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The Metabolic-Epigenetic Nexus in Type 2 Diabetes Mellitus

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Abstract

The prevalence of type 2 diabetes mellitus (T2DM) continues to rise globally. Yet the aetiology and pathophysiology of this noncommunicable, polygenic disease, is poorly understood. Lifestyle factors, such as poor dietary intake, lack of exercise, and abnormal glycaemia, are purported to play a role in disease onset and progression, and these environmental factors may disrupt specific epigenetic mechanisms, leading to a reprogramming of gene transcription. The hyperglycaemic cell *per se*, alters epigenetics through chemical modifications to DNA and histones via metabolic intermediates such as succinate, α-ketoglutarate and *O*-GlcNAc. To illustrate, α-ketoglutarate is considered a salient co-factor in the activation of the ten-eleven translocation (TET) dioxygenases, which drives DNA demethylation. On the contrary, succinate and other mitochondrial tricarboxylic acid cycle intermediates, inhibit TET activity predisposing to a state of hypermethylation. Hyperglycaemia depletes intracellular ascorbic acid, and damages DNA by enhancing the production of reactive oxygen species (ROS); this compromised cell milieu exacerbates the oxidation of 5-methylcytosine alongside a destabilisation of TET. These metabolic connections may regulate DNA methylation, affecting gene transcription and pancreatic islet β-cell function in T2DM. This complex interrelationship between metabolism and epigenetic alterations may provide a conceptual foundation for understanding how pathologic stimuli modify and control the intricacies of T2DM. As such, this narrative review will comprehensively evaluate and detail the interplay between metabolism and epigenetic modifications in T2DM.

1. Introduction

In the last five decades, the prevalence of Type 2 Diabetes Mellitus (T2DM) has increased exponentially. Current projections estimate an increase of 50% to the year 2045, stating that 693 million people will suffer from diabetes, with estimated healthcare costs of approximately US$850 billion per year (Roden and Shulman, 2019). T2DM is a complex, heterogeneous metabolic disease, characterised by deficient insulin secretion from pancreatic islet β-cells, leading to a state of impaired insulin sensitivity (insulin resistance), dysregulated blood glucose, and frequently associated life-threatening complications (Roden and Shulman, 2019). To date, the genetics of T2DM is only partially understood, with *circa* 500 T2DM-associated genomic regions identified; each of these individual loci (mostly in non-coding regions) however, only explain 20% of overall disease risk (Mahajan *et al* 2018, Fernandez-Tajes *et al* 2019). Emerging evidence suggests that parental lifestyle, intrauterine programming and early postnatal metabolic alterations may enhance disease risk susceptibility through epigenetics (Barres and Zierath, 2016). Regarding the latter, the relationship between metabolic epigenetic underpinnings, and the onset and progression of T2DM through life, is not well-defined and understood.

Chemical modification of histones and DNA, such as histone and DNA methylation, and histone acetylation, play critical roles in epigenetic gene regulation, with many enzymes that add or remove such chemical modifications sensitive to changes in intracellular metabolism. In the diabetic state, dysregulated glucose may affect one-carbon metabolism (S-adenosyl methionine [SAM] pathway), acetyl-CoA activity, JmjC and TET methylase pathways through direct action via fumarate, succinate, 2-oxoglutarate-dependent dioxygenases, and ascorbic acid; all of which are implicated in either histone modification or regulation of DNA methylation (Allis and Jenuwein 2016, Wu *et al* 2018). A dysregulated glucose metabolism in T2DM generates reactive oxygen species (ROS) via activation of protein kinase C, polyol and hexosamine pathways, causing macromolecular damage (Yuan *et al*, 2019). Moreover, histone and DNA methylation in particular are directly linked to central metabolism through critical redox intermediates such as NAD^+, SAM, and 2-oxoglutarate, and fluctuations in these intermediates caused by pathologic stimuli, may directly affect epigenetic signaling leading to a reprogramming of gene expression.

Metabolism and epigenetics trade many interacting factors, and the focus to date on the complex interplay between metabolism and DNA methylation and histone modification, and how this connection can be dysregulated, has revolved mainly around cancer disease. As such, the interplay between metabolism and epigenetic modifications in T2DM needs to be elucidated. This narrative review will examine the multifactorial interrelationship between

metabolism and regulation of epigenetics (inclusive of DNA methylation and histone modifications) in T2DM.

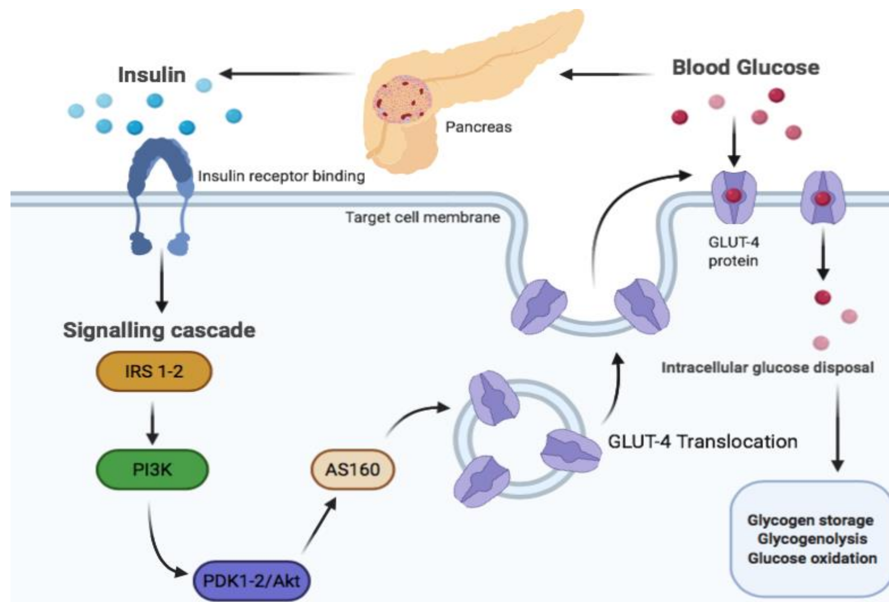
2. Diabetes Mellitus

Diabetes mellitus (*mellitus* meaning '*honey-sweet*') is a chronic disease, characterised by the relative or absolute deficiency of the hormone insulin (Guyton and Hall, 2015). Insulin is a heterodimeric peptide molecule, consisting of an A and a B chain, with 21 and 30 amino acids respectively, held together by three disulphide-bonds (Berg *et al*, 2002). In normal biochemistry, insulin is produced by the β-cells of the pancreatic Islets of Langerhans and released into the peripheral circulation by a variety of stimuli; the most important from a physiological perspective, is glucose. Insulin has a half-life of approximately 6 minutes, and although a specific transport protein for insulin has yet to be identified, it is transported in plasma with α- and β-globulins (Grayson and Woods 2013, Guyton and Hall 2015).

The major biochemical function of insulin is to facilitate uptake of glucose from plasma into the cytoplasm of target cells, such as liver, muscle and adipose tissue (Vargas *et al*, 2020). However, prior to insulin release, glucose-sensing mechanisms in pancreatic β-cells control the triggering and maintenance of secretion. These proximal events in β-cells include glycolytic reactions involving the phosphorylation of glucose to glucose-6-phosphate by the high K_M glucokinase, increased pyruvate and NADH production, and a change in the ATP/ADP ratio. Pyruvate and NADH are utilised as TCA cycle substrates aligned to mitochondrial metabolism and signalling. These signals regulate pancreatic insulin secretion by controlling distal effectors including membrane excitability, Ca^{2+} signalling, insulin granule recruitment and exocytosis (McDonald *et al* 2005, Rutter *et al* 2020).

Target cell insulin-stimulated glucose uptake is effectively regulated by the binding of insulin to a membrane receptor protein, causing the receptor to undergo conformational change, where activation of an intracellular tyrosine kinase domain ensues (Vargas *et al* 2020, Guyton and Hall 2015). The intracellular relocation of glucose transporters (GLUT-4 proteins) from the inner cell space to the outer plasma membrane subsequently facilitates cell glucose uptake (Huang and Czech, 2007) (Figure 1).

Figure 1. *Mechanism of Insulin Action on Target Cell and Principal Biochemical Actions*



Blood glucose controls the release of insulin from pancreatic β-cells (top); at target cells, insulin receptor binding activates a signalling cascade and GLUT-4 proteins move to the outer membrane to increase cell glucose disposal.

Diabetes mellitus patients have impaired glucose entry into the cell, with potential to elevate peripheral glucose to approximately 40 $mmol{\cdot}L^{-1}$ (Guyton and Hall 2015, Frayn and Evans 2019); often termed '*hyperglycaemia*', this can cause several alterations to basic cell metabolism (Frayn and Evans, 2019), including a constellation of diabetic complications such as neuropathy, nephropathy, retinopathy, artery disease and stroke (Skyler *et al*, 2017).

Diabetes manifests via a number of different types, with some less well-known types being gestational diabetes mellitus, latent autoimmune diabetes of adulthood (LADA), maturity onset diabetes of the young (MODY), and transient neonatal diabetes mellitus (TNDM). From a broad clinical perspective, alternative pathological conditions related to diabetes mellitus, include destructive pancreatic disease, chronic pancreatitis, haemochromatosis, and many forms of endocrine disease, *e.g.* Cushing's syndrome (cortisol), hyperthyroidism (thyroxine) and glucagonoma (glucagon). Common types from a societal point-of-view, are type 1 (T1DM) and type 2 diabetes mellitus. Considering the former, up to 10% of the diabetic population have T1DM (often termed insulin-dependent diabetes), caused by a destruction of pancreatic β-cells leading to complete insulin deficiency (You and Henneberg, 2016). T1DM is caused by a combination of environmental factors and genetic susceptibility, of which the latter includes the autosomal recessive inheritance of class II genes in the Human Leukocyte Antigen (HLA) system; affecting HLA protein binding to antigenic peptides and antigen presentation to T cells (Skyler *et al*, 2017). Approximately 50 additional genes outside the

HLA system, particularly the insulin gene on chromosome 11, are implicated in T1DM onset (Skyler *et al*, 2017). Type 2 diabetes is the most prevalent form of diabetes in modern society (approximately 90%), and as T2DM constitutes the primary focus of this review, a sole emphasis will be considered in the ensuing sections.

