

EXPLORING METABOLIC MEMORY FOLLOWING GLUCOSE CHANGES DURING
MEDIA REPLACEMENT IN HEK293T CELLS

by

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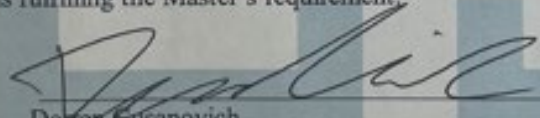
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Abbreviations

The following table lists abbreviations used throughout this thesis for reference in alphabetical order.

Abbreviation	Definition
AP-1	Activator Protein 1
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing
ATP	Adenosine Triphosphate
CTCF	CCCTC-Binding Factor
DMEM	Dulbecco's Modified Eagle Medium
FDR	False Discovery Rate
HAT	Histone Acetyltransferase
HG	High Glucose
LG	Low Glucose
NRF2	Nuclear Factor Erythroid 2-Related Factor 2
PCA	Principal Component Analysis
TF	Transcription Factor

Abstract

Metabolic memory refers to the persistence of cellular changes resulting from previous metabolic states, which may contribute to prolonged adverse effects in conditions like diabetes even after glucose normalisation. This study explores the potential mechanisms underlying metabolic memory, focusing on how changes in glucose concentration affect chromatin accessibility in HEK293T cells. Using ATAC-sequencing, we investigated whether chromatin accessibility changes previously observed in macrophages are conserved in other cell types. Cells initially cultured in high glucose (HG, 22.5mM) that were switched to low glucose (LG, 5mM) showed approximately 3000 genomic regions with increased accessibility compared to controls, while cells maintained in high glucose exhibited fewer changes. Transcription factor motif analysis identified several transcription factors potentially regulating these responses, including CTCF, AP-1, P53, and NRF2. Notably, CTCF transcription factor motifs showed increased accessibility in stable high-glucose conditions but decreased accessibility following glucose reduction, suggesting its potential role as a master regulator of metabolic memory. Pathway analysis revealed enrichment of apoptotic signalling in the HG → LG condition, while sustained high glucose was associated with decreased accessibility in developmental and patterning pathways. The experiment conducted in the reverse direction (LG → HG) yielded insufficient data quality for conclusive analysis. These findings expand our understanding of how previous metabolic environments shape the epigenome, providing insights into the molecular underpinnings of metabolic memory that could inform therapeutic approaches for metabolic diseases such as diabetes.

Introduction

The Importance of Cellular Metabolism and Glucose Homeostasis

Essential for survival, all cells must metabolise multiple organic compounds through a variety of biochemical pathways(1). These molecules can be produced through anabolic pathways (yielding macromolecules such as carbohydrates, lipids and proteins) or catabolic pathways (generating simpler molecules such as amino acids, fatty acids and monosaccharides). To preserve homeostasis in fluctuating environments, cells modulate these metabolic pathways to meet their functional demands.

Cellular metabolism is fundamental to all biological processes, supplying energy and biosynthetic intermediates required for growth, maintenance and repair(2). Glucose is a central monomer derived from the catabolic breakdown of carbohydrates through glycolysis or oxidative phosphorylation(3) and is used as substrate to produce adenosine triphosphate (ATP) through a series of enzymatic reactions. ATP is the fundamental unit of metabolic energy and is vital for almost all chemical reactions within cells. Beyond its role in energy provision, glucose also acts as a signalling molecule to regulate cell function, growth, and survival in response to environmental conditions(4).

Glucose homeostasis is vital for normal physiology. Numerous metabolic diseases, including type 2 diabetes, obesity and cardiovascular illness, have been concluded to have glucose metabolism dysregulation as a cause of their pathophysiology(5). Such illnesses are increasingly prevalent and are a current critical global health concern,

partly due to their long-term complications that persist even after blood glucose levels are normalised through clinical intervention. This suggests abnormal metabolic fluctuations may leave lasting cellular 'memories'(6).

Metabolism and the Epigenome

Building on the importance of glucose in cellular metabolism, recent findings suggest glucose availability can also influence gene expression, in part through its impact on the epigenome.

The epigenome plays a crucial role in the modulation of gene expression in response to internal and external stimuli. Recent evidence has suggested the underlying cause of the lasting effects of metabolic disturbances may arise through the encoding of various epigenetic mechanisms, such as DNA methylation, histone modification and chromatin remodelling(7).

Beyond energy provision, emerging evidence suggests that glucose-derived metabolites can also act as cofactors in various enzymatic processes, including those involved in epigenetic modifications to modulate chromatin accessibility(8). In the context of glucose metabolism, Acetyl Coenzyme A (acetyl-CoA) is a metabolite that functions as a substrate for histone acetyltransferases (HATs)(9). The role of acetyl-CoA and acetylation in chromatin regulation will be explored in greater detail later.

These links suggest that differences in glucose availability may indirectly modify the epigenetic landscape and consequently gene accessibility and gene expression, allowing them to adapt to changes in their environment. However, prolonged environmental fluctuations, such as chronic hyperglycaemia seen in diabetes mellitus, may trigger formation of epigenetic memories where these adaptive gene expression patterns are retained even after removal of the initial environmental trigger(10). In the context of nutritional availability, this phenomenon is referred to as 'metabolic memory' and is hypothesised to be a potential underlying cause of the sustained inflammatory and oxidative stress responses seen in diabetic patients(11).

The Epigenome

To fully understand how metabolic changes, such as altered glucose availability, exert lasting effects on gene regulation, it is important to understand the architecture and regulatory role of the epigenome.

The epigenome refers to a range of modifications applied to the genome that influence gene expression without altering the underlying genetic code(12). These modifications primarily include post-translational histone modifications, transcription factors, chromatin remodelling factors, non-coding RNAs and DNA methylation patterns. Collectively, they regulate gene activity by dictating the accessibility of the genome and its ability to be transcribed into RNA. These epigenetic processes are essential for regulating developmental, physiological, and metabolic pathways, ensuring the proper function of cells across different stages of life(13).

A central epigenetic mechanism that influences chromatin accessibility involves the addition or removal of chemical groups to histone proteins, which play a key role in maintaining the structural integrity of the genome. The structural integrity of the genome is maintained through interactions with histone proteins. Around 146 base pairs of DNA wrap around an octamer of histones, comprised of H3 and H4 tetramer and two peripheral H2A and H2B heterodimers. Heavy condensation of these units leads to coiling of DNA to form chromosomes. This organization, known as heterochromatin, renders the DNA largely inaccessible.

One key epigenetic modification associated with heterochromatin formation and transcriptional silencing is DNA methylation. This process involves addition of methyl groups to cytosine residues, typically at CpG dinucleotides, forming 5-methylcytosine(14). When methylation occurs in gene promoter regions, it is strongly correlated with transcriptional repression, as the presence of methyl groups can inhibit binding and recruitment of gene transcription machinery, including transcription factors and enzyme complexes(15). DNA methylation is catalysed by DNA methyltransferases (DNMTs), including DNMT3a and DNMT3b for de novo methylation and DNMT1 for maintaining methylation patterns during DNA replication(16).

In addition to DNA, histone proteins are also subject to various post-translational modifications on their N-terminal amino acid tails such as acetylation, methylation, phosphorylation, and ubiquitylation(17). These chemical modifications influence the

physical structure of chromatin and are key determinants of whether genomic regions are accessible for transcription. For example, histone acetylation is typically associated with genes that are transcriptionally active and are usually located at sites of promoters, enhancers and transcriptional regions. Histone marks that normally reflect this phenomenon include H3K36, H3K79 and H3K4me3. On the contrary, modifications like H3K9me3 are associated with heterochromatin and gene repression(18). The specific influence of each modification on chromatin accessibility and gene expression are contingent upon the number, type and location of the histone marks(19).

This study investigated how such histone modifications regulate chromatin structure in the context of metabolic through examination of how chromatin accessibility altered in response to environmental shifts in glucose levels. These modifications are particularly relevant in the context of metabolic memory, as they may underlie the transcriptional changes induced by sustained metabolic stress.

Chromatin Accessibility and ATAC-Seq

The dynamic changes in chromatin accessibility are essential to understand the regulation of gene expression and can provide valuable insights for when pathophysiology occurs in metabolic diseases. Therefore, to assess how glucose-induced epigenetic changes manifest at a chromatin level, experimental techniques like the assay for transposase-accessible chromatin using sequencing (ATAC-seq) are essential.

ATAC-seq is a powerful tool to observe chromatin accessibility that utilises a hyperactive Tn5 transposase enzyme to cleave open regions of the genome, which can then be amplified by PCR through insertion of sequencing adapters. The amplified DNA fragments can then be mapped to a reference genome for inference into which regions may be poised or silenced for transcription(20,21).

ATAC-seq requires a relatively low cell abundance so is suited for in vitro experiments of cell lines(22). Performing ATAC-seq on cells exposed to changing glucose environments can capture the rapid and dynamic changes in chromatin accessibility as a result of these metabolic conditions. The molecular mechanisms and regulatory networks of metabolic memory can then be explored by further in-depth analysis by identifying differentially accessible regions and transcription factor binding motifs(23).

