase 1 (GLS1). During the G_1 to S phase transition (through the R-point), glycolysis and glutaminolysis are upregulated, that also correlates with a decreased APC/C-CDH1 activity. Therefore, suggesting APC/C-CHD1 regulated PFKFB3 and GLS1 are crucial factors in the utilisation of nutrients during G_1 to S transition (Moncada et al. 2012). In correspondence, synchronised HeLa cells deprived of either glucose or glutamine were unable to progress from mid-to-late G_1 phase indicating the importance of the nutrients at this point in the cell cycle (Colombo et al. 2011). Tudzarova et al. (2011) correspondingly reported similar findings by silencing the PFKFB3 in HeLa cells exhibiting cell cycle arrest in mid G_1 phase.

Under limited nutrient availability during R-point the cells initiate resting state instead of progressing into the S phase. The nutrition sensitive checkpoint was first described in *Saccharomyces cerevisiae* as START. After this checkpoint, following successful evaluations of the cellular requirements, yeast becomes irreversibly committed to division (Cooper et al. 2000). In mammalian cells, epigenetic adjustments have also been observed during the transition from G_1 to S phase, including chromatin modulation that allows DNA to replicate in the process of cell division (Kheir, Lund 2010). The S phase is mainly characterised by the DNA and histone replication, in addition to the observed low rate of protein synthesis and RNA transcription. The inheritance of DNA methylation is mainly carried out by DNMT1 in S phase. During this inheritance, DNMT1 targets the hemimethylated strands and transfers the methylation pattern onto the daughter strand. Histone methylation levels have also been shown to increase in the late G_1 to S phase transition, that correlates with histone methyltransferase activity. The role of histone modification in the transition of G_1 to S phase, is controversial and remains to be elucidated (Kheir, Lund 2010). Moreover, altered global histone acetylation and cell cycle deregulation are hallmarks of cancer (Cluntun et al. 2015; Farioli-Vecchioli, Tirone 2015). The levels of hyperacetylation of histones were shown to correlate with the intracellular acetyl-CoA levels in cancer (Cluntun et al. 2015). Interestingly, IDH2, a known regulator of the intracellular acetyl-CoA levels, was also shown to be associated in cancer cells (Wise et al. 2011).

Cells can be divided into two groups: quiescent or proliferative. A population of proliferative cells may harbour subpopulations at different cell cycle phases with different proliferative characteristics (Lamerton 1974). Cells at different phases of the cell cycle can be identified by their DNA content. For instance, cells in the G_1 phase contain diploid chromosomal copies. Whereas, in S phase, cell replication increases the number of chromosomes by 2-fold, to obtain DNA contents ranging from 2-fold to 4-fold. The DNA content then remains at 4-fold for cells in G_2 and M phases. These DNA content phase-specific properties can be utilised to distinguish between cells at different stages of cell cycle using combined DNA intercalating dye-flow cytometric analysis (Cooper 2000) (Figure 5.1). Specific proteins, such as IDH2, shown in our previous data (chapter 3 and 4) to play a key role in nutrient mediated epigenetic changes, can be assessed in synchronised cells. Moreover, different nutrient regimes combined with synchronised cells can be used to identify specific nutrients required for the R-point.