2.1 Pathophysiology of Type 2 Diabetes Mellitus

Type 2 Diabetes Mellitus is a complicated, multiorgan heterogeneous disorder characterised predominantly by pancreatic β-cell dysfunction (insulin secretory defect) and tissue insulin resistance, leading to hyperglycaemia (Brunetti *et al*, 2017). T2DM aetiology is strongly associated with obesity and a complex polygenic predisposition (Skyler *et al*, 2017). On the former, body mass index is in fact a better predictor of T2DM than any of the known risk alleles (Bernstein *et al*, 2017).

The mechanism of defective pancreatic insulin secretion is complex and may be due to the following: (1) reduced β-cell mass; (2) β-cell apoptosis; (3) change in β-cell phenotype; (4) insufficient number of β-cells; (5) functional decline in β-cells (Skyler *et al*, 2017). These factors (singularly or a combination of) subsequently lead to a deterioration of pancreatic β-cell function followed by a reduction in insulin secretion and hyperglycaemia.

Insulin resistance at target tissues (*i.e.* muscle, liver, adipose) may be caused by the defective binding of insulin to receptors, a reduced number of insulin receptors on target cells, and abnormalities in the insulin signalling cascade which may be initiated by various polymorphisms such as rare variants in the insulin receptor substrate *(IRS)*-1 gene (Kaku, 2009). Generally, insulin resistance is traceable to defects in the transmission of signals from the insulin and insulin-like growth factor-1 (IGF-1) receptors to signalling of PI3K/AKT and ErkMAP kinase pathways affecting cell GLUT4 translocation (Peterson and Shulman, 2018). Although the exact underlying cause of insulin resistance is complex, and has not been fully elucidated, a number of mechanisms have been proposed involving oxidative stress, inflammation, endoplasmic reticulum stress, and mitochondrial dysfunction (Yaribeygi *et al*, 2019). However, regardless of the origins, insulin resistance prevents the efficient uptake and utilisation of cell glucose leading to a chronic state of hyperglycaemia (Kovacs *et al*, 2003).

2.2 Epigenetics and T2DM

Epigenetic modification is typically defined as heritable changes in gene regulation that occur without a change in the DNA nucleotide sequence (Bird, 2007). This definition of epigenetics

excludes effects due to non-coding RNA, which almost exclusively act at the post-transcriptional level in animals and, with few exceptions, have no heritable effect on transcription (Gibney and Nolan, 2010). Epigenetic regulation primarily includes DNA cytosine methylation and multiple histone post-translational modifications (PTM's) (Bansal and Simmons, 2018). Methylation of DNA contributes to cell-specific gene expression, and is important for embryonic development, establishing the process of imprinting, and X-chromosome inactivation in mammals (Jones 2012, Reik *et al* 2001, Bansal and Simmons 2018). Specifically, methylation occurs with the addition of a methyl group (CH_3) to the DNA base cytosine, which inactivates gene transcription. Post-translational histone modifications on the other hand, alters chromatin compaction/accessibility and influence the recruitment of transcription regulators to modify gene expression. Histone modifications include, acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, Lactylation and Serotonylation (Zhang *et al* 2019, Farrelly *et al* 2019) of amino acid residues (*e.g.* lysine or arginine), all of which regulate gene expression; achieved solely by covalently modifying the highly conserved NH_2-terminal tail of histones by a range of enzymes including histone methyltransferases, acetylases, and deacetylases (HDAC's) (Berger, 2007).

Environmental influences (nutrition, exercise, stress, alcohol) can determine the degree of transient or persistent changes occurring as either DNA methylation or histone modifications (altering gene expression) (Zhou *et al*, 2017). Early work established a possible role for epigenetics in diabetes onset, where the effect of maternal and intrauterine malnutrition and growth retardation was postulated to develop T2DM. For example, the *'thrifty phenotype hypothesis'* (Hales and Barker, 1992) proposes that undernutrition during foetal development, coupled with a low birthweight, leads to adverse and permanent changes in glucose homeostasis in the offspring (Dhawan and Naterajan 2019, Ling and Rönn 2019). It is now known that environmentally-linked epigenetic mechanisms play a principle role in the regulation of gene expression, and are involved in a plethora of relevant metabolic pathways (pancreatic and β-cell function, peripheral glucose uptake, insulin resistance) aligned to the susceptibility of the T2DM phenotype (Zhao *et al* 2012, Dhawan and Naterajan 2019, Bansal and Simmons 2018). Epigenetic alterations of numerous genes occur in human T2DM pancreatic islets. Just over a decade ago, Ling *et al* (2008) analysed the DNA methylation pattern of candidate genes for T2DM including *INS* (encoding insulin), *PDX1*, *PPARGC1A* (encoding *PGC-1α*), and *GLP1R* (encoding the *GLP-1* receptor) in T2DM compared with non-T2DM pancreatic islets, and showed hypermethylation of all genes, with a reduction in transcriptional activity and insulin secretion. *PGC-1α* is involved in regulating glucose homeostasis by co-activating *FOXO1* (Puigserver *et al*, 2013), and as such, it is possible that

promoter methylation of *PGC-1α* may play a functional role in increasing susceptibility to T2DM onset (Hawkins *et al*, 2018).

Volkmar *et al* (2012) conducted the first DNA methylation profiling of pancreatic islets from T2DM and non-T2DM controls and showed altered DNA methylation of 276 CpG sites annotated to promoters of 254 genes (*e.g. CHAC1, NIBAN, PER2*). Within a subgroup of the differentially methylated genes, concordant transcriptional changes were also present. Functional annotation of the aberrantly methylated genes highlighted pathways implicated in β-cell survival and function. In a follow-up investigation, Dayeh *et al* (2014) identified altered DNA methylation of 1,649 CpG sites affiliated to 843 genes in pancreatic islets from T2DM compared to controls, with 102 exhibiting differential gene expression (*e.g. CDKN1A, SEPT9, PDE7B*) synonymous with methylation patterns in T2DM islets. While the majority of sites showing change were in open sea areas (*i.e.* far from known genes) and the number of diabetic patients was relatively small (*n*=15), the authors went further and tested the potential functional significance of changes using an *in vitro* cell-line approach. By overexpressing *CDKN1A*, *PDE7B* and *SEPT9* in clonal β-cells, they showed the loss of methylation in these genes might contribute to a decrease in glucose-stimulated insulin secretion. Overexpression of *CDKN1A*, encoding a potent cyclin-dependent kinase inhibitor that regulates cell-cycle progression to G1, also decreased cell proliferation in clonal β-cells.

As documented by Ling and Rönn (2019), many array-based DNA methylation studies as outlined, only cover *circa* 1.5% of the methylome. As such, Volkov *et al* (2017) utilised whole-genome bisulfite sequencing (WGBS) to quantify methylation of $\sim 2.4 \times 10^7$ CpG sites (83% of all CpG sites in the human genome) and identified 25,820 differentially methylated regions (DMRs) in T2DM islets. Two of the most significant DMRs covered *PDX1*, a key transcription factor in islets that regulates insulin expression. Furthermore, 457 genes, including *NR4A3*, *PARK2*, *PID1*, and *SOCS2*, had both DMRs and expression changes in T2DM islets, and when a selection of these genes were either overexpressed or silenced in cultured β-cells, insulin secretion was impaired; reinforcing the link between DNA methylation and impaired pancreatic islet function (Volkov *et al* 2017, Ling and Rönn 2019). Aside from pancreatic islets as the main tissue of interest, other studies utilising liver, blood, muscle and adipose tissue have also identified numerous CpG sites with altered DNA methylation in patients with T2DM (Abderrahmani *et al* 2018, Dayeh *et al* 2016, Nilsson *et al* 2014, Ribel-Madsen *et al* 2012), supporting the role of DNA methylation *per se* in the pathogenesis of diabetes (Ling and Rönn, 2019).

Notably, data on genome-wide histone modifications in human pancreatic islets is scarce (Ling and Rönn, 2019), and the few studies published to date, have largely focussed on non-T2DM cohorts (Bhandare *et al* 2010, Pasquali *et al* 2014, Stitzel *et al* 2010). For example, Bhandare *et al* (2010) identified an important relationship between promoter structure, histone modification, and gene expression, and showed co-occurrences of histone modifications including bivalent marks in 31 normal human mature islets. Surprisingly however, they found little correlation between H3K4me3 levels, the usual mark of active promoters, and transcription at the insulin and glucagon genes. However, a recent animal study ascertained that knockout of islet-specific *PRMT5* (arginine methyltransferase enzyme), reduced expression of the insulin gene, and impaired glucose tolerance and glucose-stimulated insulin secretion, indicating that this methyltransferase enzyme is central to the regulation of insulin gene transcription via histone methylation-related chromatin remodeling (Ma *et al*, 2020). Others have examined histone modifications in blood of T2DM (Hou *et al* 2011, Paneni *et al* 2015), with the work of Hou *et al* (2011) demonstrating an increase in H3 acetylation of *TNF-α* and *COX-2* gene promoters in peripheral blood mononuclear cells from T2DM. That stated, there is a need for comprehensive epigenome studies in a range of human T2DM tissues, with a particular emphasis on using sorted cell fractions and single-cell analyses (Ling and Rönn, 2019) to further improve our understanding of the links between epigenetics and T2DM. For a further appraisal of epigenetic genes associated with T2DM, the reader is directed to excellent reviews by Ling and Rönn (2019), and Ahmed *et al* (2020).