Epigenetic Memory

Given the role of chromatin modifications in regulating gene expression, these same mechanisms are also implicated in long-term epigenetic memory.

Epigenetic memory refers to the persistent changes in gene expression or chromatin structure, resulting in long-lasting phenotypic alterations. These changes are believed to be both mitotically and meiotically heritable, persisting overtime across cell divisions and, in some cases, across generations(24). Understanding this phenomenon is key to understanding how cells and organisms respond to prolonged environmental stressors, such as metabolic disturbances. In response to sustained hyperglycaemia, mechanisms

of epigenetic memory may induce persisting alterations in chromatin accessibility, thereby influencing the regulation of gene expression and cellular function. These adaptations may endure despite removal of the initial glucose stimulus, offering valuable insight into the disrupted metabolic homeostasis observed in metabolic diseases(25).

The mechanisms behind epigenetic memory are thought to be primarily driven by chromatin modifications, such as DNA methylation and histone modifications, which influence gene expression and cellular responses(26). However, it is not perfectly understood how these chromatin modulations persist to provide epigenetic memory and is currently still a debated topic(13).

Epigenetic memory can be divided into three main categories: cellular memory, transcriptional memory and transgenerational memory. Cellular memory plays a critical role during cell differentiation and development by ensuring the stable inheritance of transcriptional patterns from progenitor to daughter cells, retaining lineage identity and function(27). Transcriptional memory is particularly important for adaptation to environmental stimuli, as it enables cells to "remember" previous gene expression states, promoting a more rapid and robust response upon re-exposure to similar external conditions(28,29). Transgenerational memory refers to the inheritance of epigenetic modifications across generations, which may continue to influence cellular phenotypes after removal of the original environmental stimulus(30).

This thesis explores both transgenerational and transcriptional memory in the context of hyperglycaemia as a model of sustained metabolic stress. Transcriptional memory offers a potential explanation for how glucose disturbances can lead to persisting changes to chromatin accessibility and gene expression, even after normoglycaemia is restored. In parallel, transgenerational memory may contribute to long-term propagation of metabolic dysregulation through transfer of histone modifications, induced by hyperglycaemia, to subsequent generations, potentially prolonging symptoms of metabolic disease. By examining these mechanisms, this study aims to advance our understanding of metabolic memory and its role in establishing lasting epigenetic imprints associated with chronic diseases such as diabetes.

A well-characterised example of transcriptional memory in a metabolic context involves extended expression of inflammatory genes in macrophages after exposure to hyperglycaemia(31). Even after glucose levels are normalised, pro-inflammatory genes such as *TNF- α* , *IL-6*, and *ICAM-1* remain in a transcriptionally permissive state due to sustained histone modifications, including H3K4me1 and H3K9ac at their respective promoters and enhancers(32). These epigenetic marks preserve chromatin accessibility, priming the genome for a more rapid and amplified transcriptional response upon re-exposure to metabolic stress. Such findings support the concept of metabolic memory and illustrate the role of epigenetic mechanisms in perpetuating the long-term adverse effects of metabolic disease.

Epigenetic Memory and Metabolic Diseases

Despite its evolutionary role in cellular adaptation to recurring stimuli, epigenetic memory can also be maladaptive in heterogeneous environments and is thought to contribute to increased susceptibility and persistence of complex diseases such as diabetes.

The impact of metabolic environments on epigenetic modifications has gained significant attention, particularly in the context of metabolic diseases such as diabetes, obesity, and fatty liver disease. These conditions have become increasingly prevalent with industrialisation and modern lifestyle consumerism(33).

Health concerns and disease complications related to diabetes persist in individuals regardless of lifestyle changes and scientific advancements that act to normalise blood glucose levels. Recent studies suggest that epigenetic memory could explain why complications in diabetic individuals persist even after glucose levels are normalised. This phenomenon, often referred to as "metabolic memory," is thought to be driven by lasting epigenetic changes, including DNA methylation, histone modification, and chromatin remodelling, which continue to affect gene expression long after the initial metabolic disturbance has been resolved(34).

A deeper understanding of how metabolic cues influence gene regulation requires an exploration of the fundamental components and mechanisms that define the epigenome. As previously discussed, metabolism of glucose, fatty acids and lactate lead to the production of various metabolites that influence epigenome-modifying

enzymes, ultimately remodelling the chromatin landscape and influencing chromatin accessibility to regulate gene expression in response to changes in the metabolic environment(35,36).

For instance, metabolic conditions like hyperglycaemia have been shown to induce epigenetic modifications such as histone acetylation that regulate gene expression, particularly in pathways related to inflammation and oxidative stress(37).

Histone acetylation requires the transferral of an acetyl group to histone proteins post-translationally utilising a histone acetyltransferase (HAT) enzyme(38). Substrates required for histone acetylation are derived from multiple metabolic pathways, including glycolysis, fatty-acid metabolism and the citric acid cycle. The availability of acetyl-CoA is correlated with cellular glucose levels, as glucose is metabolised to pyruvate during glycolysis, which is then converted to acetyl-CoA in the mitochondria. Increased levels of glucose, and therefore acetyl-CoA, promote histone acetylation. Histone acetylation is thought to weaken histone-DNA interactions by neutralising the positive charge on histone proteins, resulting in a more relaxed and open chromatin state(39).

Additionally, ATP is generated through the metabolism of glucose to acetyl-CoA, supporting the energy demands of chromatin modification enzymes. Thus, fluctuations in glucose concentrations can directly influence chromatin accessibility and gene expression, linking metabolism to epigenetic regulation. This metabolic-epigenetic link enables cells to modulate gene expression in response to nutrient status and may

contribute to long-term chromatin accessibility and transcriptional changes associated with metabolic memory(40,41).

Multiple studies by Kowluru et al discuss how previous hyperglycaemic environments lead to prolonged epigenetic modifications, including DNA methylation, histone methylation and histone acetylation that subsequently resulted in extended upregulation of pathways involved in inflammation and oxidative stress(42,43).

These modifications can persist even when glucose levels return to normal, leading to prolonged disease complications such as atherosclerosis, retinopathy, and vascular dysfunction(37). Studies investigating metabolic memory have shown upregulation of genes involved in fibrosis, inflammation and oxidative stress is sustained in human umbilical vein endothelial cells, initially cultured in high glucose, for weeks even after transferral to normal glucose conditions(44).

The findings of these previous studies provide a mechanistic link between metabolic state and gene regulation, helping explain how cells "remember" their previous metabolic environment.

Investigating Metabolic Memory in Immune Cells

To explore whether metabolic memory influences immune function, our lab previously examined chromatin accessibility in macrophages exposed to glucose shifts. Immune cells, particularly macrophages, are central to the development and persistence of

metabolic memory. Hyperglycaemia induces a pro-inflammatory response in immune cells, which may contribute to the long-term complications observed in diseases like diabetes(45).

Cells were cultured in low-glucose (5mM, LG) media for a week before the media was replaced with the same LG media or high-glucose media conditions (22.5mM, HG)(46). After media change, cells were incubated for 3 hours followed by ATAC-sequencing to assess genome-wide chromatin accessibility.

Pair-wise correlations and PCA plots revealed the most distinguished condition was the control group with PC1 capturing 31% of data variability, while both the LG and HG conditions clustered together. Add Specifically, 2,055 loci became more accessible with the addition of fresh HG, and 2,001 loci showed increased accessibility with the addition of fresh LG media compared to the control. Conversely, some loci exhibited decreased accessibility, with 1,687 loci decreasing upon addition of HG media and 1,578 loci decreasing with LG media.

These findings indicate that the addition of fresh media had the largest effect on differential chromatin accessibility, regardless of glucose concentration, as both LG and HG conditions shared around 50% of their differentially expressed peaks. Alongside suggesting changes in chromatin accessibility are responsive to metabolic conditions, these findings also highlight the importances of controlled experimental conditions, particularly when executing cell sequencing methods following cell culture.

The act of cell culture is an indispensable skill that is required to execute most scientific laboratory research(47). Frequent replenishment of media and cell passaging is a necessary protocol to keep cells at optimal health and maximise cell propagation. Basal cell culture medium and its supplements vary depending on the specific cell line's ideal requirements, which has been widely researched emphasising the importance of optimising cell culture protocols to provide a physiologically appropriate microenvironment for in vitro studies(48,49).

The previous study's results emphasise the importance of precise planning and control of cell culture prior to any experimental procedures as any extraneous influence may affect the reliability and reproducibility of downstream analysis, including but not limited to sequencing-based assays.

Glucose-Specific Changes in Chromatin Accessibility

While initial analysis highlighted the broad impact of media replacement on chromatin accessibility, further analysis investigated these identified specific genomic regions that were changing in response to glucose concentration. This analysis identified 175 peaks that were differentially accessible and associated with glucose-dependent chromatin remodelling. Pathway analysis revealed enrichment in functions relevant to macrophage biology, including plasma cell regulation(50).

In addition, transcription factor motif enrichment analysis captured CTCF as a significantly enriched transcription factor that increases in accessibility under metabolic memory conditions. Other candidate memory genes identified near or within these peaks were *Ext1* and *DOCK11*, relevant to macrophage polarisation. Of genes involved in glucose homeostasis, the chromatin accessibility around *SSBP3* was identified as potentially upregulated in response to the changing glucose environment(46).