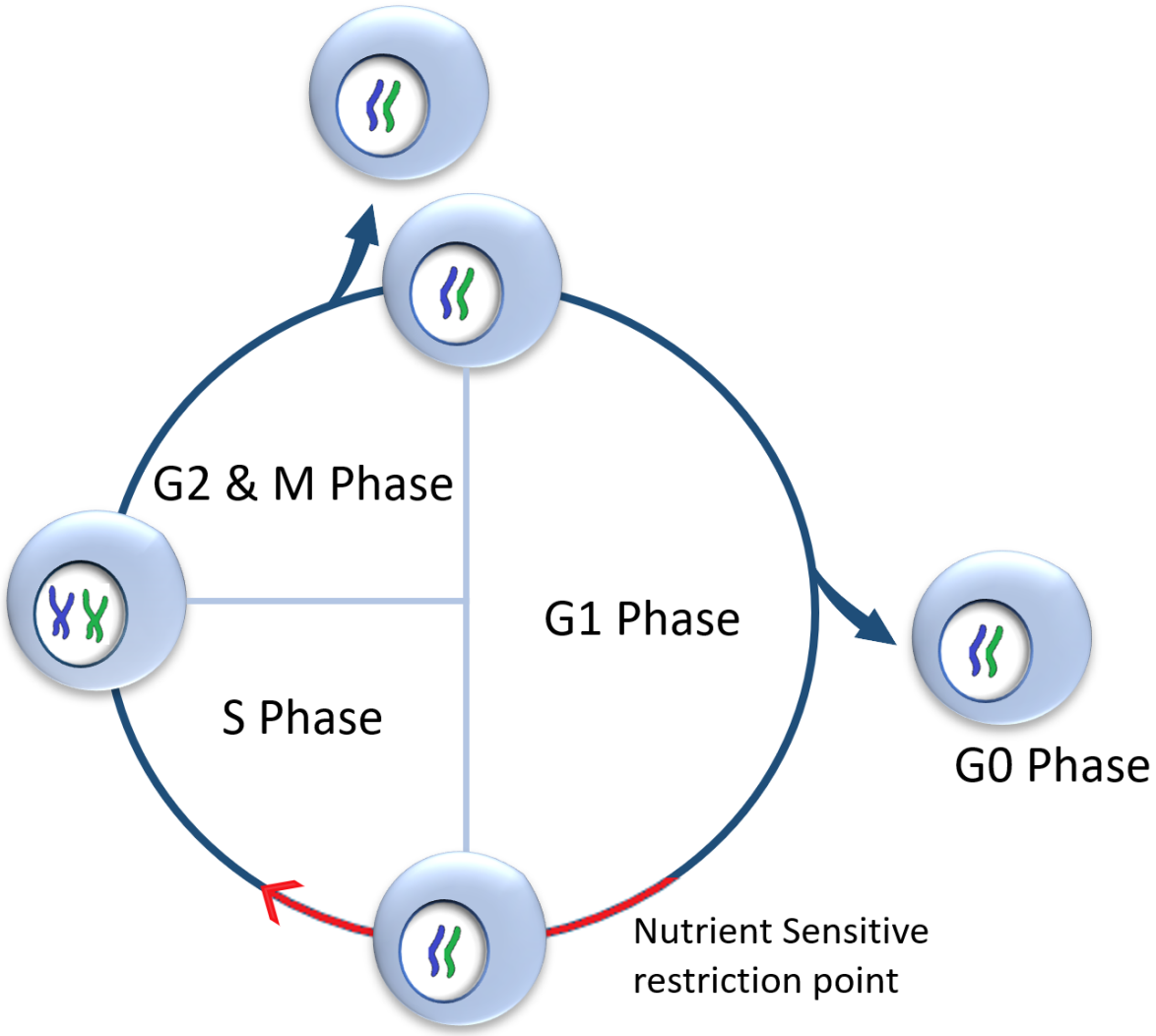


Figure 5.1: The nutrient sensitive point in the cell cycle. Cell cycle phases include G_1/G_0, S, G_2 and M, and are regulated by binding of cyclin to several cyclin-dependant kinases partners, subsequently modulating various checkpoints. These checkpoints play an important role in normal and pathological cellular types. Cell cycle-regulated transcription can be grouped into three main waves; $G_1 \rightarrow S$, $G_2 \rightarrow M$ and $M \rightarrow G_1$. The nutrition sensitive transition of $G_1 \rightarrow S$, is dependent on cellular and environmental availability of the nutrients required for DNA synthesis and maintaining genomic structural integrity. Cells deprived of nutrients induce cell cycle arrest at G_1 phase, thereby, inhibit proliferation. The $G_1 \rightarrow S$ transition induces transcriptional activation during the late G_1 phase, subsequently promoting cell's entry into S phase. Once in S phase, cells turn-off the transcriptional wave allowing for DNA replication. Therefore, this point commits the cells to division, and after the completion of this restriction point the cells continue to grow irrespective of the nutrients available (Bohnsack, Hirschi 2004; Bertoli et al. 2013).

5.2. Aims of Chapter 5: Cell cycle model/HeLa cells

- Evaluating the regulation of IDH2 and histone acetylation
- Characterising the role of acetate on cell cycle progression through the nutrient sensitive restriction point

5.3. Results

Cell cycle deregulation and altered global histone acetylation are hallmarks of cancer (Cluntun et al. 2015; Farioli-Vecchioli, Tirone 2015). In this chapter, we initially investigated the regulation of histone acetylation and IDH2 expression in synchronised HeLa cells. The potential impact of acetate nutrient regime on cell cycle was evaluated using synchronised HeLa cells.

5.3.1. The regulation of histone 4 acetylation during cell cycle

The modulation of histone acetylation in synchronised HeLa cells was assessed by western blotting. The data obtained showed a significant increase in histone 4 acetylation status in cells synchronised with NCZ (Figure 5.2A), displaying a 1.75-fold increase at 12 hours post NCZ release, as assessed by densitometry analysis (Figure 5.2B). The level of histone acetylation was normalised to total histone content of the cells.

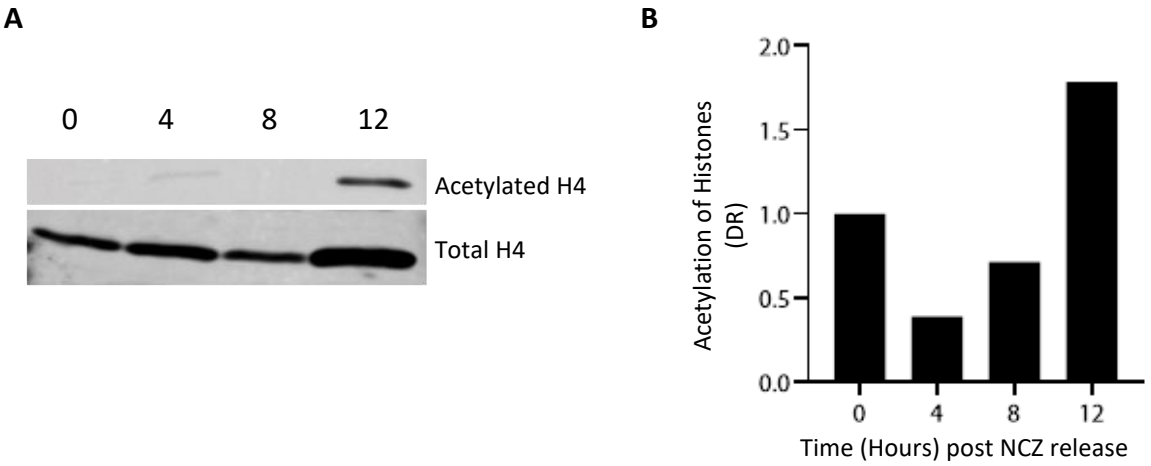


Figure 5.2: The regulation of histone 4 acetylation during cell cycle. (A) Western blotting analysis of the NCZ synchronised HeLa cells at 12 hours showed an increase in histone 4 acetylation relative to total histone 4. (B) Densitometric assessment of the histone acetylation analysis by western blotting. (DR; Densitometry ratios, Post NCZ release time points are demonstrated as 0, 4, 8 and 12 in the figures, H4; Histone 4). Results are representative of n=1.