3. Metabolism and Epigenetic Control of Gene Transcription

3.1 Metabolism

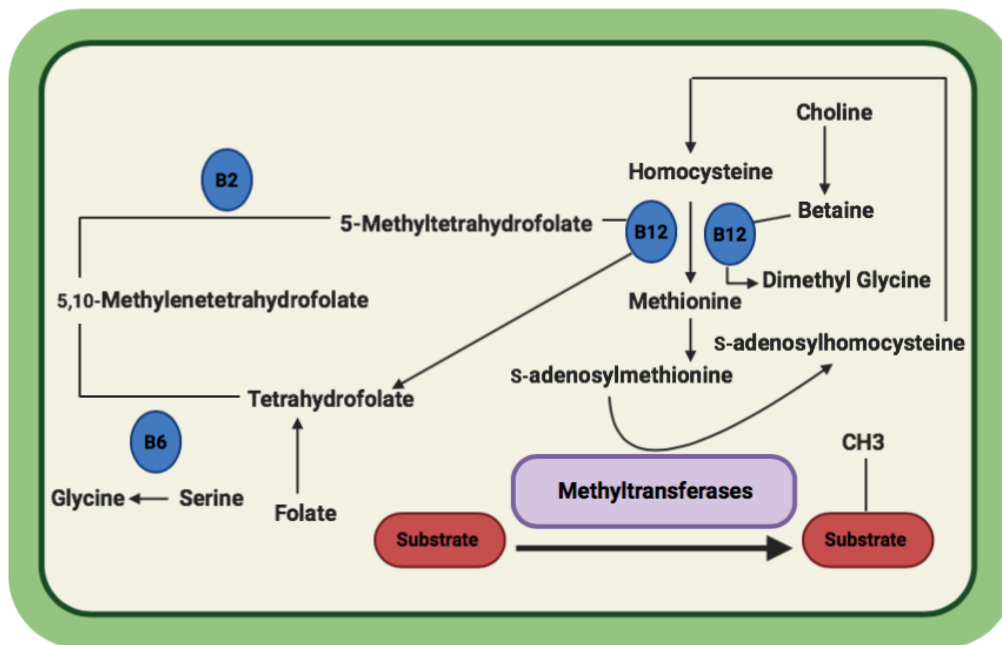
Metabolism may be defined as the set of life-sustaining chemical modifications within cells, and these chemical modifications may be catalysed by specific enzymatic reactions in order to maintain cell and tissue homeostasis in response to environmental conditions (Etchegaray and Mostoslavsky, 2016). Many aspects of epigenetic modifications require intermediates of cell metabolism for customary enzymatic function; conversely, any change to cell metabolism can alter specific acetyltransferases and methyltransferases conferring widespread fluctuations in epigenetic modification patterns (Keating and El-Osta, 2015). In addition to overall cell metabolic changes, the epigenome may also be modified by intracellular metabolite localisation, for example mitochondria (specifically the TCA cycle) produce a plethora of key regulatory molecules, which are consumed as co-substrates for numerous transcriptional and epigenetic processes (Shaughnessy *et al* 2014, Keating and El-Osta 2015). To the contrary, TCA enzymes may block enzymatic action. This interplay between metabolism and epigenetics, termed *'metaboloepigenetics'* (Donohoe and Bultman, 2012),

and the prospect of metabolic intermediates controlling gene expression is no doubt exciting, however connections to date are not fully understood, and limited to only a few disease states such as cancer (Miranda-Goncalves *et al*, 2018). As T2DM is a disease of metabolic dysfunction, it is theoretically plausible that hyperglycaemia interrupts enzymatic function, causing a modification of epigenetic signatures and gene expression. As such, this connection will be explored as per the following.

3.2 One-Carbon Metabolism and DNA/Histone Methylation

One-carbon metabolism is a biochemical pathway that utilises nutrients for multiple biological functions (Mentch and Locasale, 2016). Two major components of one-carbon metabolism comprise the folate and methionine cycles, that essentially transfer single carbon units to acceptor molecules. *S*-adenosylmethionine (SAM) provides the activate methyl (CH_3) donor group as the main substrate for methyltransferase (DNA methyltransferase – DNMT, and histone methyltransferase - HMT) reactions on DNA, and on arginine and lysine residues of histones, leading to DNA methylation and PTM's (Mentch and Locasale 2016, Keating and El-Osta 2015). Tetrahydrofolate (THF) when methylated on its N-5 atom (N^5-MTHF - methyltetrahydrofolate) also acts as a methyl donor. However, unlike SAM, the transfer potential of the methyl donor group of N^5-MTHF is not sufficiently high for the majority of methylation reactions (Kaelin and McKnight, 2013). SAM is synthesised by methionine adenosyltransferase (MAT) using methionine and adenosine triphosphate (ATP) as substrates. The methyl group of SAM is chemically activated by the positive charge on the adjacent sulfur atom, causing the SAM methyl group to be more reactive compared to the methyl group on N^5-MTHF (Kaelin and McKnight, 2013). When SAM donates its methyl group, it is converted to *S*-adenosylhomocysteine (SAH). One caveat worth highlighting is that SAH is a potent inhibitor of DNMTs and HMT's. As such, the SAM/SAH ratio can dictate methyltransferase activity *in vivo* (Wong *et al*, 2017). That said, SAH is physiologically controlled at low concentrations via hydrolysis to homocysteine (via S-adenosylhomocysteine hydrolase - SAHH), which is recycled to methionine via the remethylation pathway, by the transfer of a CH_3 from N^5-MTHF or catabolised to amino acids or glutathione. As such, the SAM/SAH ratio serves as a biosensor of the cells metabolic state, and any fluctuations in the SAM/SAH ratio by a change in nutrient availability, can thus modulate the activity of DNMT and HMT affecting DNA promoter methylation and/or histone methylation (Mentch 2015, Etchegaray and Mostoslavsky 2016) (Figure 2).

Figure 2. *Metabolism and Methyltransferases*

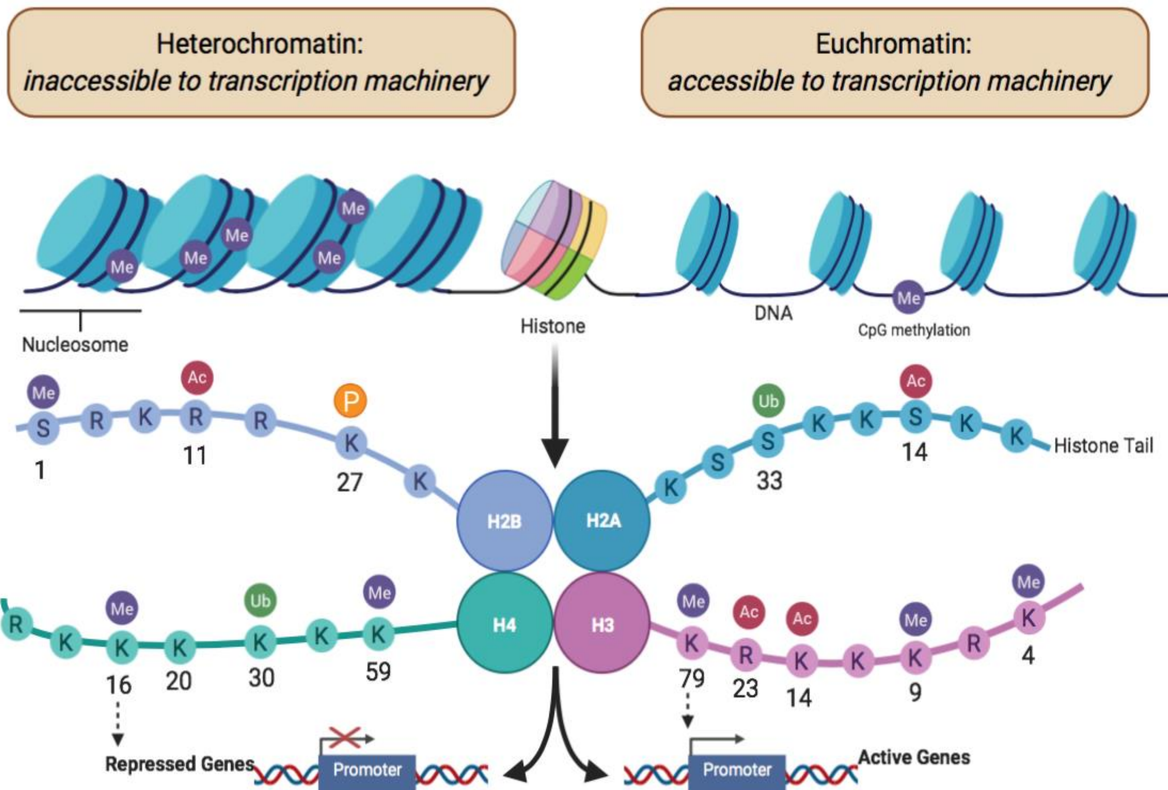


DNA and histone methyltransferases use S-adenosylmethionine as a methyl donor, a reaction that involves upstream vitamin B12 activity and the production of S-adenosylhomocysteine. Redrawn from Kaelin and McKnight (2013).

DNA methylation occurs in two contexts aided by three DNA methyltransferase enzymes. DNMT3A and DNMT3B are *de novo* enzymes that deposit CH_3 marks on previously unmodified cytosines (Okano *et al*, 1999). DNMT1, in contrast, is a maintenance methyltransferase, which recognises hemimethylated DNA to form a symmetrically modified duplex (Grurnbaum *et al*, 1982). Methylation of DNA occurs with the addition of CH_3 at the C^5 position of the nucleoside cytosine, forming 5-methylcytosine (5mC); most frequently at CpG dinucleotides. In fact, over 80% of cytosine residues in CpG dinucleotides are methylated at position 5. Methylation is notably absent in CpG-rich regions (CpG islands) near the 5'-prime end of genes which contain promoter regions with active transcription. However, when promoter-associated CpG islands are methylated, transcription is silenced (Van den Veyver, 2002). More recently, it has been clearly shown by a number of genome-wide and functional studies that DNA methylation in the gene body facilitates transcription (Wu *et al* 2010, Neri *et al* 2013, Irwin *et al* 2014). The inhibitory functions of DNA methylation on gene expression are mediated by the presence of methyl groups, and methyl-CpG-binding proteins (*i.e. MeCP2*) which allosterically prevent the binding of transcription factors or enhancers to the gene promoter region (Kirchner *et al* 2013, Hawkins *et al* 2018). The positive effects on the other hand seem to be associated with transcription-coupled deposition of DNA methylation, linked to the reading of H3K36me3 (Greenberg and Bourc'his, 2019).