These findings suggest that chromatin accessibility in the RAW264.7 macrophage model is sensitive to changes in glucose concentration in media, highlighting a potential mechanism for metabolic memory. The enrichment of CTCF suggests this key transcription factor as a potential regulator of histone modifications and epigenetic memory, providing insight into the molecular mechanisms behind metabolic disease such as diabetes.

However, immune cells are not the only cell type with links to metabolic memory. Fibroblasts, endothelial and epithelial cells are also subject to the adverse effects of hyperglycaemia(44). It is plausible that other cell types show similar cellular changes in chromatin accessibility and expression in response to changing environmental stimuli, portrayed as a change in media composition during cell culture.

To determine whether the chromatin accessibility changes observed in macrophages were specific to immune cells or represent a broader, more universal mechanism, we extended our investigation to HEK293T cells — a human embryonic kidney-derived

epithelial cell line. HEK293T cells were selected due to their well-characterised genome and widespread use in chromatin accessibility and epigenetic studies. Their epithelial origin and metabolic profile contrast with that of macrophages, making them an ideal model for exploring whether the phenomenon of metabolic memory, particularly in response to glucose fluctuation, is conserved across divergent cell types(51). This approach allowed us to assess whether the glucose-induced epigenetic changes seen previously are generalisable or if they exhibit cell-type specificity.

Building on our previous laboratory results conducted in RAW264.7 macrophage cells, this thesis aimed to extend our understanding of metabolic memory in the context of chromatin accessibility. Using ATAC-seq, we examined chromatin accessibility changes in HEK293T cells subjected to shifts in glucose concentrations. Specifically, we investigated 1) whether chromatin accessibility changes observed in macrophages are conserved in other cell types or universal across cell lines and 2) whether the direction of the glucose concentration change—either an increase or a decrease—influenced the changes observed in chromatin accessibility. Through this, we aim to further elucidate the epigenetic mechanisms underlying metabolic memory and expand our understanding of how past and current nutrient environments shape gene regulation.

Materials and Methods

Cell Culture

HEK293T (adherent) cells were stored in liquid nitrogen and cultured in-house. Cells were maintained in low-glucose (LG, 5.0 mM) Dulbecco's Modified Eagle Medium (DMEM: Gibco, cat. no. 11885), supplemented with 10% foetal bovine serum (FBS) and 1x Penicillin-Streptomycin. Cells were cultured at 37°C in a 5% CO₂ incubator for one week. For condition groups, media was aspirated and replaced with either the same LG media or high-glucose (HG, 22.5mM) Dulbecco's Modified Eagle Medium (DMEM: Gibco, cat. no. 11995), as specified. All media was pre-warmed for a minimum of 30 minutes in a 37°C water bath prior to use.

HEK293T cells were thawed by gentle rotation in a 37°C water bath for 2 minutes, or until minimal ice remained. Approximately 1 mL (~1 million cells) was transferred to a 15-mL conical tube containing 5 mL LG media or 5mL HG media and centrifuged at 500 x *g* for 5 minutes at 23°C. The supernatant was carefully aspirated and the cell pellet was resuspended in 5mL LG media and transferred to a TC-treated T25 flask.

HEK293T cells were passaged at 80% confluency using Trypsin Blue Stain (0.4%) at a 1:10 ratio.

After one week of cell culture, as described previously, HEK293T cells were lifted using Trypsin Blue Stain (0.4%) and counted on a haemocytometer with Trypan Blue staining. An appropriate number of cells were seeded onto 6-well plates to ensure optimal cell density for the following experimental day.

For the cells initially cultured in LG media, two 6-well plates were designated for DNA isolation (800,000 cells/well) and the remaining two for RNA isolation (800,000 cells/well). The final well volume was adjusted to 2mL for all plates (Figure 1). Each plate contained two control samples, two low-glucose (LG) replacement samples and two high-glucose (HG) replacement samples. Plates were incubated overnight at 37°C in a 5% CO₂ incubator. The following day, all plates were removed from the incubator and the old media was aspirated from the HG and LG conditions. The media was not aspirated from the control group. 2mL of appropriate media (HG or LG) were added to the respective conditions and no media was added to the control group. After media replacement, all plates were returned to the 37°C, 5% CO₂ incubator for 3 hours, as determined by a previous in-house pilot study.

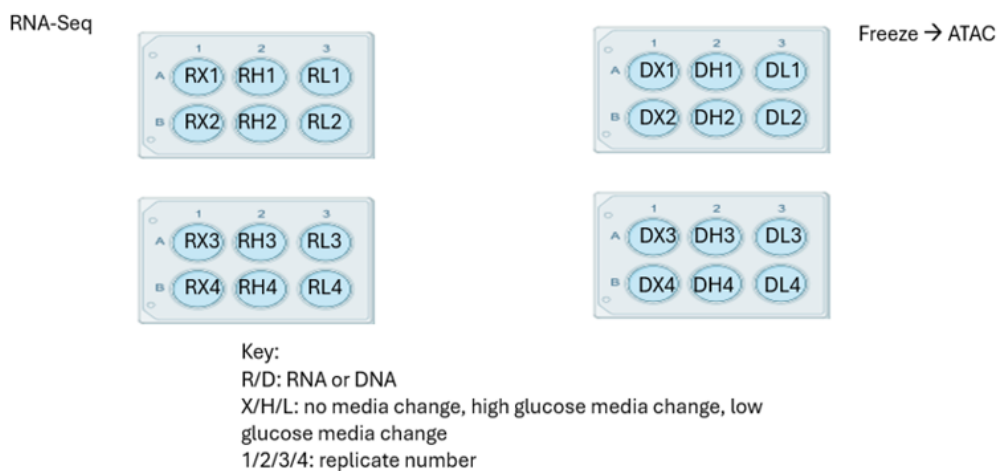


Figure 1: Schematic of the 6-well Plate Layout for Cells Initially Cultured in Low-Glucose (LG) Media. HEK293T cells were divided into 4 x 6-well plates. Two were dedicated to ATAC-Seq, where nuclei were frozen after isolation. The remaining two 6-well plates underwent RNA extraction for subsequent RNA-sequencing. Each row of the plates contained a full replicate set of each condition (control, HG media change or LG media change). In total, each condition had 4 biological replicates (n=4).

After the three-hour incubation period, nuclei were isolated and frozen from two of the 6-well plates (12 samples: 3 conditions, 4 replicates) for later ATAC-seq, as described below. The remaining two plates underwent RNA extraction, where RNA concentration was measured using a Nanodrop spectrophotometer, and RNA quality determined by TapeStation analysis on Agilent TapeStation.

For the cells initially cultured in HG media, they were seeded onto two 6-well plates (800,000 cells/well). Final well volume was brought up to 2mL for both plates. This experiment did not have parallel RNA plates as RNA extraction was not performed for this experiment. Plates were incubated overnight at 37°C in a 5% CO₂ incubator. Each condition had 3 biological replicates (n=3) for the control group, where no media was aspirated/replaced, the HG condition, where 2mL media was replaced with the same HG media that cells were cultured in and the LG condition, where the HG media was replaced with 2mL LG media. After media replacement, all plates were returned to the 37°C, 5% CO₂ incubator for 3 hours.

After the three-hour incubation period, nuclei were isolated (9 samples: 3 conditions, 3 replicates) and ATAC-seq was performed, as described below.

Nuclei Isolation and Freezing of Isolated Nuclei

Prior to cell lysis and nuclei isolation, the following reagents were prepared for both cell lines: Omni Resuspension Buffer (52) (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂), 2x Omni Tagmentation Buffer (20 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 20%

dimethylformamide), Freezing Buffer (53) (50 mM Tris-HCl pH 8.0, 5 mM Mg(OAc)₂, 25% glycerol, 0.1 mM EDTA, 5 mM dithiothreitol, 2% protease inhibitors (v/v)), Lysis Buffer (1X Omni Resuspension Buffer, 0.1% Igepal-CA630, 0.01% digitonin, 0.1% Tween-20) and Stop Solution (1X Omni Resuspension Buffer, 0.1% Tween-20).

After the 3-hour incubation period following media change, HEK293T cells were removed from the incubator and placed on ice. Media was aspirated from both plates and 200 μ L of lysis buffer was added directly to each well. The plates were incubated on ice for 3 minutes. Then, 1mL of stop solution was added and nuclei were scraped using a Corning cell scraper into solution. The samples were then transferred to a 15mL conical tube. Nuclei concentration was determined using a Countess cell counter, following a 1:50 dilution (5 μ L nuclei, 45 μ L of 2x Omni Tagmentation Buffer, 50 μ L Trypan Blue). The required volume for ~1.5 million cells was calculated.

After centrifugation at 500 x *g* (10 min, 4°C), the pellet was resuspended in 1mL of freezing buffer and transferred to 2mL Corning cryogenic vials. Aliquots were flash-frozen and stored at -80°C for up to 48 hours before nuclei were processed for ATAC-sequencing.

ATAC-Sequencing Library Preparation

The Omni-ATAC protocol was modified for ATAC-Seq on frozen DNA samples from the HEK293T cell line(21).