5.3.2. The regulation of IDH2 and Cdh1 during cell cycle

Following cell synchronisation, the degree of synchronisation was assessed by measuring cell cycle progression. Cdh1 was previously shown to regulate G_1 to S phase transition (by our group's principal investigator Colombo et al. 2011), hence, it was employed to examine the cell cycle progression in our study. Post NCZ release, the protein expression of Cdh1 was analysed by western blotting of samples collected at different time points; 0, 4, 8 and 12 hours. The data obtained showed a significant and time-dependant increase of Cdh1 protein abundance in cells synchronised with NCZ (Figure 5.3A and B).

Findings from Chapter 3 and 4 suggested that the expression changes in IDH2 can affect acetylation of histones. Therefore, to characterise the role of IDH2 in cell cycle, total proteins were extracted from synchronised HeLa cells at 0, 4, 8 and 12 hours and analysed by western blotting. The data obtained showed no significant difference of IDH2 expression at different time-points of NCZ synchronised HeLa cells (Figure 5.3A). In addition, IDH2 was also assessed for acetylation status using IDH2 acetylated specific antibody. The data obtained showed a significant and time-dependant increase in IDH2 acetylation in cells synchronised with NCZ (Figure 5.3A and C).

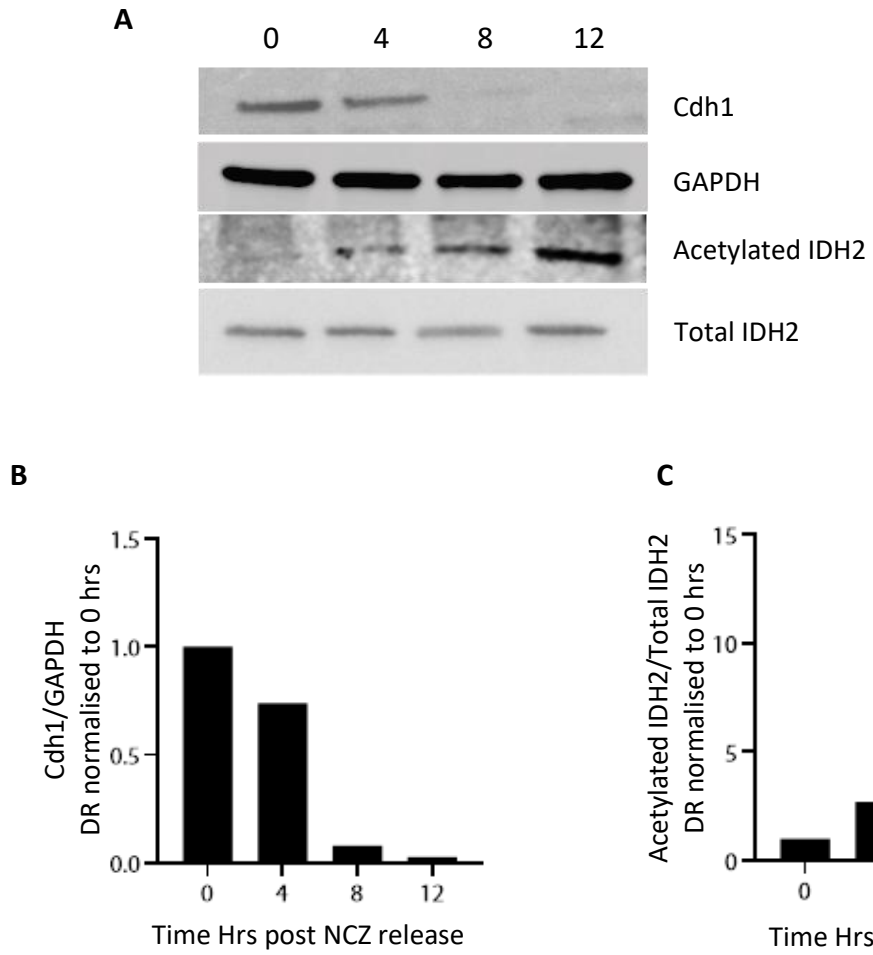


Figure 5.3: Analysis of IDH2 expression and acetylation status during the cell cycle. (A) Western blotting analysis of the NCZ synchronised HeLa cells at 0, 4, 8 and 12 hours, showed a gradual decrease in Cdh1 expression. Chd1 was used to characterise the G_1 to S phase transition during cell cycle progression. IDH2 protein abundance analysis showed no significant changes between different time-points. Post-NCZ release, the acetylation status of IDH2 was increased in a time-dependent manner as observed in western blotting analysis of synchronised cells at 4, 8 and 12 hours. (B and C) Cdh1 expression and acetylated IDH2 in synchronised HeLa cells are demonstrated in the schematic graph, generated using the densitometry ratios. Cdh1 protein expression was normalised to GAPDH, and IDH2 acetylation levels were normalised to total IDH2 protein content (IDH2 protein content normalised to GAPDH). (Post NCZ release time points are demonstrated as 0, 4, 8 and 12 in the figures). Results are representative of n=1.