Methylation of histone H3 and H4 is aided by the recruitment of HMT enzymes to covalently add CH_3 from SAM onto the side-chain nitrogen atoms of mainly lysine and arginine residues (Vanzan *et al* 2017, Wong *et al* 2017). Lysine methyltransferases contain a conserved domain Su(var)3-9, Enhancer of zeste, and Trithorax (SET) responsible for the disposition of CH_3 specifically at H3K4, H3K9, H3K27, and H3K36 (Vanzan *et al* 2017). The consequence of histone methylation is determined by the specific histone residue modified, the number of methyl groups added (mono-, di-, or tri-methylation), and the location within the N-terminal regions of either H3 or H4 (Kaelin and McKnight 2013, Etchegaray and Mostoslavsky, 2016); manifested through a state of euchromatin (lightly packed DNA promoting transcription) or heterochromatin (condensed DNA suppressing transcription; Figure 3) (Bansal and Simmons, 2018). For instance, methylation of H3K4, H3K48, H3K36, and H3K79 leads to transcriptional activation, while methylation of H3K9, H3K27, and H4K20me1, cause compaction of chromatin and transcriptional repression. Regarding arginine residues, H3R17me2 represents activation, while H3R2me2 is associated with transcriptional repression (Etchegaray and Mostoslavsky, 2016).

Figure 3. *Chromatin and Regulation of Gene Transcription*



Gene transcription is regulated by chromatin accessibility. The nucleosome comprises 147 base pairs of tightly coiled DNA around each (two copies) of the 4 core histone proteins H2A, H2B, H3, and H4 (Rudolph and Luger 2020, Keating and El-Osta 2015). R, Arginine; K, Lysine; S, Serine; Me, Methylation; Ac, Acetylation; Ub, Ubiquitination; P, Phosphorylation. H4K16me and H3K79me characterise a closed and open chromatin state, leading to repressed and active gene transcription respectively.

3.3 Histone Acetylation

The tricarboxylic acid (TCA) cycle is a series of enzymatic energy producing reactions in a closed-loop, that controls mitochondrial oxidative metabolism; it begins with the reaction that combines the two-carbon acetyl-CoA (generated from glucose, fat or amino acid oxidation) with a four-carbon oxaloacetate to generate the six-carbon citrate (Martinez-Reynes and Chandel, 2020). The most prominent signalling function of acetyl-CoA in particular, is likely related to its ability to transfer an acetyl group to lysine amino acids on the N-terminal tails of histone to yield acetylation (Martinez-Reynes and Chandel 2020, Vanzan *et al* 2017). Acetylation (t1/2 = 3 minutes) can occur in any of the four histones controlled by a HAT enzyme, and the activity of HAT is sensitive to fluxes in intracellular acetyl-CoA concentration (~10-fold under normal physiological conditions and within the K_m range of HAT's), determined in part by glucose availability (Kaelin and McKnight 2013, Wong *et al* 2017). HTA's typically belong to three main families: GNAT (Gcn5-related N-acetyltransferase), MYST (MOZ, Ybf2/Sas3, Sas2, Tip60), and p300/CBP. These enzymes catalyse the abstraction of a proton from the ε-amino group of lysine, which is subsequently primed for nucleophilic attack on the

keto-carbon of acetyl-CoA; this intermediate structure decomposes, releasing free coenzyme A and acetyl-lysine. HAT enzymes are not specific for lysine residues on histone, but salient for pan-acetylation reactions (Cry and Domann, 2011).

The established role of histone acetylation is regulation of gene transcription. For example, acetylation (*i.e.* H3K56ac, H3K9ac, H3K27ac, H4K91ac, H4K5ac) at specific gene loci is implicated in transcriptional activation by opening chromatin structure, however the mechanism is under debate, and may include: (1) histone acetylation inherently involves the charge moving from positive to neutral, thus weakening the interaction of the nucleosome with the DNA backbone; this opens chromatin leading to active transcription, (2) histone acetylation serving as a docking station for the recruitment of transcription regulators (Vanzan *et al* 2017, Wang *et al* 2018).

3.4 Histone Lactylation

Lactate is an abundant metabolite produced during glycolysis, where glucose is converted to two pyruvate molecules; these can either be fluxed into lactate or transported into mitochondria forming acetyl-CoA (Izzo and Wellen, 2019). Using pro-inflammatory (M1) macrophages, Zhang *et al* (2019) identified 28 lactylation sites on core histone lysine residues that directly stimulates gene transcription (*e.g.* H3K18la = M2 anti-inflammatory gene activation) from chromatin. Through a combination of functional experiments and using isotopically labelled glucose ($^{13}C_6$-glucose), it is postulated that lysine lactylation depends solely on glucose flow through glycolysis – *i.e.* as glycolysis increases, intracellular lactate rises, paralleling an increase in histone lactylation. The authors imply that an endogenous '*lactate timer*' in M1 macrophages controls histone modification and stimulation of M2 gene expression to promote intracellular homeostasis. Whilst this novel data is intriguing, caveats remain: 1. the data warrants independent validation; 2. While the authors show that lactyl-CoA is a lactyl-group donor for lysine lactylation, the enzymes that produce lactyl-CoA from lactate, and the cell concentration of lactyl-CoA remain unknown (Izzo and Wellen, 2019).

4. Metabolic Regulation of DNA and Chromatin Dynamics in T2DM

4.1 Metabolite-Dependent Dioxygenases in the Regulation of Histone/DNA Demethylation and Histone Deacetylation

TCA cycle metabolites modulate DNA and histone demethylation. Methylated cytosine residues are demethylated in two sequential steps: oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), catalysed by the ten-eleven translocation (TET 1-3) dioxygenase proteins (TET proteins share a similar C-terminal domain with a cysteine rich

region and a double-stranded beta-helix, N-terminal regions are less conserved - Veland and Chen, 2017) followed by decarboxylation to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). 5caC is recognised and excised by the base excision repair enzyme thymine DNA glycosylase (TDG's) to restore unmethylated cytosine (He *et al* 2011, Rodriguez *et al* 2017). TET proteins control these oxidation reactions, and in doing so utilise α-Ketoglutarate (α-KG*),* oxygen and Fe^{2+} as co-factors (Figure 4). α-KG is produced from isocitrate by mitochondrial enzymes isocitrate dehydrogenase 2 (IDH2) and 3 (IDH3) in the TCA cycle, and its involvement in TET activity is dependent on the intracellular ratio of α-KG to succinate, fumarate or 2-hydroxyglutarate (2-HG) (Martinez-Reyes and Chandel, 2020). That said, intracellular α-KG concentrations are estimated to be in the low millimolar range, well above the α-KG K_m values of the known TET proteins (Chowdhury *et al*, 2011). A caveat, however, is that such K_m values are typically determined via *in vitro* scenarios using purified enzymes in the absence of endogenous inhibitory molecules such as ROS, fumarate and succinate (Kaelin and McKnight, 2013). On the latter, the TCA cycle intermediate molecules fumarate and succinate are potent inhibitors of TET activity, causing hypermethylation of DNA. Intracellular fumarate and succinate concentration accumulate due to inactivation of succinate dehydrogenase and fumarate hydratase respectively when glycolysis switches to a state of oxidative phosphorylation, thus outcompeting α-KG to downregulate TET activity (Xu *et al* 2016, Wang *et al* 2018).

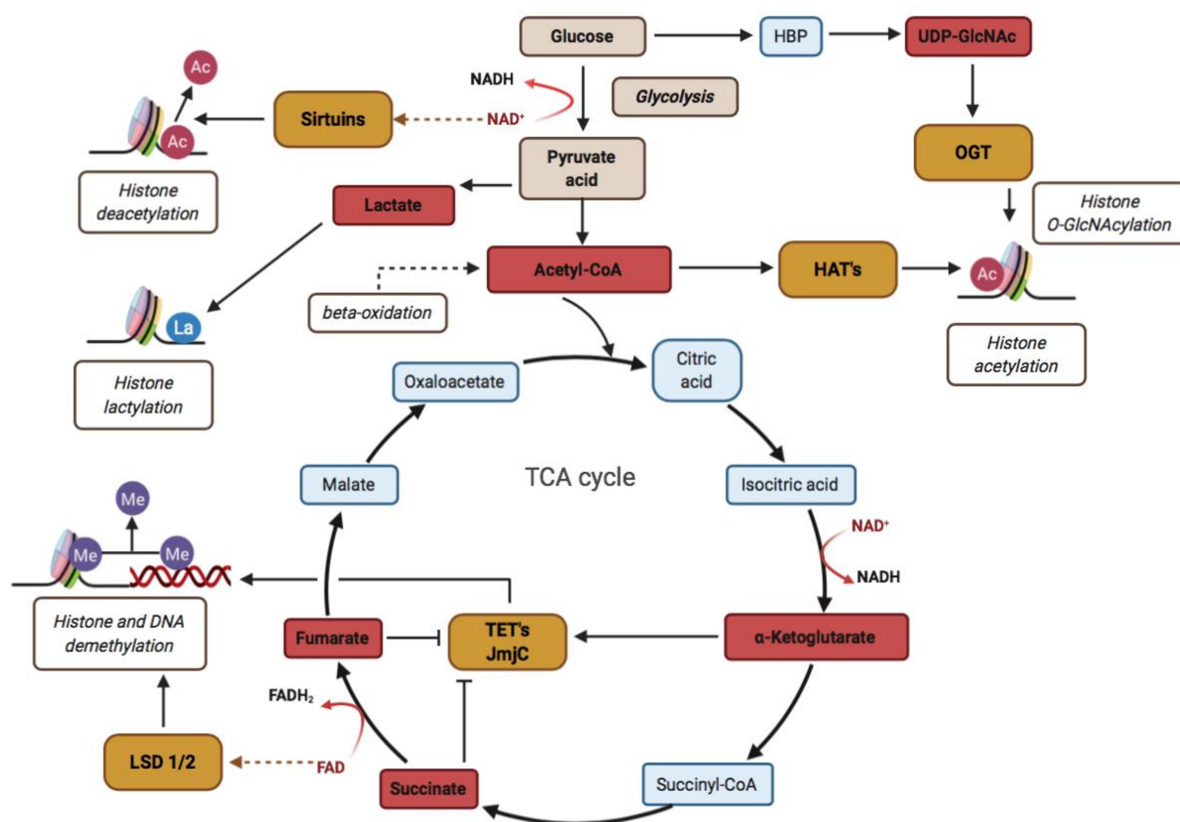
α-KG-related hydroxylation reactions further depend on the regeneration of Fe^{2+} and involves L-ascorbic acid (vitamin C) by inducing the reduction of oxidised Fe^{3+} to Fe^{2+} to restore TET activity (Martinez-Reyes and Chandel, 2020). Vitamin C is an essential water-soluble micronutrient that exists as ascorbate anion under physiological pH conditions, however, humans cannot synthesize ascorbate due to a mutant and non-functional enzyme, L-gulonolactone oxidase (*Gulo*), which catalyses the last step of ascorbate biosynthesis (Young *et al*, 2015). As such, humans are required to supplement with ascorbate through dietary sources (Etchegaray and Mostoslavsky, 2016). In addition, intracellular ROS activity leading to a state of oxidative stress, may also dictate ascorbate concentration and activity in cells. The role of ascorbate as a co-factor for TET dioxygenases to sustain and complete the hydroxylation of 5mC to 5hmC is supported by DiTroia *et al* (2019) and Blaschke *et al* (2013), the latter showing rapid and global DNA demethylation in embryonic stem cells. As such, TET proteins can respond under physiological changes to agonists such as α-KG, Fe^{2+}, ascorbic acid or antagonist molecules such as fumarate and succinate, and may be further deregulated by an altered and dysfunctional metabolism (Kaelin and McKnight, 2013).