Prior to library preparation, the following buffers were prepared on ice: RSB washing buffer (1X Omni Resuspension Buffer, 0.1% Tween-20, 0.1% BSA), PBS containing 0.04% BSA (PBSB), 2X Omni Tagmentation Buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl₂ and 20% Dimethyl Formamide), Transposition Mix (1X Omni Tagmentation Buffer, 0.01% digitonin, 0.1% Tween-20) and qPCR master mix (1X NEB mastermix, 0.5X SYBR Green, 1.25 μM Ad1 primer from PMID: 24097267 (54)).

Frozen nuclei were removed from -80°C and thawed in a 37°C water bath for ~2 minutes before being immediately placed on ice. The nuclei were transferred to a 15mL conical tube, gently mixed with 1mL PBS and centrifuged at 500 x g for 10 minutes in a pre-chilled (4°C) swinging-bucket centrifuge. After aspirating the supernatant, the pellet was resuspended in 100μL of PBSB and counted using a Countess cell counter. A 1:50 dilution was performed (2 μL nuclei, 23μL PBS, 25 μL of 2x Omni Tagmentation Buffer and 50 μL Trypan Blue). A total of 20,000 nuclei were diluted with PBSB to a final volume of 6.6μL.

To this, 12.4μL of transposition mix and 1μL of 0.3 μg/μL in-house Tn5 transposase were added to each sample. Tagmentation was performed on a thermocycler at 37°C for 30 minutes. Tagmented DNA was purified using Zymo DNA Clean and Concentrator-5 columns (Zymo, cat. no. D4004) according to the manufacturers guidelines to elute a final volume of 12.5μL purified DNA.

Next, 5 μ L of the purified DNA was amplified by qPCR using 16.88 μ L of qPCR master mix and 3.13 μ L of reverse primer (final concentration: 1.25 μ M). The reverse primers were added separately to each sample (Ad1 primer from PMID: 24097267(54)). Samples were amplified on a Bio-Rad CFX Connect Real-time cycler using the following parameters: 72°C for 5 minutes; 98°C for 30 seconds; 8-12 cycles of 98°C for 10 seconds, 63°C for 30 seconds and 72°C for 1 minute. Samples were removed once the amplification curve showed signs of plateau.

Following amplification, 25 μ L of Qiagen Buffer EB was added to the qPCR products, bringing the total volume to 50 μ L. Double size selection clean-up was then performed using AMPure XP beads (Beckman Coulter, cat. no. A63881) according to the Beckman's protocol. DNA fragments larger than ~1500 bp (0.4X AMPure XP beads) and smaller than ~100 bp (1.5X AMPure XP beads) were removed. The final ATAC-Seq library product (20 μ L) was collected in 1.5mL Lobind tubes, quantified using a Qubit 1X dsDNA HS Assay Kit (Invitrogen, cat. no. Q33231) and 1 μ L of the product was used to run on a 6% polyacrylamide gel electrophoresis (PAGE) gel to visualise distinct ATAC banding prior to sequencing.

ATAC-Sequencing

Prior to sample preparation, Illumina hybridization buffer (HT1) was thawed at room temperature and 0.2N NaOH and 200mM Tris-HCl (pH 7.5) were prepared. ATAC libraries were diluted to 2nM, based on an average fragment size of 350bp. The samples were pooled together and 10 μ L of the pooled library was mixed with 10 μ L 0.2N

NaOH in a 1.5 mL Lobind tube to denature the libraries. After a brief vortex, the pooled 2nM library was centrifuged at 280 x *g* for 1 minute at 23°C and incubated for 5 minutes at room temperature. Following incubation, 10µL of 200 mM Tris-HCl was added to the denatured library, which was then vortexed briefly and centrifuged at 280 x *g* for 1 minute at 23°C.

Next, 970µL of pre-chilled HT1 was added to the denatured library and the mixture was vortexed briefly and centrifuged at 280 x *g* for 1 minute at 23°C. For use with the Illumina NextSeq 500/550 High Output Kit v2.5 (150 Cycles) (Illumina cat. no. 20024907), the 20pM library was further diluted to 1.8pM by combining 117µL of the 20pM library and 1183µL of pre-chilled HT1. The 1.8pM library was then inverted to mix, pulse centrifuged and kept on ice.

ATAC-Seq libraries were sequenced on an Illumina NextSeq 500 Platform using the following program: Read 1: 76 cycles; Read 2: 76 cycles; Index 1: 8 cycles (Ad2.X primers).

RNA Isolation and Quality Analysis

Prior to handling RNA, all equipment and the lab bench was sterilised with RNase Away. RNA was extracted from samples using the Zymo Quick-RNA Miniprep Kit following the manufacturer's guidelines. Lysis buffer was added directly to the plate containing HEK293T cells. RNA concentration was quantified using a nanodrop spectrophotometer and RNA quality was assessed by TapeStation analysis.

RNA-seq library preparation was attempted in-house, following the TRACE-seq protocol. However, due to time restrictions and reagent limitations, RNA-Sequencing was unable to be completed before the deadline of this thesis.

ATAC-Seq Data Analysis

Data analysis was completed in collaboration with a colleague.

Preprocessing. Initially, base call (BCL) files were converted into FASTQ format, and sequencing clusters were demultiplexed and assigned to their respective samples based on index adapter sequences introduced during library preparation, using the bcl2fastq2 software. Next, paired-end reads were processed with Trimmomatic to remove adapter sequences and low-quality bases, applying the parameters “LEADING:3”, “TRAILING:3”, “SLIDINGWINDOW:4:10”, and “MINLEN:20”. To limit the maximum fragment size to 2000 bp and trim one base from the 3' end of each read, the parameters “-X 2000” and “-3 1” were applied. The trimmed reads were subsequently aligned to the mm10 mouse reference genome using Bowtie2. After alignment, only properly paired reads (identified by SAMtools using the “-f3” and “-F12” flags) that mapped to assembled nuclear chromosomes with a MAPQ score of ≥ 10 were retained. Finally, duplicate reads were identified and removed using the “MarkDuplicates” tool from the Picard suite.

Peak Calling. Peaks were identified using the MACS2 peak-calling algorithm, applied to BED-formatted ATAC-seq reads. MACS2 models read enrichment using a Poisson

distribution, but in this case, the default fragment size model was disabled “-nomodel”. Instead, a fixed shift-extension model “--shift -100 --extsize 200” was used to better reflect Tn5 transposase insertion patterns in ATAC-seq. After peak calling, the peaks were sorted by significance, renamed, and merged using BEDTools to create a unified set of accessible chromatin regions.

Differential Analysis. To identify differentially accessible peaks between experimental groups, we used edgeR, a statistical method specifically designed for analysing count-based genomic data, such as that produced by ATAC-seq. The analysis began with raw count data, representing the number of reads mapped to each peak region for every sample. We first filtered out lowly accessible peaks that were unlikely to provide meaningful signal. To ensure fair comparison across samples with varying sequencing depths, we applied TMM normalisation (Trimmed Mean of M-values), which adjusts for differences in library size and composition.

We then used the glmQLFit() function to fit a generalized linear model (GLM) to each peak, which allowed us to model variability across biological replicates and prepare the data for statistical testing. To compare chromatin accessibility between conditions, we used glmQLFTest() to perform a quasi-likelihood F-test, which offers a robust approach for identifying statistically significant changes while accounting for both biological and technical variation.

Each peak was assigned a log fold change (logFC), a p-value, and a false discovery rate (FDR). We considered peaks to be differentially accessible if they had $FDR \leq 0.05$ and $\log FC \geq 2$ or ≤ -2 , corresponding to at least a fourfold change in accessibility. This allowed us to prioritize peaks that were not only statistically significant but also exhibited potentially biologically relevant changes in accessibility between conditions.

To calculate average concordance between biological replicates, we computed pairwise Pearson correlations on \log_2 CPM-normalized ATAC-seq counts (TMM normalization) using the correlation matrix (cor.mat). For each condition, we extracted all possible pairwise combinations of biological replicates and calculated the average Pearson correlation coefficient to assess within-group concordance.

Results

The Effect of Changing Media on Chromatin Accessibility in HEK293T Cells: High-Glucose (HG) → Low-Glucose (LG)

An initial study conducted in RAW264.7 cells revealed subtle but reproducible differences in chromatin accessibility in response to a change in glucose concentration from low glucose (5.0mM, LG) to high glucose (22.5mM, HG). These changes suggested a potential glucose-dependent regulatory mechanism influencing chromatin structure in the context of metabolic memory, prompting further investigation. To determine whether this phenomenon was cell-type specific or part of a broader mechanism of glucose sensing and metabolic memory, we performed a similar experiment in a different cell line, HEK293T cells. By comparing responses across these cell types, we aimed to assess the robustness and generalisability of glucose-induced chromatin changes as a potential layer of epigenetic regulation in metabolic memory.

To investigate the relationship between glucose concentration and chromatin accessibility, pairwise correlations were calculated between the peaks of all samples. For the first experiment, cells were initially cultured in HG media for one week, after which media was aspirated and replaced with either the same HG media (HG → HG condition), different LG media (HG → LG condition) or the media was unchanged (control condition). Each condition had three biological replicates (Figure 2).