5.3.3. The effect of acetate nutrient regime on cell cycle checkpoint in HeLa cells

In order to characterise the role of acetate in cell cycle, HeLa cells were cultured in low glucose levels to deplete the cellular glycogen storage and synchronised using NCZ. Following NCZ release, cells were then incubated with acetate or glucose nutrient regimes, for 0, 4, 8 and 12 hours. The regulation of cell cycle checkpoint was assessed by cell sorter. The data obtained showed that acetate nutrient regime induced cell cycle progression by promoting the transition of $G_1 \rightarrow S$ phase. Indicating that the substitution of glucose with acetate, is sufficient to meet the nutrition demand of R-point and progress through cell cycle.

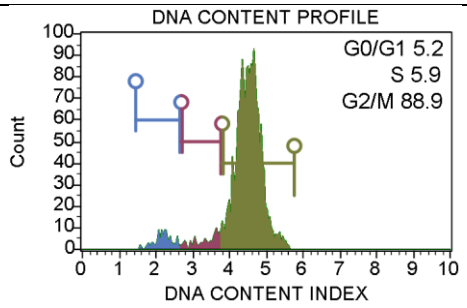
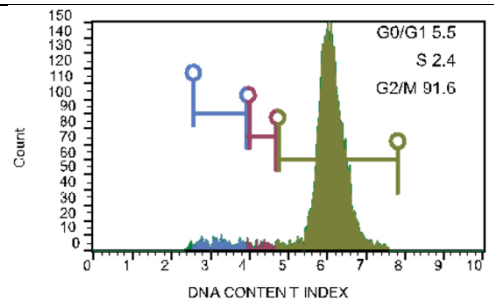
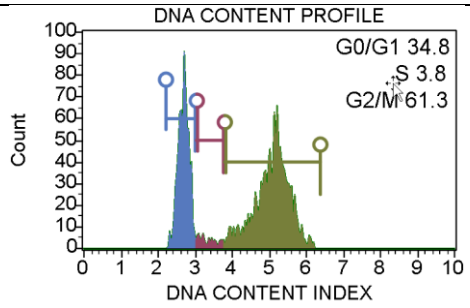
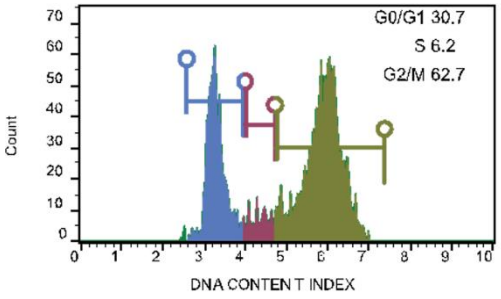
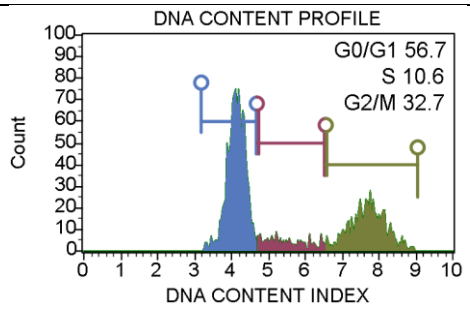
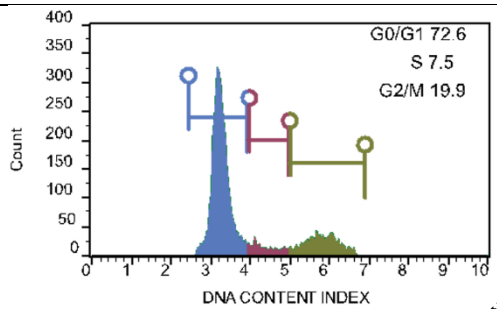
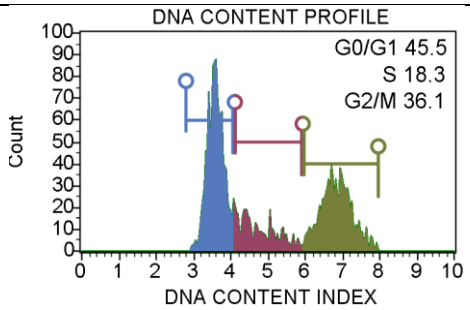
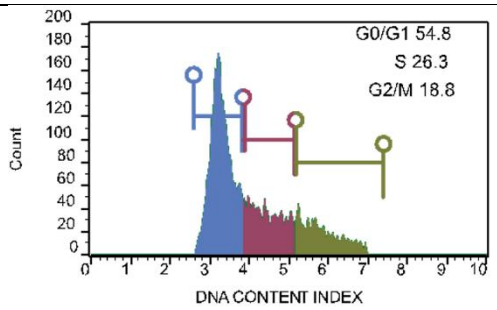
Post NCZ release	Glucose released HeLa cells	Acetate released HeLa cells
0 h		
4 h		
8 h		
12 h		

Figure 5.4: Cell cycle coordination of HeLa cells in glucose containing media and glucose-negative acetate containing media. The proportion of cells in different phases is calculated based on Propidium Iodide, and therefore DNA content of the cell. Results obtained from two-nutrient regimes (glucose and acetate) showed increasing percentage of cells in S and G_2/M phase.