Regarding histones, active demethylation is tightly associated with products of cell metabolism and may be carried out via two pathways: lysine-specific demethylases (LSD), and through the JumonjiC (JmjC) domain proteins (Lu and Thompson, 2012). On the former, LSD1 incorporates the co-factor flavin adenine dinucleotide (FAD^+), which is reduced to $FADH_2$ in mitochondria (Miranda-Goncalves *et al*, 2018) following demethylation of H3K4me1, H3K4me2, H3K9me1 and H3K9me2 (Etchegaray and Mostoslavsky, 2016). The paralog LSD2 also utilises FAD^+ to demethylate H3K4me1 and H3K4me2 (Karytinos *et al*, 2009), and interestingly, LSD2 possesses an E3 ubiquitin ligase which promotes the degradation of O-GlcNAc transferase (OGT): an enzyme necessary to add the sugar moiety to substrates through a posttranslational state of *O*-GlcNAcylation (Etchegaray and Mostoslavsky, 2016). Similar to the workings of the TET isoenzymes, the *circa* 30 JmjC domain-contain histone demethylases operate through Fe^{2+} and α-KG co-factors to remove methyl groups from arginine and trimethylated lysine (H3K4, H3K9, H3K27, H3K36 and H4K20) in an oxidative reaction producing hydroxymethyl lysine. Moreover, JmjC is also inhibited by the TCA intermediates fumarate and succinate (Wang *et al* 2018, Rodriquez *et al* 2017, Etchegaray and Mostoslavsky 2016).

Histone deacetylation (removal of an acetyl group) precedes a closed chromatin configuration, leading to gene silencing, and to a large extent, deacetylation reactions are metabolic responsive (Wang *et al* 2018, Miranda-Goncalves *et al*, 2018). The glycolytic substrate nicotinamide adenine dinucleotide (NAD) is a salient redox cofactor in the deacetylation activity of sirtuins, which are a subgroup of the highly conserved HDAC enzymes. Seven sirtuins are distributed within mammalian cells, with SIRT1, 2, 6 and 7 localised to the nucleus (Miranda-Goncalves *et al* 2018, Imai *et al* 2000). As NAD is subject to oxidation during the course of normal cell metabolism, it yields NAD^+ and NADH, and any adjustment in the NAD^+/NADH ratio can subsequently affect sirtuin activity (Figure 4). For example, when cell energy is positively charged (*e.g.* increased glucose flux), the NAD^+/NADH ratio drops, and this metabolic sensor inhibits sirtuin activity to regulate gene expression (Wong *et al*, 2017). In contrast, when cell NAD^+ concentration is enhanced (increased NAD^+/NADH ratio) through AMP-activated kinase (AMPK) in a scenario such as a calorie restrictive state, sirtuin activation ensues: when cells are deprived of ATP and other metabolic substrates, SIRT1 and 6 genes are upregulated leading to histone (H3K9ac and H3K14ac) deacetylation (Canto *et al* 2009, Etchegaray and Mostoslavsky 2016).

The aforementioned supports a clear interconnectedness between active metabolic pathways, and the intricate balancing of chromatin dynamics to achieve a state of normal cell functioning, summarised in Figure 4.

Figure 4. *TCA Cycle and Glycolytic Regulation of Chromatin and DNA (De)Methylation*



Products of glycolysis (cytosol) and the tricarboxylic acid cycle (TCA; mitochondria) are required for chromatin and DNA modifications. Acetyl-CoA synthesised from pyruvate acid and fatty acid oxidation provides substrate for histone acetyltransferases (HATs). Histone deacetylation requires sirtuin molecules via the co-factor nicotinamide adenine dinucleotide (NAD.) Lysine specific demethylases (LSD) utilise flavin adenine nucleotide (FAD), while α-ketoglutarate controls the ten-eleven translocation (TET) enzymes and Jumonji C domain-containing (JmjC) demethylases. Fumarate and succinate blocks TET and JmjC molecules. Cell glucose can be metabolized through the hexosamine biosynthetic pathway (HBP) generating uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc), a key substrate used by O-GlcNAc transferase (OGT) for histone O-GlcNAcylation. Lactate is an end product of glycolysis yielding histone lactylation. Key intermediate metabolites involved in the control of histone and DNA modification shown in red. Molecules affected by key metabolites shown in orange.

4.2 Metabolic Regulation of Epigenetics in T2DM

Metabolism *per se* regulates epigenetics, and any fluctuation in metabolic homeostasis can cause perturbations in gene transcription, cell growth and differentiation, and in DNA replication and recombination (Lu and Thompson, 2012). The primary characteristic underpinning the pathophysiology of T2DM is dysregulated glycaemia, and as such it is conceivable that alterations in epigenome-related metabolism may facilitate disease progression. A complex relationship exists between hyperglycaemia and epigenetic and

transcriptional modifications, however to date, work in this area is scant. Gaikwad *et al* (2010) ascertained the effect of hyperglycaemia on histone H3 modification in diabetic rats, and showed for the first time, an increase in histone H3 acetylation and gene expression of the *Fbn1* gene following hyperglycaemia, suggesting that the change in expression of the *Fbn1* gene is epigenetically regulated. The translational relationship between environmental stimuli and epigenetics on a clinical phenotype was ascertained by Volkmar *et al* (2012), where they performed the first comprehensive DNA methylation profiling in T2 diabetic and non-diabetic pancreatic islets cells. 276 CpG loci affiliated to promoters of 254 genes were differentially methylated in diabetic islets; dysregulation of these genes was linked to β-cell functionality, cell death and adaptation to metabolic stress. That said, the same methylation modifications were not present in blood cells, nor in non-diabetic islet cells experimentally induced by high glucose. However, in other studies, pancreatic β-cells exposed to hyperglycaemia display a hypermethylated state (Iskikawa *et al* 2015, Yang *et al* 2012), while work in aortic endothelial cells and vascular smooth muscle cells suggest that constant exposure to hyperglycaemia can instil permanent epigenetic marks that persistently change gene expression (El-Osta *et al* 2008, Villeneuve *et al* 2008). This 'metabolic memory' phenomenon is understood to be responsible for the progression of diabetic complications, even in the presence of a strict glycaemic control (Reddy *et al*, 2015).

Examining the relationship between gene methylation and long-term glycaemic control was the focus of work by Ortiz *et al* (2018), where a greater percentage methylation of the *FKBP5* intron 2 at a single CpG-dinucleotide region was positively related to a higher HbA1c (8.3%) in T2DM patients (r = 0.535, p = 0.003). In a follow-up study by Roshanzamir and Hassan-Zadeh (2019), the effects of glycaemia on DNA methylation relating to pro-inflammatory genes (*IL-1β* and *IL1R1*) was ascertained. Hyperglycaemia elicited a hypomethylation state in *IL-1β* and a hypermethylation state in *IL1R1.* Further analysis using Sanger sequencing discovered that two selected and specific CpG sites in *IL-1β* are affected by hyperglycaemia, while only one CpG site (from three) in *IL1R1* was affected. These data suggest that DNA methylation at specific CpG sites aligned to a selected metabolic pathway may have the potential to be utilised as an epigenetic marker of chronic inflammation and T2DM development. However, there are major caveats around assuming changes in methylation at single, or even a few CpGs, are functionally important, particularly if the absolute changes are not great, so this work must be treated with caution until linked to further mechanistic evidence.

With a greater emphasis on mechanisms *per se*, Pinzon-Cortes *et al* (2017) analysed global DNA methylation and hydroxymethylation in peripheral blood mononuclear cells (PBMC's) of well- and poorly-controlled T2DM patients, with the latter showing an increase in 5hmC. 5mC

and 5hmC were tightly correlated with glycated haemoglobin, suggesting a direct relationship between hyperglycaemia and epigenome modifications. This work raises the intriguing possibility that TET isoenzymes are activated in the presence of an increased glucose load, and indeed this mechanistic possibility was confirmed by Dhliwayo *et al* (2014) through a series of RNA expression and enzymatic assays, demonstrating that TET enzymes are activated by hyperglycaemia inducing a state of genomic cytosine demethylation. Moreover, this work also observed that demethylation via the TET-dependent oxidation pathway (5mC-5caC) is prevented by inhibiting poly(ADP-ribose) polymerase (Parp), suggesting that hyperglycaemia activates Parp enzyme activity, which in turn stimulates TET's leading to DNA methylation. In a related mechanistic study, Yuan *et al* (2019) examined fasting blood from a T2DM cohort using a HPLC-MS/MS approach to quantify global 5mC and 5hmC, while using qPCR to detect expression of histone deacetylate *SIRT6* and *TET* activity. Genomic 5mC decreased, while 5hmC increased in the presence of an upregulation in *TET2, TET3* and *SIRT6*. Correlational analysis revealed a positive association between *SIRT6* and *TET2* activity ($r = 0.277$, $p < 0.001$); collectively these data indicate that hyperglycaemia stimulates *SIRT6* and *TET2* (perhaps via NAD^+ and α-KG activation respectively) expression, thereby oxidising 5mC to 5hmC. Furthermore, and in support of *SIRT6* playing a pivotal role in metabolic regulation, a pharmacologically-based approach ascertained that *SIRT6* inhibition improves glycaemic control in T2DM (Sociali *et al*, 2017).