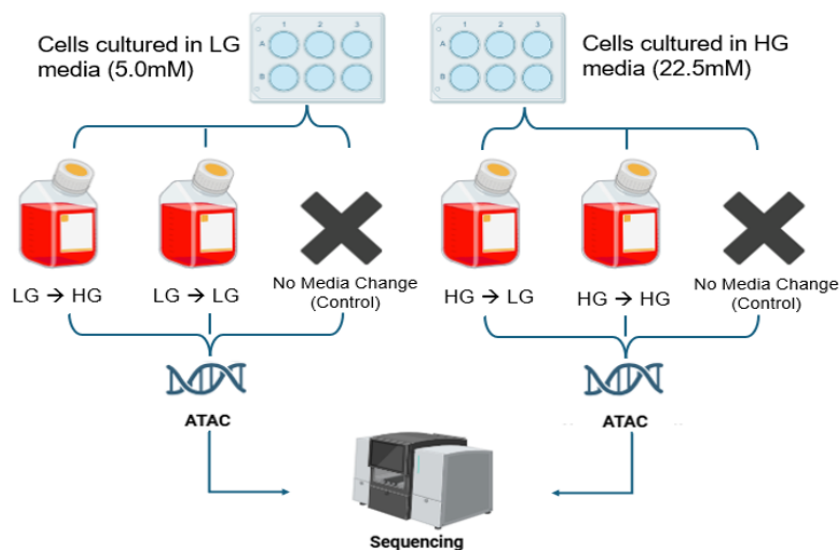


Figure 2: Schematic of Media Switch Protocol in HEK293T cells to Assess The Effect of Fresh Media on Chromatin Accessibility. HEK293T cells were cultured for one week in either low-glucose (LG; 5.0 mM) or high-glucose (HG; 22.5 mM) DMEM. Cells were then either maintained in the same media, switched to media of the opposing glucose concentration, or left unchanged as a control. Following the media switch, cells were incubated for three hours before harvesting for ATAC-sequencing to assess changes in chromatin accessibility. Cells initially cultured in LG; n = 4, cells initially cultured in HG; n = 3)

Nuclei from HEK293T cells were isolated and Assay for Transpose-Accessible Chromatin using Sequencing (ATAC-Seq) was performed to assess genome-wide chromatin accessibility. This method involves fragmentation of the genome at regions of open chromatin using the Tn5 transposase enzyme. Tn5 attaches sequencing adapters to either ends of the fragments – this process is called ‘tagmentation’. Next-generation sequencing is performed on the DNA fragments and the resulting data were mapped to a reference genome. The reads dictating the accessible genome regions accumulate and are referred to as peaks. Peaks were identified using the MACS2 algorithm, which applies a Poisson distribution to model read enrichment. To account for Tn5 transposase insertion patterns in ATAC-seq data, a fixed shift-extension model “--shift - 100 --extsize 200” was used instead of the default “--nomodel”. Peaks were then

ranked by significance and merged using BEDTools to create a unified set of peaks corresponding to any identified accessible chromatin regions.

The results are presented in a heat map (Figure 3A), The X and Y-axes depict sample names, where “HG → HG” represents a high-glucose media switch, “HG → LG” represents a low-glucose media switch and “Control” refers to the control group, where samples were not subjected to a fresh media change. The number following these conditions is indicative of biological replicate number. Sample correlation is determined by Pearson’s correlation coefficient (r), represented by colour, with red indicating higher correlation and blue indicating lower correlation. The dendrogram shows hierarchical clustering between samples as another visual representation of correlation on chromatin accessibility profiles between samples.

Principal Component Analysis (PCA) was also performed to highlight the two primary sources of variation within the data (Figure 3B). Both the heatmap and PCA plot demonstrated high reproducibility and data quality within each condition, with biological replicates of the same condition clustering closely together (HG→HG: average correlation = 0.938; HG→LG: 0.935; Control: 0.940).

The hierarchical dendrogram in Figure 3A also revealed that the two most correlated condition groups were the “Control” (no media change) and the “HG → HG” (HG media change) groups. The most distinguished condition was the “HG → LG” group (LG media change).

This was further supported by PCA analysis, which showed that PC1 captured 32% of variance within the data, with the “HG → LG” condition clearly separated from the other two groups on the vertical axis. This suggests significant differences in chromatin accessibility between these conditions.

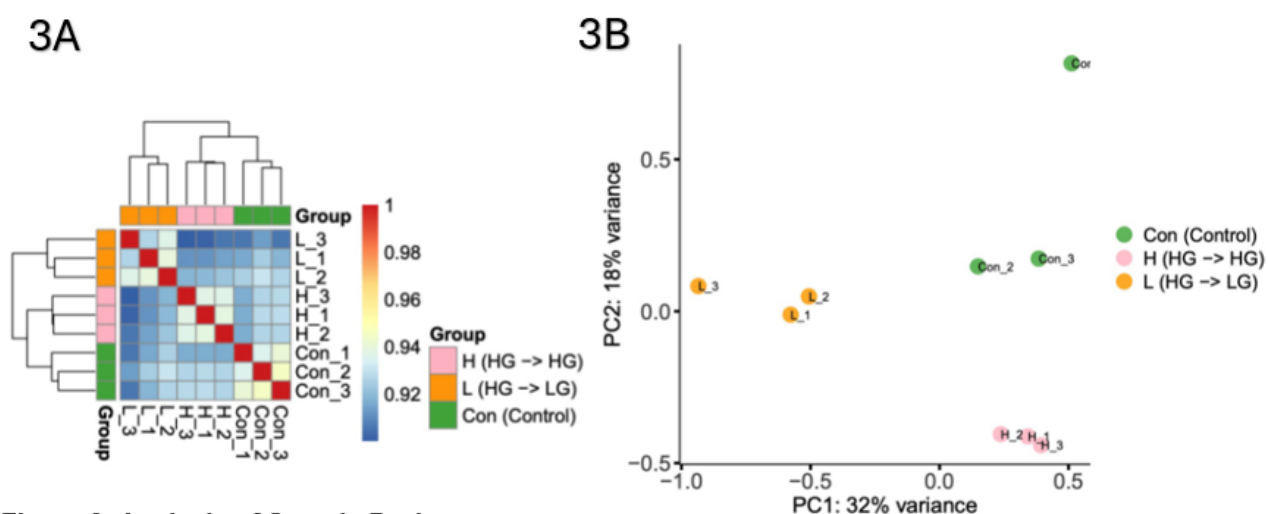


Figure 3: Analysis of Sample Peaks.

3A) Heatmap of pairwise correlations for HEK293T cells cultured in high-glucose (HG) media, followed by media replacement with either the same HG or low-glucose (LG) conditions. The X and Y axes represent sample names: “H” for the HG → HG condition, “L” for the Hg → LG and “Con” for control (no media change). Hierarchical clustering was performed using Pearson’s correlation coefficient to assess the similarity of chromatin accessibility profiles between conditions.

3B) PCA plot displaying ~4000 of the most variable peaks. Each point represents an individual sample, with colours indicating the different condition groups.

Differential Chromatin Accessibility Across Conditions

For the HG → LG experiment, we examined the frequency of differentially accessible peaks between conditions by calculating log fold changes (logFC) for each condition to provide a broad overview of how many peaks were changing in response to media change.). Peaks with $\log_{2}FC \geq 2$ or ≤ -2 and $FDR \leq 0.05$ were considered significantly different, ensuring both statistical and biological relevance. Average concordance

between biological replicates was assessed using pairwise Pearson correlations on log₂ CPM-normalized counts.

Figure 4A shows that, compared to the control group, nearly 1200 peaks exhibited decreased accessibility when cells were maintained in fresh HG media (HG → HG) during media replacement. In comparison, these same samples showed approximately 500 peaks increased in accessibility compared to the control group.

Figure 4B illustrates the differential accessibility in the “HG → LG” group, where samples were subjected to a glucose reduction upon media replacement. Compared to the control group, the “HG → LG” condition showed approximately 3000 peaks with increased accessibility. On the other hand, only ~500 peaks exhibited decreased accessibility. These findings are further confirmed in Figure 4C, which compares the “HG → LG” group to the “HG → HG” condition, highlighting a greater number of peaks with increased accessibility when they receive fresh media with a different glucose concentration (HG → LG) compared to media replacement of the same one initially used in cell culture (HG → HG). This suggests a significant response to media change.