5.4. Discussion

The progression of cells through the division cycle is regulated by checkpoints. It was previously proposed that R-point represents the nutrient-sensing point, and the sequential transition from the late G_1 to S phase is largely regulated by nutritional status of the cell. This R-point marks a critical point in cell cycle, after which cells commit to proliferation independently of growth factors and extracellular stimuli (Jia et al. 2009). If growth factors were deprived prior to R-point, cells exit the cell cycle into a state of quiescence known as G_0 (Yao et al. 2008). Cancer development and progression are associated with uncontrolled cell division caused by a series of changes in the activity of cell cycle regulators. Therefore, the characterisation of the mechanisms involved in the regulation of cell cycle, specifically the R-point, are important in highlighting important differences between normal cell proliferation and cancer initiation (Meeran, Katiyar 2008).

5.4.1. Histone acetylation regulation in cell cycle

It was previously shown that cancer cells display higher rate of glycolysis and hyperacetylation of histones (Cluntun et al. 2015). Approximately 10% of glucose taken up by cancer cells is utilised for biosynthesis, and the remaining is mostly converted into lactate (Heiden et al. 2009). Lactate levels of synchronised HeLa cells were previously demonstrated to increase during the R-point (Colombo et al. 2011). Following these findings, we assessed the regulation of histone acetylation in synchronised HeLa cells. The data obtained showed a hyperacetylation state of histones following the R-point. These findings suggest that various nutrients may be required at the R-point in order to regulate the histone acetylation. Liu et al. (2015) demonstrated an increased rate of aerobic glycolysis which was associated with open chromatin configuration and histones hyperacetylation in cancer cells. In addition, the same group also demonstrated that inhibiting glycolysis with pharmacological interventions reverted the open chromatin configuration (i.e. open to close) resulting from the deacetylation of histones. Therefore, suggesting a close relationship between metabolism and chromatin dynamics.

5.4.2. IDH2 protein abundance and acetylation in cell cycle

Moreover, findings from other chapters (Chapter 3 and 4), suggested an inverse relationship between the IDH2 expression levels and histone acetylation status. For example, low IDH2 expression levels correlated with histone hyperacetylation in INS-1 cells (Chapter 3), whereas, high IDH2 expression levels correlated with histone hypoacetylation in HeLa cells (Chapter 4). Thus, we investigated the IDH2 expression in synchronised HeLa cell. Surprisingly, no changes in the protein expression level of IDH2 were observed. Other studies have reported that IDH2 downregulation can accelerate cell cycle progression and induce a pro-proliferative state in osteosarcoma cell lines (YI et al. 2016). However, our data indicate that IDH2 expression modulation may not an integral part of the cell cycle. There is substantial evidence that indicate acetylation of IDH2 can regulate its catalytic activity (Yu et al. 2012; Zou et al. 2017; Smolková, Ježek 2012). SIRT3 deacetylase, is located in the mitochondria, that modulates protein acetylation thereby regulating the function of various mitochondrial protein (Lombard et al. 2007). Moreover, overexpressed SIRT3 has also been shown to deregulate cell cycle progression, resulting in G_1 cell cycle arrest (Wang et al. 2016). Collectively, these findings suggest that protein acetylation may play a key role in the modulation of IDH2 catalytic activity in cell cycle.

Therefore, we carried out IDH2 acetylation analysis at a different time points in HeLa cells after NCZ release. The data obtained showed a gradual and significant increase in the acetylation status of IDH2 at 4, 8 and 12 hours post NCZ release. This data suggests that the IDH2 protein abundance remains constant, whereas, IDH2 acetylation is significantly increased during the cell cycle. These findings in combination with previous data from this chapter, indicates that IDH2 acetylation and histone acetylation are implicated in the R-point. The activity of IDH2 can regulate the intracellular acetyl-CoA levels by modulating mitochondrial citrate levels (Schug et al. 2015). Supporting evidence from Wellen et al. (2009) also showed a direct connection between the histone acetylation and ACLY-mediated citrate conversion to acetyl-CoA. We also observed from our data, that the acetylation of IDH2 occurred before the acetylation of histones in the synchronised HeLa cells. Therefore, suggesting the activity (known to be modulated by acetylation) of IDH2 can possibly modulate histone acetylation as a downstream effect.

5.4.3. The regulation of cell cycle by acetate

Our previous data (from Chapter 3) showed that acetate downregulates IDH2 expression and increases histone acetylation. Therefore, according to these findings and to assess our second aim, we characterised the impact of acetate nutrient regime on cell cycle. Colombo et al. (2011) previsouly demonstrated the impact of specific nutrient regimes, i.e. glucose, on distinct stages in the cell cycle. The absence of glucose was shown to inhibit cell cycle progression at mid-to-late G_1 phase. Based on these findings, the cells in our study were deprived of glucose, and synchronised with NCZ, thereafter, released into different nutrient regimes, glucose (control) or acetate. The flow cytometric analysis of synchronised HeLa cells showed that acetate induced progression from G_1 to S phase, independently of glucose. In addition, resolving cell cycle distributions at the remaining phases i.e. G_1, S and G_2/M phases, identified similar cell distribution percentages amongst stages in both nutrient regimes. Thus, indicating that the substitution of glucose with acetate, is sufficient to meet the nutrition demand of R-point and progress through cell cycle. In support of our data, Morrish et al. (2010) demonstrated that c-Myc, a cell cycle regulator, induces glycolysis and increases mitochondrial synthesis of acetyl-CoA, subsequently upregulating histone acetylation. Moreover, 50% of intracellular acetyl-CoA was shown to be generated by mitochondria during cell cycle (Morrish et al. 2009). Collectively, these findings and ours indicate that acetyl-CoA generation, IDH2 acetylation and histone acetylation are intrinsically regulated during the cell cycle.