A constant state of hyperglycaemia is purported to link diabetes to cancer onset, however until recently, this supposition lacked molecular evidence. Wu *et al* (2018) demonstrates that hyperglycaemia adversely effects the DNA 5-hydroxymethylome. Specifically, in PBMC's extracted from T2DM patients with poor metabolic control (HbA1c 10.7 $\pm$ 1.9%), an increase in glucose impedes AMPK-mediated phosphorylation at serine 99, thereby destabilising TET2 - leading to the dysregulation of both 5hmC and the tumour suppressor function of TET2. As such, this work describes an environment-to-epigenome signalling pathway, the glucose-AMPK-TET2-5hmC axis, which links the concentration of extracellular glucose to the dynamic regulation of 5hmC. Interestingly, treatment with the T2DM drug metformin, seems to protect AMPK-mediated phosphorylation, stabilise TET2, and restore 5hmC. As outlined by the authors, this novel '*phospo-switch*' and model, represents a new paradigm towards a diabetes oncogenic state. An observation worth highlighting at this juncture, is the contrasting data by Wu *et al* (2018) compared to Dhliwayo *et al* (2014) and Yuan *et al* (2019); while the former illustrates that hyperglycaemia destabilises TET isoenzymes, the latter shows that hyperglycaemia activates TET's. More work is clearly required in this important area of investigation, specifically linking metabolism *per se* to the modification of DNA methylation in β-cell's via TET enzymes. To our knowledge, no TET enzyme has been isolated in terminally

differentiated β-cells; it is therefore plausible, that epigenetic changes in the T2DM model may likely be caused by changes in 5mC in stem cells that differentiate into terminally differentiated cells in the pancreas or blood. This paradigm is also likely synonymous with models explaining the developmental origins of disease, where modifications to DNA methylation may originate early in stem cells prior to appearing in terminally differentiated cells. Further work however, through rigorous experimentation is needed to confirm this supposition.

More recently, and as discussed, Zhang *et al* (2019) describes a previously unknown histone modification, lactylation, derived from cell metabolic lactate, and translating to disrupted gene expression. Indeed, lactate has been linked to PTM's now for some time, with Latham *et al* (2012) demonstrating that lactate inhibits HDAC activity and promotes gene transcription in cultured HCT116 cells. As lactate is an oxidised product of glucose, and hyperlactatemia is common in T2DM (Aleksander *et al*, 2016), it is theoretically plausible that a dysregulated glucose metabolic state in diabetes may increase susceptibility to histone lactylation and transcriptional modification.

Whether an adverse glycaemic control is a cause or consequence of epigenetic regulation, has been the subject of recent debate. Kim (2019) highlights this salient perspective, and posits that DNA methylation in particular may be regarded as both a cause and consequence of T2DM. Numerous studies infer a driver role for DNA methylation in T2DM development, partially caused by an accumulation of errors leading to an altered transcriptional response (Kim, 2019). While aberrantly methylated genes in T2DM are linked to β-cell dysfunction leading to a perturbation of insulin and glucagon secretion (Dayeh and Ling, 2015), other data reveals a differential methylation pattern (compared to non-T2DM) in tissues aligned to adipose-tissue, skeletal muscle and liver (Barres *et al* 2013, Nilsson *et al* 2014, Nilsson *et al* 2015). On PTM's, there is accumulating evidence for a plethora of genes (*IGFR, InsR, IRS1/2, PI3K, Akt, GLUT4, FoxO, PGC-1α, PPAR, AMPK, MAPK*; Emamgholipour *et al* 2020) proposed to regulate insulin resistance and glucose homeostasis. In early work as already mentioned above, Bhandare *et al* (2010) determined the histone modification profile of human pancreatic islets by mapping genome-wide locations of histone marks associated with gene activation – H3K4me1, H3K4me2, H3K4me3, and gene repression – H3K27me3. Although promoters of the highly transcribed insulin and glucagon genes are only sparely occupied by H3K4me2 and H3K4me3, on a global scale, there were notable relationships between promoter structure, histone modification and gene expression. In an interesting set of studies, Backe *et al* (2018 and 2019) have shown that preserving H3K27 and H3K4 methylation using the lysine demethylase inhibitor GSK-J4 improves β-cell function, and by knockout of the H3K4 demethylase *KDM5B*, insulin sensitivity and glucose homeostasis may be improved;

highlighting the potential role of *KDM5B* in diabetes related metabolism. Most recently, Ma *et al* (2020) investigated the role of protein arginine methyltransferase 5 (*Prmt5*) in β-cells and found that islet-specific knockout of *Prmt5* decreases insulin gene expression, hypothesising a contributory role in impaired glucose tolerance. Collectively, the aforementioned work points towards epigenetics in the regulation of T2DM onset through malfunctioning biochemical pathways aligned to pancreatic insulin secretion and subsequent glucose control.

Divergently, Geach (2017) claims that although methylation is a relatively stable biochemical process, timeline modifications to the methylome can occur during disease progression. A deregulated metabolism is central to the concept that DNA methylation ensues as a consequential function of T2DM (Kim, 2019). For example, aging is a time-dependent deterioration in multiple biological organs, including pancreatic islets, and any related change may decrease intracellular metabolism. Notably, aging independently modifies the healthy methylome leading to a state of hypermethylation (Bell *et al*, 2019). It has been proposed that the aging diabetic may be more susceptible to a higher incidence of whole genome hypermethylation, initiating an upregulation in genes aligned to proinflammatory pathways, and a corresponding downregulation of basal transcriptional machinery, as well as a compromised DNA-damage-repair system (Kim 2019, Levine *et al* 2018). For example, the mitochondrial cytochrome *c* oxidase polypeptide 7A1 (*COX7A1*) gene is hypermethylated across the lifespan and has the potential to be associated with age-related T2DM (Rönn *et al*, 2008).

A cornerstone in the adverse progression of T2DM is the onset of vascular diabetic complications, and it is widely recognised that epigenetic dysregulation contributes to these phenomena. An epigenetic profiling study using EWAS, shows significant changes (compared to controls) in DNA methylation at loci involved in fibrosis from renal tubes in humans with chronic kidney disease including diabetic nephropathy. Differences in methylation were rarely observed in promoters, but mostly overlapped with enhancer regions, and differentially methylated loci were enriched in binding sequences aligned to renal transcription factors (Ko *et al*, 2013). A further EWAS profile of blood DNA methylation shows predictive changes associated with the decline of renal function in diabetic nephropathy (Chu *et al*, 2017). A main driver of renal dysfunction during diabetes is the cytokine transforming growth factor *(TGF*) *β1*, where it promotes the expression of a select number of extracellular matrix pro-fibrotic genes, by interacting with histone modifying proteins to enhance euchromatin (Rodriguez *et al*, 2017). Under conditions of high glucose, Sun *et al* (2010) examined the role of H3Kme in *TGF-β1*-induced gene expression of collagen-α1[I] and plasminogen activator inhibitor-1, observing an increase in chromatin marks (H3K4me1, H3K4me2, and H3K4me3), and a

decrease in repressive marks (H3K9me2 and H3K9me3) at gene promoters. *TGF-β1* also increased expression of the H3K4 methyltransferase SET7/9 and recruitment to these promoters. Similarly, hyperglycaemia induces H3K18, H3K27ac and H3K4me3 at the collagen I gene promotor via myocardin related transcription factor A (*MRTF-A*) recruitment of p300-HAT and WDR5 linked HMT, enhancing tubulointerstitial fibrosis (Xu *et al*, 2015). As shown by Okabe *et al* (2012) vascular endothelial cells under transient stress by high glucose accumulate Set7 in the nucleus, increasing pro-inflammatory gene expression. In terms of diabetic retinopathy, H3K4me1 is enhanced at the promotor region of *Keap1* which is accompanied by an increase in SET7/9. This is important as in the hyperglycaemic state, an increase in H3K4me1 and subsequent binding of Sp1 at the *Keap1* promotor in the retina, can impede the movement of the transcription factor *Nrf2* to the nucleus, thus impairing the transcriptional activity of a salient cell regulator (Mishra *et al* 2014, Kowluru and Mishra 2017). Likewise, a glucose stimulated increase in H3K420me3 and H3K9ac at the promotor/enhancer region of *Sod2*, can downregulate the expression of this mitochondrial protective protein leading to a compromised retina. Targeting these epigenetic modifications may open up the possibility for therapeutic targets to retard the development and progression of diabetic complications (Zhong and Kowluru, 2011).

T2DM can be denoted by a state of oxidative stress; defined by the modification of DNA, RNA, lipids and proteins, and exacerbated by the production of ROS (Rehman and Akash 2017, Krause *et al* 2014). Chronic hyperglycaemia can generate ROS due to overactivation of metabolic pathways aligned to protein kinase C, polyol, hexosamine, and glucose autoxidation (Brownlee, 2005). The role of mitochondria in the pathogenesis of T2DM is well-established, and prominent sources of ROS include electron leakage at complexes I-III in the electron transport chain, $p66^{Shc}$ and monoamine oxidase (Kaludercic and De Lisa, 2020). Enhanced ROS production is inextricably linked to initiation, progression and clinical consequences of diabetes, including retinopathy, neuropathy, nephropathy and cardiovascular disease (Kietzmann *et al,* 2017). Enzymatic and non-enzymatic antioxidant molecules scavenge ROS, however, T2DM and chronic hyperglycaemia can deplete systemic and intracellular ascorbic acid, increasing oxidative stress (*i.e* DNA damage) leading to poor glucose control (Christie-David *et al*, 2015). Indeed, recent work demonstrates that ascorbic acid supplementation improves glycaemic control in T2DM (Mason *et al,* 2019). Of interest, hyperglycaemia and ROS can activate the H3K4 methyltransferase SET7, leading to a reduction in Nrf2 antioxidant enzyme expression and activity (Kietzmann *et al*, 2017). There are two aspects of interest linked to the above: 1). Ascorbic acid is a known co-factor regulating TET isoenzymes, and 2). ROS-induced DNA damage may interfere with epigenetic process. On the first, *in vivo*