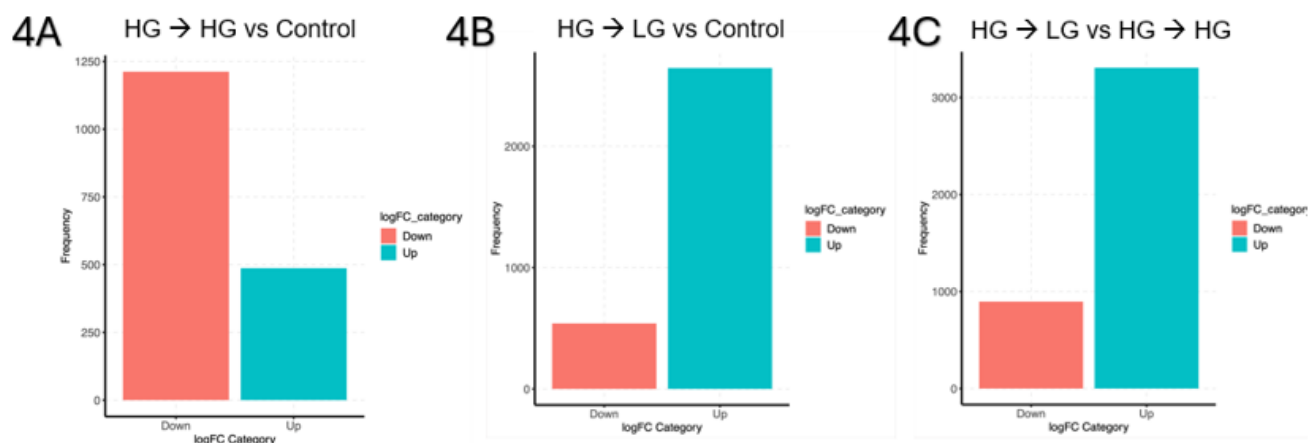


Figure 4: Bar Charts Visualising Differentially Accessible Peaks. In all panels, the X axis represents log fold changes in differential accessibility, with "Down" indicating a decrease and "Up" indicating an increase in accessibility. The conditions are as follows: "HG → HG" refers to samples that underwent media replacement with the same high-glucose (HG) media, "HG → LG" refers to samples with a media change to low-glucose (LG), and "Control" refers to the control group with no media change.

4A) Differentially expressed peaks in the HG → HG condition compared to the control.

4B) Differentially expressed peaks in the "HG → LG" condition compared to the control.

4C) Differentially expressed peaks in the "HG → LG" condition compared to the "HG → HG" condition.

Transcription Factor Motif Analysis

To gain further insight into the regulatory mechanisms underlying the observed chromatin accessibility changes, we performed transcription factor motif discovery using the HOMER known motif enrichment algorithm to identify enriched transcription factor motifs in the differential peaks in each experimental condition compared to the control group. Similar to the previous experiments in RAW264.7 macrophages conducted by our group, the transcription factor motif for CTCF was identified as a potential regulator of chromatin accessibility (Table 1).

CTCF is a zinc-finger transcription factor with key roles in chromatin organisation and therefore gene expression and overall 3D structure of the genome(55,56). In the "HG →

LG” condition, peaks containing the CTCF transcription factor motif showed a significant decrease in accessibility ($P = 1 \times 10^{-9}$). In contrast, CTCF transcription factor motifs were seen to be enriched in the “HG → HG” condition, with a highly significant increase in accessibility ($P = 1 \times 10^{-51}$). This suggests potential roles for CTCF in chromatin reorganisation following a decrease in glucose concentration and maintaining chromatin structure when glucose levels are stable. These findings are also consistent with previous reports highlighting CTCF’s role in chromatin looping and transcriptional regulation during metabolic stress, including glucose deprivation(57). Additionally, studies have shown CTCF transcription factor motif enrichment in open chromatin regions under high-glucose conditions in human stem cells, further supporting its involvement in nutrient-responsive chromatin dynamics(58,59).

Additional significant transcription factor motifs that underwent significant accessibility alterations included P53, BORIS and AP-1. BORIS (Brother Of the Regulator of Imprinted Sites) is a transcription factor and a paralogue of CTCF and also has roles in regulating chromatin structure(60). P53 and AP-1 (Activator Protein-1) are involved in regulating stress responses, cell cycle control, and apoptosis.

1






<u>Transcription Factor Motifs that Differentially Decreased in Accessibility Compared to the Control</u>				
Motif	Name	Description	<i>P</i>	
			HG → LG	HG → HG
	CTCF	Related to tumour suppression, epigenetic memory and gene organisation	1 x10 ⁻⁹	-
	P53	Regulates cell cycle arrest, DNA repair, and apoptosis	-	1 x10 ⁻³
<u>Transcription Factor Motifs that Differentially Increased in Accessibility Compared to the Control</u>				
	CTCF	Related to tumour suppression, epigenetic memory and gene organisation	-	1 x10 ⁻⁵¹
	NRF2	Related to stress response	1 x10 ⁻⁹⁵	-
	ATF3	Involved in the regulation of cell growth, apoptosis, invasion and collagen synthesis.	1 x10 ⁻⁹⁵⁹	1 x10 ⁻⁴

Table 1: Differential Transcription Motifs Found in Recorded Peaks. Key transcription factor motifs were identified in peaks showing differential accessibility in response to media change, compared to the control condition. Statistical significance for changes in accessibility is reported for both the “HG → LG” and “HG → HG” conditions.

Pathway Enrichment Analysis

Alongside investigating relevant transcription factor motifs, we also performed pathway enrichment analysis to identify likely biological pathways associated with the responses of the different conditions.

In the “HG → LG” condition, enriched pathways largely included those related to regulation and binding of transcription machinery, as shown in Figure 5. Notably, apoptotic signalling pathways and negative regulation of apoptosis were also enriched in the “HG → LG” condition, indicating potential stress responses associated with glucose reduction.

For cells in the “HG → HG” condition, a greater number of pathways were associated in genomic regions that decreased in accessibility (Figure 6), many of which were linked to transcriptional regulation. Additionally, pathways involved in regionalisation, development, and pattern formation were also found to be associated with decreased chromatin accessibility.

This is consistent with previous studies reporting that prolonged hyperglycaemic stress can disrupt normal cellular differentiation programs and alter cell fate decisions(61–63). The observed downregulation of developmental and patterning pathways under sustained high-glucose conditions suggests that chronic exposure may impair the epigenetic landscape required for maintaining cellular plasticity or initiating differentiation.

These findings they raise the possibility that persistent hyperglycaemia-induced chromatin alterations could compromise cellular identity or function. This provides a testable hypothesis that sustained high glucose may form metabolic memories of maladaptive states by reducing accessibility at involved regions. Future studies could explore whether these changes are reversible and which transcription factors or chromatin modifiers are involved to offer further insight into metabolic memory and its influence on cell fate.

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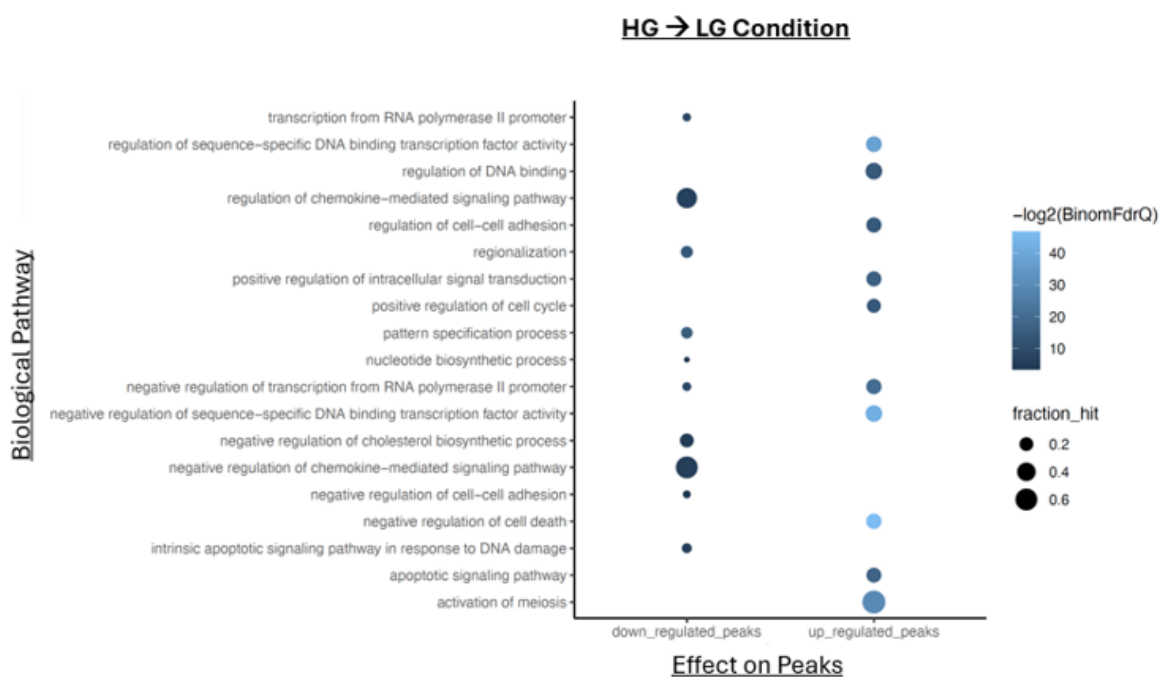


Figure 5: Pathway Enrichment Analysis in the HG → LG condition. Key biological pathways are shown on the Y axis. The X axis represents whether these pathways were enriched or depleted in terms of chromatin accessibility. Data points indicate the fraction of cells affected (point size) and the magnitude of the difference in accessibility compared to the control group (point colour).