5.5. Conclusion

In conclusion, our data showed that IDH2 acetylation and histone acetylation are closely regulated during G_1, S and G_2/M phases. Moreover, IDH2 protein abundance was not found to be modulated during cell cycle. Additionally, differential nutrient regimes suggested that substitution of glucose with acetate, is sufficient to meet the nutrition demand of R-point and progress through cell cycle.

CHAPTER 6: GENERAL DISCUSSION, CONCLUSIONS AND FUTURE STUDIES

6.1 General Discussion

The trans-generational epigenetic inheritance has emerged as a new area of research and challenged the central dogma of biology that genetic code is the sole basis for biological inheritance. The area of epigenetics has provided new insights into the underlying biological mechanisms that can be involved in transmitting traits to subsequent generations (Zakhari 2013; Rissman, Adli 2014). DNA methylation and histone acetylation are important processes in epigenetic transcriptional regulation. In addition, different tissue types display different epigenetic profile in their genomes. Thus, epigenomics require the characterisation of variants not only amongst different individuals, but also among different tissues, disease types and developmental stages (Reichetzeder et al. 2016). DNA methylation has long been considered a crucial component in the epigenetic network and is the most widely studied mechanism (Zhang 2015). The pathophysiological role of DNA methylation is poorly understood. However, several advances have improved our understanding of the role of DNA methylation in cancer and diabetes (Etchegaray, Mostoslavsky 2016). Therefore, in order to achieve our aim of characterising the epigenetic changes and gene regulation by nutrients, we utilised cancer and diabetes cellular models to investigate the novel mechanisms that led to functional consequence. Cell proliferation and insulin synthesis were assessed in the human derived cancer HeLa cell and rat pancreatic β-cell INS-1, respectively. Moreover, certain aspects of DNA methylation are well understood, but the cues that designate the *de novo* methylation of specific CpG islands are not well-documented, except for the CpG islands rich regions that has been shown to attract *de novo* methylation (Edwards et al. 2017). Therefore, our study characterised a novel mechanism by which nutrient can regulate the methyltransferase-mediated *de novo* methylation and elucidates the impact of galactose and acetate nutrient regimes on IDH2 deregulation and DNA methylation. Finally, we assessed the significant impact of nutrient mediated epigenetic changes and PTMs in cell cycle regulation by demonstrating novel pathway of regulating histone acetylation and $G_1 \rightarrow S$ phase transition by IDH2 acetylation. Moreover, we suggest that acetate without glucose, can meet the nutrient requirements of the nutrition sensitive restriction point in cell cycle.

6.1.1 Differential nutrient regime deregulates IDH2 expression and its DNA methylation profile

Previously, IDH2 expression abnormalities were reported in multiple cancers: presenting as downregulation and upregulation, and in some cancers downregulation at early stages followed by upregulation in advanced stages (Lv et al. 2012; Green, Beer 2010; Fu et al. 2010). These findings require further investigations to elaborate on the role of IDH2 in cancer. In diabetes, previous studies have shown that IDH2 knockdown inhibits insulin secretion under high glucose conditions (Macdonald et al. 2013). However, the mechanisms leading to the regulation of IDH2 in diabetes remains to be elucidated. In our study, we used cellular models of cancer (HeLa cells) and diabetes (INS-1 cells) in order to investigate the role of nutrients, such as galactose and acetate, in cancer and diabetes, respectively. Additionally, we conducted a study on the effect of acetate on cell cycle regulation. In the **3rd Chapter** of this thesis, we applied well characterised INS-1 pancreatic β-cell line in the presence of acetate. The data obtained showed acetate-mediated downregulation of IDH2 mRNA and protein expression, that correlated with increased promoter DNA methylation and resulted in the reduction of total α-KG levels. IDH2 was also assessed in HeLa cancer model in the **4th Chapter**. Using a methylation sensitive enzyme, we demonstrated that galactose-mediated a reduction in IDH2 DNA methylation status, whereas increased the status of hydroxymethylation. These results were associated with changes in mRNA and protein expression of IDH2. Collectively, downregulation of IDH2 in INS-1 cells was associated with a decrease in insulin content. Moreover, IDH2 upregulation in HeLa cells was associated with decreased proliferation. Suggesting IDH2 expression is associated with improved disease outcomes and it can serve as a potential therapeutic target in diabetes and cancer. Therefore, to assess whether IDH2 can serve as a therapeutic target we overexpressed IDH2 in INS-1 cells, which partially but significantly reverted some of the key acetate-induced changes, that has been associated with diabetes. DNA hydroxymethylation is a reversible modification, that can be used as a therapeutic to upregulate gene expression without inducing changes in the genomic sequence. Therefore, dCas9-Tet was used to induce hydroxymethylation and thereby upregulate hydroxymethylation-mediated IDH2 expression. The data showed that hydroxymethylation was able to upregulate IDH2 significantly. Therefore, suggesting that the ability to drive the upregulation of IDH2 using dCas9-TET can serve as a possible future treatment in diabetic and cancer patients. Moreover, because of the involvement in the regulation of citrate and

α-KG, IDH2 is believed to play a crucial regulatory role in DNA methylation and histone/protein acetylation. In support of this statement, IDH2 was also concluded in our study as a crucial step of epigenetic regulation.