data demonstrate that ascorbic acid enhances TET-mediated 5mC oxidation (Yin *et al* 2013, Blaschke *et al* 2013). However, and notwithstanding that glucose availability influences α-KG production, where one would expect an increase in 5hmC through functional TET activation, it is indeed plausible that a compromised ascorbic acid concentration in T2DM may partially inactivate TET, leading to a hypermethylated state. This supposition may also give rise to an oncogenic cell environment. Secondly, DNA bases are directly modified by ROS, where the hydroxyl free radical (·OH) can play an integral role through its production by Fenton reactions that involve the reduction of H_2O_2 by either ferrous (k ~ 76 M^{-1} s^{-1}) or copper ions (k ~ 4.7 x 10^3 M^{-1} s^{-1}) (Davison, 2016). ·OH-mediated DNA modifications are initiated by hydrogen abstraction or by ·OH interfering with a DNA base (Davison, 2016); an example is the oxidation of 5mC to 5hmC (Kietzmann *et al*, 2017). Moreover, ·OH radical production can directly affect DNA methylation through oxidation of guanosine to 8-oxo-2'-deoxyguanosine (8-oxodG). Usually, the 8-oxodG residues are removed by 8-oxoguanine DNA glycosylase (OGG1) via the short-patch base excision repair (BER) pathway (Kreuz and Fischle 2016, Kietzmann *et al* 2017). However, if 8-oxodG accumulates, methylation of adjacent cytosines is attenuated, causing hypomethylation and transcriptional activation (Le and Fujimori, 2012). Zhou *et al* (2016) infers that OGG1 promotes DNA demethylation by recruitment of TET1 to the oxidised lesion, thus suggesting a model in which oxidative stress recruits OGG1/TET1 complex proteins to 8-oxodG, facilitating the conversion of 5mC through to 5caC close to sites of ROS-induced damage.

In a similar line of investigation, and in a meticulous study by Sulkowski *et al* (2020), an unexpected connection between 2-HG, succinate, fumarate, and DNA repair has been identified. The TCA cycle metabolites inhibit the lysine demethylase KDM4B, resulting in aberrant hypermethylation of H3K9 at loci in close proximity to DNA double-strand breaks. This conceals a local H3K9 trimethylation signal that is essential for the proper functioning of the DNA homology-dependent repair (HDR) pathway. Consequently, the recruitment of salient HDR transcriptional regulators - Tip60 and ATM, is substantially impaired at DNA break sites leading to reduced end resection. Although this work originates from an oncometabolite perspective, T2DM sufferers have less abundant succinate dehydrogenase (2.4-fold) and fumarate hydratase (3-fold) in islets cells (Haythorne, *et al* 2019), and by inference, more active succinate and fumarate respectively (Yang *et al*, 2012). It is also noted that in the presence of a high glucose concentration, fumarate in particular, contributes to a progressive deterioration of β-cell function (Adam *et al*, 2017). It is thus plausible, that a disruption in chromatin signalling and DNA repair as outlined, may further link T2DM to the pathogenesis of cancer. Whilst facets of the aforementioned may be perceived as hypothetical, its

postulates should be subject to scientific enquiry through rigorous experimental testing in a T2DM setting.

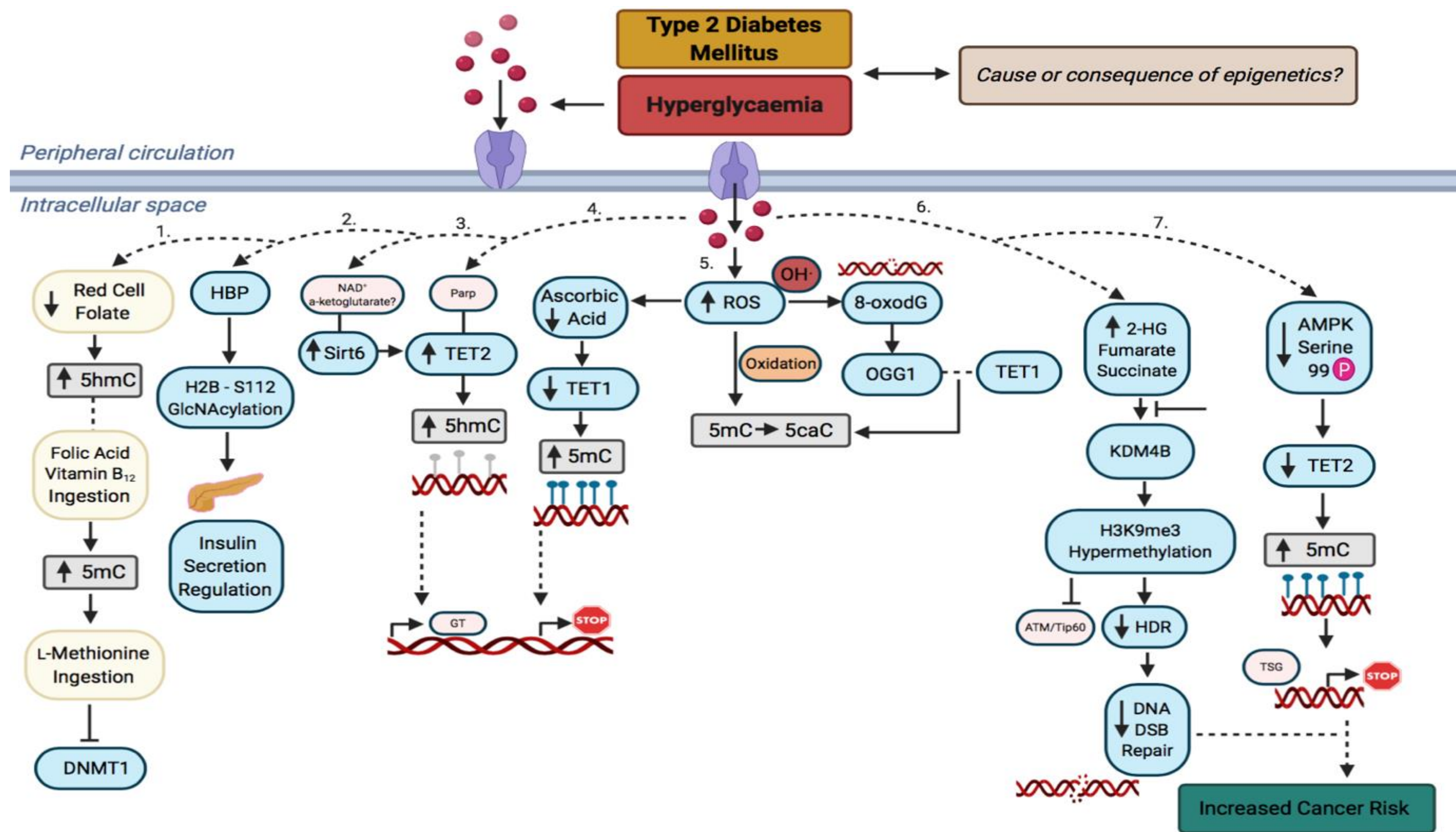
As an alternative to glycolysis, glucose fluxes through the hexosamine biosynthetic pathway to produce the substrate *O*-GlcNAc prior to histone *O*-GlcNAcylation (Xu *et al*, 2016). Alterations in protein *O*-GlcNAc metabolism are associated with hyperglycaemia and subsequent diabetic complications (Love and Hanover, 2005). Using *in vitro* and living cell models, Fujiki *et al* (2011) postulates that histone H2B is GlcNAcylated at residue S112 by glucose, and nuclear histone GlcNAcylation appears to fluctuate in response to extracellular glucose. In pancreatic β-cells, hyperglycaemia leads to the hyperGlcNAcylation of *NeuroD1* and *PDX-1*. The former, a transcription factor involved in the regulation of insulin gene expression interacts with OGT in high glucose. Moreover, increased nuclear localisation of *O*-GlcNAcylated *NeuroD1a* can enhance DNA binding and glucose-dependent insulin synthesis (Andrali *et al* 2007, Ma and Hart 2013). Regarding *PDX-1*, *O*-GlcNAcylation augments DNA binding in the HR2 region of the promoter aligned to *FFA1/GPR40*, thus stimulating *GPR40* gene transcription and insulin secretion (Kebede *et al*, 2012). Conversely, and over the long-term, *O*-GlcNAcylation leads to impaired insulin secretion and pancreatic cell apoptosis (Ma and Hart, 2013). Regarding liver, overexpression of OGT seems to impair the transcription of insulin-responsive genes leading to a state of insulin resistance. This occurs by phosphatidylinositol 3,4,5-trisphosphate recruiting OGT and subsequently catalysing the modification of the insulin signalling pathway by *O*-GlcNAc (Yang *et al* 2008). In a recent study, Kronlage and colleagues (2019) determined the effects of glucose and posttranslational modifications by β-linked *O*-GlcNAc on histone deacetylase 4 (HDAC4). Data shows that the (cardioprotective) N-terminal proteolytic fragment of HDAC4 is enhanced in T2DM, and also *in vitro* under high-glucose and high *O*-GlcNAc conditions. The authors assert that *O*-GlcNAcylation of HDAC4 at serine-642 is cardioprotective in T2DM.

Nutrient-sensing also regulates epigenetics (Etchegaray *et al*, 2016). Folate is an extensively studied nutrient (Friso *et al*, 2017), and T2DM patients have a decreased erythrocyte folate concentration, that positively correlates with hypomethylation in 236 liver-specific CpG sites. Hypomethylation in this case may be explained by a methyl donor supply-consumption imbalance (Nilsson *et al*, 2015). To partially address this hypothesis, Li *et al* (2018) postulate that folic acid supplementation (20 ug/ml water – 10 weeks) improves insulin resistance, hypermethylates DNA, and induces corresponding gene transcriptional changes in obese mice fed a high-fat diet. Methylome profiling identified differentially methylated regions corresponding to 3787 genes following folic acid ingestion. In a vitamin B_{12} and/or folic acid focussed study, vitamin B_{12} supplementation induced methylation changes in 589 differentially

methylated CpGs and 2892 regions, compared to 169 differentially methylated CpGs and 3241 regions in the vitamin B_{12}/folic acid combined group. Aligned to B_{12} supplementation, changes were also identified in the regulation of T2DM genes (*TCF7L2* and *FTO*) through methylation of the miRNA marker miR21 (Yadav *et al*, 2018). We have also reported widespread changes to DNA methylation in response to folic acid supplementation, though T2DM-related genes were not specifically examined (Irwin *et al*, 2019). Navik *et al* (2019) provides the first evidence that dietary L-methionine supplementation prevents H3K36me2 methylation in a diabetes rodent model. Interestingly, L-methionine was also found to inhibit DNMT1 expression. Regarding other DNA methyltransferase activity, DNMT3 mediates insulin resistance in cultured mouse and human T2DM adipocytes, by methylating specific *cis*-regulatory elements in the *Fgf21* gene (downregulating expression), while DNMT3 deficiency (via knockout) seems to confer protection from diet-induced insulin resistance and glucose intolerance (You *et al*, 2017). Collectively, these data provide a clear supposition that nutrient ingestion has the potential to modify and regulate DNA methylation and chromatin in a diabetes state, and as such, this emerging domain may have therapeutic and translational potential.