6

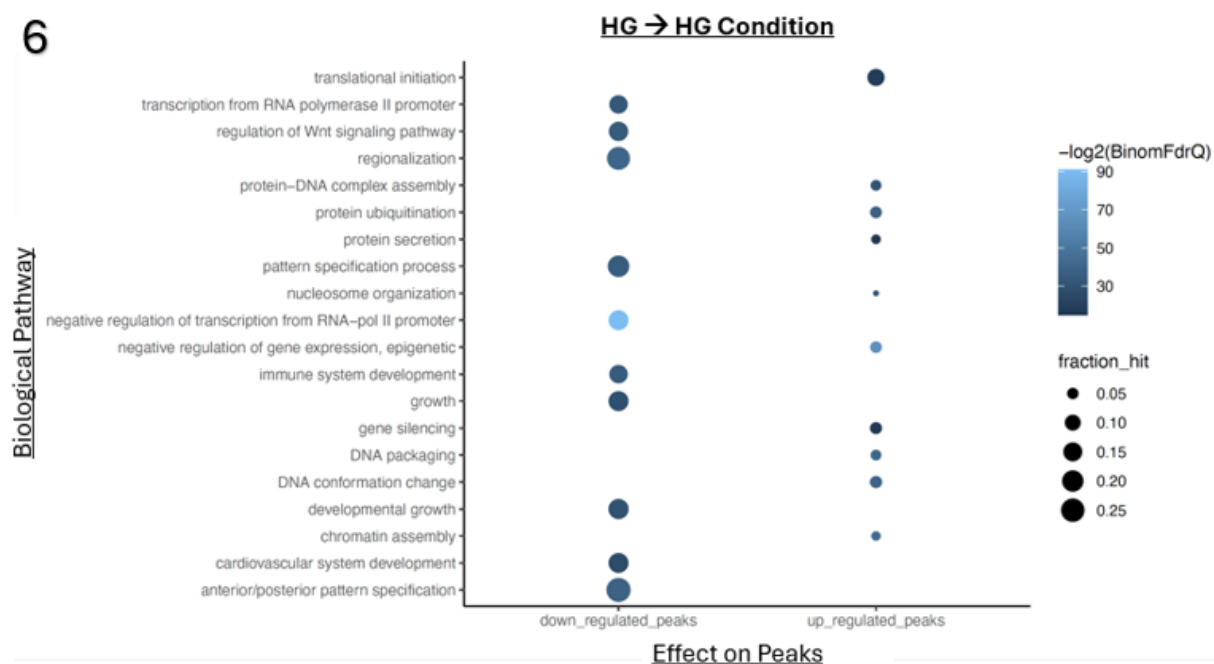


Figure 6: Pathway Enrichment Analysis of the “HG → HG condition. Key biological pathways are shown on the Y axis. The X axis represents whether these pathways were enriched or depleted in chromatin accessibility. Data points indicate the fraction of cells affected (point size) and the magnitude of the difference in accessibility compared to the control group (point colour).

The Effect of Changing Media on Chromatin Accessibility in HEK293T Cells: Low-Glucose (LG) → High-Glucose (HG)

To further investigate the impact of media and glucose changes on chromatin accessibility, the experiment was repeated in the reverse direction of media change. In this setup, HEK293T cells were initially cultured in low glucose (LG, 5.0 mM) for one week and then subjected to either a fresh replacement with the same LG media (LG → LG), a switch to high glucose (HG, 22.5 mM), or no media replacement (control) (Figure 2). ATAC-seq was performed as described previously.

To investigate the relationship between media change in the opposite direction on chromatin accessibility, pair-wise correlations between the peak data from all samples was calculated as stated above. Results are presented in a heat-map and PCA plot (Figure 7).

In each condition, two of the four biological replicates clustered together; however, the replicate pairs within each condition did not group with one another as shown by the hierarchical dendrogram, suggesting limited concordance across replicates (Figure 7A). This may indicate high variability between replicates, possibly due to experimental design or handling issues.

Data points that deviated significantly from the expected patterns were removed as potential outliers (Figure 7B). This included Control2, LG → HG_2 and LG → HG_3.

Principal Component Analysis (PCA) revealed that PC1 accounted for 18.47% of the variance, with PC2 capturing 14.9%. Interestingly, even after outlier removal, the control condition appeared to contain another possible outlier (Control_1), although initial PCA analysis did not raise concerns about this data point indicating it may not deviate as much from the other control replicates as Control_2. As a result of this, most of the biological variation between samples is represented by PC2, where experimental conditions appear stratified. This indicates that media changes, whether from LG to HG or vice versa, led to chromatin accessibility profiles that differed from the control group, as samples clustered away from the control condition.

Although preliminary data suggest some biological variation between the conditions, the reproducibility of the LG → HG samples was too low to draw any significant conclusions. The experiment must be repeated to achieve robust biological replicates to obtain valid analysis before drawing definitive conclusions.

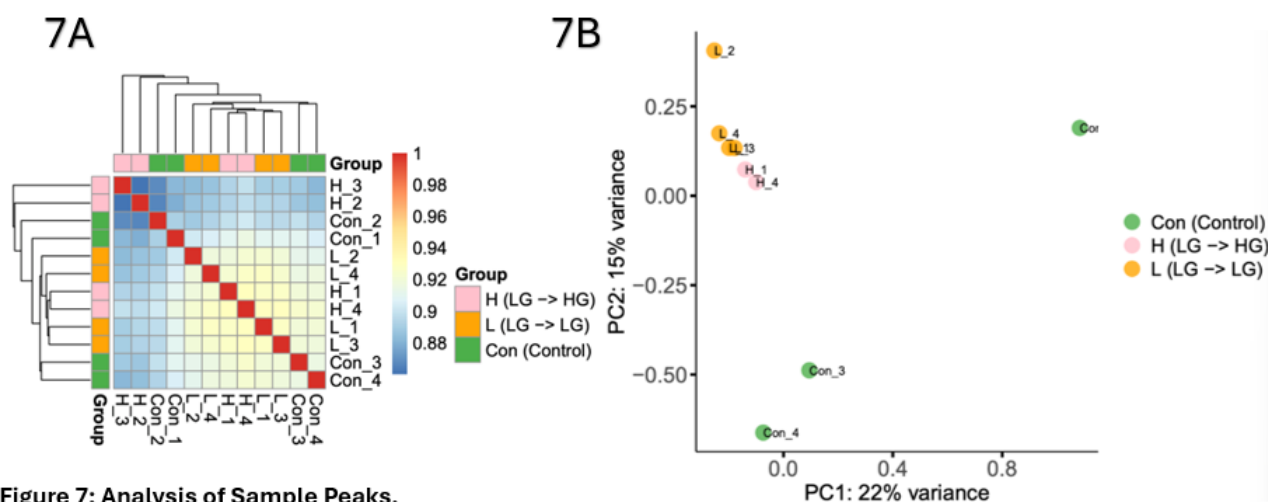


Figure 7: Analysis of Sample Peaks.

7A) Heatmap showing pairwise correlations of sample peaks for HEK293T cells cultured in low-glucose (LG) media, followed by media replacement with either the same LG or high-glucose (HG) conditions. The X and Y axes represent sample names, where "H" indicates the LG → HG condition, "L" denotes the LG → LG condition, and "Con" refers to the control group (no media change). Hierarchical clustering was performed using Pearson's correlation coefficient to assess the similarity of chromatin accessibility profiles between conditions.

7B) PCA plot following outlier removal displaying the most variable peaks in the HEK293T cells following the media transition. Each point represents an individual sample, with colours indicating the different condition groups.

Discussion

The concept of 'metabolic memory' refers to the persistence of long-term cellular changes that result from previous metabolic states, such as variations in nutrient availability like glucose concentration(64). This phenomenon has been proposed to be a contributing factor of the prolonged adverse effects seen in diabetic patients, even after blood sugar glucose normalisation(34). Specifically, the lingering cellular alterations in gene expression and chromatin accessibility after metabolic disturbances, such as hyperglycaemia, can form metabolic memory and may help explain continued complications of diseases such as diabetes(64). In this study, we aimed to investigate the role of altering glucose concentration on chromatin accessibility in HEK293T cells and the potential involvement of metabolic memory.

Metabolic Memory in Response to Glucose Changes

This study extends prior observations from a previous experiment by our group conducted on RAW264.7 macrophages, which reported differential chromatin accessibility changes following media replacement, irrespective of glucose concentration(46). It was concluded that the process of media replacement itself – rather than solely glucose levels - was the most significant factor on altering the chromatin landscape. This raised further questions and warrants further investigation into the molecular and mechanistic underpinnings of metabolic memory and the role of epigenetic modifications in response to media change.

We investigated this in the present study, where the same experiment was repeated on a different cell line, HEK293T cells. We aimed to investigate whether the glucose

influence on chromatin accessibility, including regulatory transcription factor motif activity and potential activation of biological pathways, is isolated to macrophage cells and whether the direction of change in glucose levels, from LG to HG and vice versa, effect different genomic regions in the context of metabolic memory.