6.1.2 Nutrients regulate histone and IDH2 acetylation

Another aspect of this thesis was to investigate the changes in histone acetylation. In **Chapter 4**, the data obtained showed that galactose nutrient regime significantly reduced histone acetylation in HeLa cells in a time-dependent manner. These findings suggest that 'The Warburg effect', usually observed in cancer cells, indeed exceeds the requirement for protein and nucleotide synthesis, and includes widely epigenetic modifications. Additionally, in **Chapter 3** acetate nutrient regime induced significant increase in histone acetylation in INS-1 cell. Interestingly, findings from **Chapter 3** and **Chapter 4**, demonstrated that histone acetylation inversely correlated with IDH2 expression levels. Suggesting that IDH2 may play a key role in the regulation of histone acetylation. Moreover, cell cycle regulation also involves the heritable epigenetic changes including histone acetylation, and therefore in **Chapter 5**, we studied the role of IDH2 and histone acetylation at different stages of the cell cycle. Histone acetylation was shown to be upregulated during the late G_1 to S phase transition. Surprisingly, IDH2 protein expression was not regulated in the cell cycle. Other than protein expression, protein acetylation is recognised as a major regulator of protein function. IDH2 is one of the proteins shown to be downregulated by acetylation. Subsequently, we carried out IDH2 acetylation analysis at different time points in HeLa cells after NCZ release. The data collected showed a gradual increase in the acetylation status of IDH2 during the late G_1 and S phase, mimicking the pattern observed in histone acetylation. Combined together, the findings from the **three chapters** (3, 4 and 5) collectively suggest that the changes in metabolism associates with the changes in epigenetics, and therefore supporting our findings of mitochondrial activity and IDH2 expression inversely correlating with changes in DNA methylation and histone acetylation.

Multiple metabolic pathways including glycolysis and β-oxidation can lead to changes in acetyl-CoA, and therefore regulate the acetylation of histones (Etchegaray, Mostoslavsky 2016). Peleg at al. (2016) also suggested that the level of histone acetylation is susceptible to metabolites such as acetyl-CoA, is highly regulated in yeast and provides evidence that acetyl-CoA connects metabolism to growth and survival (Cai et al. 2011). In cancer cells,

instead of being converted into α-KG by IDH2, the glycolysis-derived pyruvate enters the truncated TCA cycle, and is thereafter converted to citrate, subsequently exported into cytosol by tricarboxylic transporter. Thereafter, the citrate is converted back to acetyl-CoA by ACLY, thereby, regulating histone and protein acetylation (Hatzivassiliou et al. 2005; Latham et al. 2012). Likewise, this regulation may explain the observed acetate-induced histone acetylation in INS-1 cell by the downregulating of IDH2 and reducing α-KG content. Furthermore, IDH2 mediated irreversible oxidation and decarboxylation of isocitrate into α-KG is a crucial step in the TCA cycle determining the level of mitochondrial citrate (Zou et al. 2017). These findings combined with ours indicate that galactose may be decreasing the pool of acetyl-CoA by regulating IDH2 expression in the HeLa cells and therefore leading to deacetylated histones. Conversely, acetate may be increasing the pool of acetyl-CoA directly and by regulating IDH2 expression and therefore leading to acetylated histones.

6.1.3 IDH2 expression is not correlated with lactate production in INS-1 cells

The connection between IDH2 and lactate production has previously been demonstrated (Wen et al. 2015). In our study of the diabetic model, lactate levels did not correlate with IDH2 expression levels in INS-1 cells. Whereas, lactate production strongly correlated with IDH2 expression in HeLa cells. These findings combined suggest that there might not be a direct connection between IDH2 and lactate production. However, we did not assess protein expression, PTMs or epigenetic regulation of LDHA in both nutrient regimes, acetate and galactose. Therefore, to overcome these limitations, further investigation will be required. Another outstanding question regarding the association between α-KG levels and the 5hmC status in INS-1 cells. However, an increased level of 5mC can presumably reduce the level of 5hmC in the cell, further hindering the detection of 5hmC (Ecsedi et al. 2018). Although, NGS high-throughput sequencing can identify 5hmC levels with increasing accuracy and sensitivity (Pang et al. 2016). An investigation focusing on the 5hmC levels in β-cells can provide additional insights into gene regulations and subsequently additional targets for treatment interventions. Our efforts to relate 5hmC levels on IDH2 promoter region and consequently increasing the expression were evident in **chapter 4**.

6.1.4 Regulation of DNMT3a in differential nutrient regimes

DNMT3a participates in the *de novo* promoter methylation of genes (Robertson et al. 1999). DNMT3a regulation was assessed under acetate **(Chapter 3)** and galactose **(Chapter 4)** nutrient regime. In contrast to galactose, acetate induced DNMT3a protein abundance. Further investigation, in both chapters using different cell lines, showed that by inhibiting proteasomal degradation by MG132, DNMT3a protein abundance was increased in a time-dependent manner, suggesting DNMT3a can be regulated by PTMs. This mechanism is conserved between the two species, as suggested by INS-1 and HeLa cells (rat and human). Moreover, DNMT3a was shown to be conversely regulated by IDH2 expression and mitochondrial function. Subsequently, leading to increased methylation of DNA promoter regions of key metabolic and mitochondrial related genes.