In summary, it is evident from examining the available literature, that metabolism influences epigenetics in T2DM, and an overview of these connections are outlined in Figure 5. However, it is also clear that published work in this domain is not extensive, and a greater emphasis should be placed on further research to ascertain the effect of (1) enhanced ROS production and their subsequent effect on molecular oxidation; (2) the link between ROS and TET and JmjC activity; (3) mitochondrial TCA cycle intermediates and their regulation of histone protein alterations and DNA methylation in T2DM.

Figure 5. *T2DM, Hyperglycaemia and Epigenetics*



T2DM is characterised by hyperglycaemia, affecting intracellular metabolism and epigenetics. It is currently unknown if epigenetic modifications are a cause or consequence of T2DM/hyperglycaemia. Hyperglycaemia may cause: 1. Compromised red cell folate leading to hypomethylation, however, folic acid and vitamin B_{12} supplementation causes hypermethylation. 2. Protein O-GlcNAcylation may control pancreatic insulin secretion. 3. Nicotinamide adenine dinucleotide (NAD^+) and α-ketoglutarate may upregulate sirtuin (SIRT6) molecules, activating TET2 leading to a hypomethylated state. 4. Poly (ADP-ribose) polymerase (Parp) may activate TET2 causing hypomethylation. Gene transcription is activated. 5. Enhanced reactive oxygen species (ROS; ·OH; hydroxyl free radical) production oxidises 5mC, and damages the DNA base guanine. 8-oxoguanine DNA glycosylase (OGG1) through the base excision repair pathway recruits TET1 to the active site of damage, leading to a hypomethylated state. ROS may decrease ascorbic acid concentration, affecting co-factor regulation of TET1, increasing hypermethylation. Gene transcription may be deactivated. 6. An increased concentration of 2-hydroxyglutarate (2-HG), fumarate and succinate may inhibit lysine demethylase KDM4B causing histone hypermethylation at H3K9. This aberrant hypermethylation blocks ATM/Tip60 affecting Homology-Dependent Repair (HDR), ultimately compromising DNA repair, rendering the cell susceptible to tumour growth. 7. AMPK-mediated phosphorylation at serine 99 is hindered, downregulating TET2 leading to 5mC; this may destabilise the tumour suppressor function of TET2, increasing the risk of cancer. HBP, hexosamine biosynthetic pathway; TET, ten-eleven translocation isoenzymes; GT, gene transcription; TSG, tumour suppressor genes; 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; 5caC, 5-carboxylcytosine; DNMT, DNA methyltransferase. Grey lollipop, hypomethylation; Blue lollipop, hypermethylation. Circle shaded in red = circulatory glucose concentration.

5. Conclusion and Future Perspectives

Unambiguous crosstalk exists between metabolism and epigenetics, with subsequent heterogeneous effects spanning insulin secretion regulation, to the control of DNA DSB repair; the latter potentially rendering the cell susceptible to genotoxic stress and tumour growth. However, what is not particular clear is the *extent* to which metabolites have the potential to interact with DNA methylation and histones. As outlined, metabolic pathway intermediates are involved in epigenetic regulation, and moderate evidence, albeit limited, is available for α-KG, ROS, NAD^+/sirtuins, *O*-GlcNAc, fumarate, and succinate. As such, there is still much work to be completed incorporating the entire gamut of known regulatory molecules, particularly as blood glucose in T2DM can follow an oscillating cycle in any 24-hour period. There is a need to understand if cofactors and enzymes fluctuate over time, and if so how are they sensed, and are they controlled by temporal and spatial aspects of intracellular pathways (Kaelin and McKnight, 2013). It is known that disruption of natural circadian rhythms leads to glucose intolerance and interferes with pancreatic β-cell function, causing diabetes; transcription in pancreatic β-cells also displays circadian oscillations (Etchegaray and Mostoslavsky, 2016). However, it is unknow whether metabolic circadian variation has any influence on DNA (de)methylation and histone modifications in the T2DM model. The intriguing prospect that metabolites emanating from the mitochondrial genome might influence epigenetic responses in the nuclear genome is also exciting, and one that warrants immediate investigation (Wiese and Bannister, 2020).

The link between ROS production, metabolism and epigenetics in particular deserves further enquiry. ROS have the potential to play a significant role in shaping the epigenetic landscape, not only from the perspective of adversely controlling DNA methylation and PTM's, but as modulators of epigenetic mechanisms through redox signalling, with essential implications for localised gene transcription. Recent work by Gurdon and colleagues (2020) performed at the University of Cambridge, postulates that transcription factor binding to a chromatin site can remain for hours or days, thereby stabilising gene expression. No data exists to determine the role of hyperglycaemia-induced ROS, and their potential to oxidise the plethora of transcription factors aligned to epigenetic regulation. Whilst speculative, if key regulating transcription factors are oxidised, and become loosely bound to DNA, the effect of a reduced residence time, on either the eu- or hetero-chromatin state might be significant. There is also a gap in knowledge relating to how ROS affect chromatin and methylation in cells other than pancreatic islets in T2DM.

While whole tissue antioxidant supplementation-based studies in T2DM have yielded mixed outcomes, specifically regarding DNA damage, a more targeted antioxidant approach may be necessary to control hyperglycaemia-induced ROS/oxidative stress. To date, no data exists around the effect of new mitochondrially targeted antioxidants on any aspect of epigenetics in T2DM. To this end, MitoQ, a lipophilic cation-based antioxidant may be considered (Williamson and Davison*,* 2020). Likewise, there is a dearth of data interrogating the SAM pathway, and its control of methyl donor biochemistry (in the context of T2DM), inclusive of methyltransferase enzyme activity. In addition to L-methionine, folic acid and vitamin B_{12} ingestion, consideration should also be afforded to vitamin B_6 and to the micronutrient's choline and betaine. Additionally, supplementing with ascorbic acid to ascertain effect on TET isoenzymes should also be prioritised. Experimentation involving dietary/drug strategies will help to accurately define the molecular mechanisms associated with the regulation of key epigenetic enzymes, thus supporting novel therapeutic approaches in T2DM.

The effect of hyperglycaemia on whole epigenome regulation has primarily been determined on pancreatic β-cells. Now with the advent of targeted gene editing using CRISPR-Cas9, there is an opportunity to ascertain the effect of glycaemia on epigenetics across a range of animal and human cell lines through novel *in vitro* experimentation. Whole gene knockout, or DNA editing directed at metabolic TCA cycle intermediates and epigenetic related enzymes is now possible. However, caveats exist with respect to using this technology clinically. While experiments show that the CRISPR-Cas9 tool can make '*off target*' gene mutations downstream from the primary target site, a new series of studies by Alanis-Lobato *et al* (2020) on the preprint server bioRxiv, also reveals that CRISPR-Cas9 editing can cause superfluous changes to the genome, at or near the target site of interest. That stated, a new technique termed prime editing, that can precisely edit DNA sequences is available (Anzalone *et al*, 2019). However, imperfect random edits still arise with this technology, and delivering the large prime editing system into some cell types may be challenging (Platt, 2019). While these genome editing technologies have considerable promise, extreme caution is justified.

Lastly, and following the first sequence of the human genome in 2003, and to the present day, there has been a plethora of modern advances in genomic technology. Indeed, first generation sequencing, initially described by Fred Sanger in 1977 (Sanger *et al*, 1977), has been comprehensively superseded by a range of Next Generation Sequencing (NGS) technologies, and this has led to an exponential increase of elucidated genetic causes of common and rare disease states (Behjati and Tarpey 2013, Lohmann and Klien 2014). NGS is largely utilised as a formidable tool to detect human variants, and to date, is regarded as the most robust methodology to explicate disease causing mutations, and *de novo* mutations in sporadic

individuals; the latter of which was impossible by conventional Sanger sequencing without knowing the candidate gene (Lohmann and Klien 2014). There now exists, a number of well-established NGS platforms that can be utilised to further ascertain the interplay between metabolism and epigenetics. Aside from the opportunity to determine heterogeneous epigenetic patterns using bisulfite sequencing (DNA methylation) for example, with recent advances in single-cell technologies, it is possible to determine chromatin accessibility and corresponding transcriptomics at a single-cell resolution using sc-assay for transposase-accessible chromatin (ATAC)-seq and scRNA-seq respectively (Wang *et al*, 2020). Other single-cell epigenome profiling techniques exist through chromatin immunoprecipitation sequencing (ChIP-seq), DNase I hypersensitive site sequencing (DNase-seq), cleavage under targets and release using nuclease (CUT&RUN), and cleavage under targets & tagmentation (CUT&Tag) (Ma and Zhang, 2020). Using scRNA-seq tools, one can also spatially resolve transcriptomics to identify cell subpopulations and transcriptional variations related to human tissues in disease such as T2DM (Roth *et al*, 2020).

An epigenome is interpreted in the view of present knowledge, and with rapid developments in NGS technologies, tomorrow's epigenome may look slightly different; as such, and with recent developments in other '*omic*' approaches like metabolomics and proteomics, there is much work to be done in conjunction with *in silco* methods and bioinformatics to mine for novel epigenetic players that regulate metabolism and *vice versa*. To this end, this comprehensive dialogue should be viewed as a starting point for further exploration into the complex bidirectional connection between metabolism and epigenetic architecture in T2DM.

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