6.1.5 Concluding remarks

In conclusion, our study considerably enhances the understanding of the role of nutrients in DNA methylation and histone acetylation. Furthermore, functional consequence in pathological conditions, including diabetes and cancer were elucidated. The role of nutrients in the cell cycle was also illustrated in this study: the findings from this study can be used to enhance our understanding of the diseases that deregulate cell cycle, in addition to our understanding of the normal proliferating cells. IDH2 was shown to participate in the development of diabetes-related complication (i.e. downregulation of insulin production), regulation of the 'The Warburg effect' in cancer model and regulation of restriction point in the cell cycle. The nutrient-mediated DNMT3a regulation correlated with the observed DNA methylation changes in *IDH2*, *insulin* and *NIX*, further explaining the connection between nutrients and epigenetics. Molecular interventions including *IDH2* overexpression was able to revert some of the changes implicated by nutrient regimes, suggesting IDH2 can serve as a target for future therapeutic interventions. Moreover, dCas9-Tet mediated 5hmC upregulation in *IDH2* promoter region was demonstrated as a potential therapeutic tool. The anti-proliferative and apoptotic properties of galactose were demonstrated in HeLa cells, that essentially reversed 'The Warburg effect' commonly observed in many cancers. Moreover, acetate demonstrated a negative effect on the INS-1 cells and suggested an underline cause of the increasing prevalence of T2D in the population.

6.2 Thesis conclusions

1. In the presence of acetate, *IDH2* downregulation inversely correlated with DNA promoter methylation. Furthermore, DNMT3a protein abundance was significantly increased and mitochondrial activity decreased in acetate nutrient regime.

2. *IDH2* overexpression reverted the effect of acetate on DNMT3a protein abundance, histone acetylation and cellular ATP levels in INS-1 cells.

3. Acetate led to the downregulation of *insulin* gene expression, correlating with increased DNA promoter methylation, ultimately resulting in decreased insulin content of INS-1 cells.

4. Galactose mediated upregulation of *IDH2* and *NIX* expression in HeLa cells, correlated with DNA hydroxymethylation and inversely correlated with DNA methylation.

5. Reduced proliferation and increased apoptosis of HeLa cells correlated with upregulation of IDH2, NIX and mitochondrial activity. Furthermore, a significant decrease was observed in DNMT3a protein abundance and histone acetylation in galactose nutrient regime.

6. dCAS9-Tet mediated increase of *IDH2* DNA hydroxymethylation upregulated the protein expression.

7. Acetylation of IDH2 and histones was found to be regulated during cell cycle, specifically during the nutrition sensitive restriction point.

6.3 Future Studies

The identification of PTM-mediated stabilisation of DNMT3a correlated with changes in DNA methylation status of various genes implicated in mitochondrial and cellular activity. Following these findings, we speculated the acetylation mediated DNMT3a protein stability and the functional consequence can affect global DNA methylation, and therefore can be assessed using immunopurification combined mass spectrometry and MethylSeq. Furthermore, the role of lysine acetylases and deacetylases in regulating DNMT3a can be investigated using proximity ligand assay. In addition to investigating mitochondrial regulations in our study, to further characterise mitochondrial role in diabetes and cancer, the basal respiration, maximal respiration, proton leak and spare capacity can be assessed using the gold standard methodology, seahorse Cell mito stress.

We successfully upregulated IDH2 using dCAS9-Tet mediated hydroxymethylation. dCAS9 regulates gene expression without gene editing/direct genetic changes in the genome, therefore, serving as an ideal tool for genetic intervention in diabetic and cancer patients. Moreover, epigenetic changes can be essentially reversed, suggesting the dCAS9 mediated modification can be 'tuned' for individuals and serve as a precision medicine tool. We also demonstrated overexpression of IDH2 using expression plasmid vector. Gene therapies have already been initiated in humans and showed extensive success (George et al. 2017). Non-integrating gene therapy is another therapeutical application that can also serve as a method to increase the expression of IDH2, using non-integrating vectors with low immune response rate. Adenoviral-mediated therapy has previously been shown to be successful in diabetic mice, reversing the signs of diabetes (Slosberg et al. 2001). Speculatively, for future studies, gene therapy to overexpress IDH2 in pancreatic cells may induce *insulin* gene expression leading to reversion of diabetes via epigenetic modifications. Mitochondrial dysfunction has been considered as a major contributor to the development of T2D (Lu et al. 2010). Given IDH2 upregulation can be achieved by galactose nutrient regime, it is possible that the addition of galactose to pancreatic β-cells can reduce 5mC and increase 5hmC on *IDH2*, and subsequently restore the dysfunctional IDH2 protein expression and increase mitochondrial activity. Additionally, the *insulin* gene can be modulated in a similar manner to *IDH2*, and therefore restoring insulin content of the cells.

To further characterise cell cycle regulation, synchronised cells in the absence of nutrient will need to be utilised as an experimental negative control. Moreover, the production of acetyl-CoA from acetate is carried out by ACSS1, 2 and 3 (Lyssiotis, Cantley 2014) and can be selectively blocked using pharmacological interventions in the presence of acetate to ensure acetate is sufficient for cell cycle progression in the absence of glucose.

The role of nutrients and metabolic pathways involved in the pathogenesis and treatment of diabetes and cancer can be further investigated in a clinical context, using animal models to assess the nutrient combined drug therapy or nutrient therapy alone.

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