In this experiment, we used two glucose conditions: low glucose (LG, 5mM), representing normoglycaemia, and high glucose (HG, 22.5mM), which mimics a hyperglycaemic state. It has been well documented that hyperglycaemia induces persistent changes in chromatin accessibility, particularly in enhancer regions and their neighbouring elements, which are critical for gene regulation. Research has shown enhanced gene expression in pathways related to diabetes, suggesting high glucose exposure can lead to lasting epigenetic changes that are linked to the pathophysiology of diabetes(65).

Therefore, in this study we hypothesised that the transition from low to high glucose (LG → HG) would result in an increase in chromatin accessibility in regions of the genome associated with pathways that are overexpressed in diabetic individuals. A media switch from high to low glucose (HG → LG) would potentially invoke metabolic memory, so similar alterations to chromatin organisation may be recorded which would reflect the prior glucose exposure.

The results from Figure 3 support previous findings that chromatin accessibility changes occur as cells transition between glucose conditions. More peaks exhibited increased

accessibility in the “HG → LG” condition compared to the “HG → HG” condition, implying that the glucose reduction resulted in unwinding of chromatin and promoted accessibility in certain genomic regions. These findings suggest a compensatory response where chromatin regions that are typically condensed during high glucose exposure become more accessible when glucose is depleted, possibly reflecting the cell's adaptation to nutrient availability.

Transcription Factors Involved in Metabolic Memory

To provide further insight into the enriched motifs and transcription factors that may play a role in chromatin modulation and metabolic memory in response to media changes, the altered chromatin regions were subjected to transcription factor motif analysis. Key transcription factors were identified and are discussed below:

CCCTC-binding factor (CTCF)

CTCF is a well-documented insulator protein that regulates chromatin organisation by controlling long-range gene interactions involving enhancers and silencers. Studies have shown that CTCF is involved in chromatin looping during metabolic stress, such as glucose deprivation, and may play a role in shaping the response to nutrient changes(58,59). For example, Gao et al (2019) discusses how inducing metabolic shifts affect CTCF-mediated chromatin looping, ultimately affecting chromatin architecture and transcriptional regulation(57). Other research has produced results that further support enrichment of the CTCF transcription factor motif in open chromatin regions of human stem cells (HSCs) when they are subjected to diabetic conditions of high glucose(66).

Consistent with our previous data and published studies, the present study demonstrated CTCF undergoes accessibility changes in both experimental conditions. In the HG → LG experiment, the CTCF transcription factor motifs were enriched in the “HG → HG” condition and depleted in the “HG → LG” condition. This suggests activity of CTCF is dependent on glucose concentration, which may enable it to facilitate long-term changes of chromatin structure in response to past metabolic states. If CTCF's binding is altered by glucose levels, it could potentially drive metabolic memory by maintaining certain regions of the genome in a more open or compact state, even after the glucose environment has shifted.

Together, published literature, previous data and this experiment has led to the thought that CTCF could be a potential master regulator of metabolic memory, particularly in the context of epigenetic remodelling and long-term chromatin accessibility following removal of a metabolic stimulus.

Activator Protein-1 (AP-1)

AP-1 is a well-known transcription factor complex composed primarily of proteins from the JUN, FOS, and ATF subfamilies, which plays a pivotal role in regulating gene expression in response to various cellular stressors, including metabolic shifts(67).

Transcription factor motif analysis in the “HG → LG” condition revealed enrichment of the canonical AP-1 binding sequence, indicating that members of the AP-1 complex may be activated in response to glucose depletion.

AP-1 is known to modulate a range of cellular responses, such as proliferation, differentiation, and apoptosis, particularly under conditions of oxidative or nutrient stress(68).

While some ATF family proteins like ATF2 are known to bind AP-1 transcription factor motifs and participate in this complex to regulate apoptosis, the enrichment seen here does not specifically implicate ATF(69). Instead, it more broadly suggests AP-1 complex activity. This activation may reflect a coordinated transcriptional response to the stress of glucose withdrawal, potentially priming the chromatin landscape for adaptive gene regulation. In the context of metabolic memory, AP-1 could act as a central mediator of chromatin remodelling, linking environmental glucose changes to persistent epigenetic states that influence gene expression even after the stressor is removed.

Nuclear Factor Erythroid 2-Related Factor 2 (NRF2)

NRF2 is a transcription factor that mediates the oxidative stress pathway in response to various environmental stressors, including changes in metabolism, to maintain cellular homeostasis(70). NRF2 has also recently been implicated in erasing metabolic memory by reversing high-glucose-induced epigenetic modifications. A study showed that overexpression of endogenous NRF2 or use of an agonist of the NRF2 pathway appears to reverse the HG-induced memories stored in the epigenome and have even proposed the use of the NRF2 pathway in resolving some of the chronic adverse effects seen in diabetic individuals(65).

Our findings show that NRF2 transcription factor motif accessibility increased in the “HG → LG” condition. This may suggest that NRF2 contributes to selective remodelling of chromatin in response to removal of the metabolic stress stimulus, being high glucose. This could partially explain the divergence in chromatin accessibility patterns between “HG → LG” and “HG → HG” conditions. While NRF2 may play a role in mitigating some aspects of metabolic memory, its effects are likely context-dependent and integrated with other regulatory factors, such as CTCF(71). These findings highlight the complexity of transcriptional responses to glucose fluctuations and support the relevance of glucose-induced chromatin modifications in shaping metabolic memory.

P53

Similar to AP-1, another well-characterised stress-responsive protein, P53, was also identified as a transcription factor motif of interest. P53 is involved in cell cycle regulation and apoptosis, particularly in the context of glucose metabolism and nutritional stress(72). P53 showed decreased accessibility in the “HG → HG” condition compared to the control group. This is consistent with literature that has published evidence that P53 signalling can be activated by glucose deprivation and therefore makes it a relevant transcription factor for metabolic memory(73).

Cells in the “HG → HG” condition still received the same HG media. This suggests that P53 activity is reduced in response these sustained hyperglycaemic conditions as cells may have already adapted to their environment. Therefore, these cells may no longer require apoptotic signalling or chromatin remodelling under these conditions.

Biological Pathway Analysis

Further enrichment analysis revealed that the “HG → LG” group showed activation of pathways involved in apoptotic signalling and the negative regulation of cell death. This finding is consistent with the transcription factor motif analysis discussed above, particularly with the role of P53.

In contrast, the “HG → HG” condition indicated downregulation of pathways associated with regionalisation, development and pattern specification. This could indicate cells are experiencing disruptions in normal cellular patterning and differentiation, which is being driven by prolonged hyperglycaemic stress. These observations support the findings of previous studies that link high glucose exposure with alterations in cell fate and regulatory pathways(61).

Alternatively, the epigenetic marks and transcription factors within the cells that regulate chromatin accessibility may "remember" the prior high-glucose environment. This memory could lead to a diminished influence on the regulatory networks controlling mitosis, potentially as a result of metabolic memory and high-glucose-induced epigenetic imprints.

Although transcription factor motif analysis identified CTCF, AP-1, NRF2 and P53 as potential motifs of interest, this only suggests that genomic regions where these transcription factor motifs reside become more or less accessible in response to change in glucose levels in media. However, It does not provide confirmation of whether these

transcription factors are actually binding to those regions. Therefore, we can only make tentative inferences about which factors may be influencing these changes in the epigenome. To obtain direct evidence of transcription factor occupancy and validate their involvement, future experiments could employ ChIP-Seq or CUT&Tag, which would confirm the physical binding of these factors to their respective motifs under different glucose conditions.

Similarly, while ATAC-seq offers insight into changes in chromatin accessibility, it does not directly indicate whether active transcription or gene expression within these genomic regions are affected. Initially, we planned to complement this data with a parallel RNA-sequencing protocol as part of the LG → HG study to correlate perceived changes in the chromatin landscape with gene expression. However, due to time constraints and reagent limitations, this analysis was not completed. Future studies could revisit this approach or perform additional parallel ATAC/RNA-seq experiments to determine whether gene expression is changing in the dynamic chromatin regions identified by ATAC-seq.

Nevertheless, the fact that our data suggests chromatin modifications persist and potentially influence long-term gene expression highlights the potential role of epigenetic memory in metabolic adaptation, which could have therapeutic implications for diseases such as cancer and diabetes, where cells often experience fluctuating glucose levels.

Experimental Limitations and Future Directions

Several limitations of this study must be addressed when interpreting our data and resolved in future experiments.

The quality of data in the LG → HG experiment was compromised, likely due to technical issues related to the handling of the biological replicates, particularly in the LG → HG condition. These issues may have contributed to significant cell injury or cell death. Additional stress to the cells may have resulted from the extended protocol, as cells were simultaneously harvested for RNA isolation, which required freezing the nuclei for subsequent ATAC-seq. Such procedural variations between the two experiments raises concerns about the validity of the data. The additional workload and freezing of nuclei may require further refinement of the protocol before its final execution. Future experiments could consider alternative methods of processing the parallel samples, such as keeping the nuclei on ice during RNA extraction to minimise potential stress. Additionally, this experiment was conducted with four replicates, compared to the HG → LG experiment, which used three replicates. The increased number of replicates may have contributed to the challenges in sample handling.

Due to the poor quality of the data, we were unable to perform transcription factor motif and pathway analysis for comparison with the HG → LG experiment to assess whether differential chromatin accessibility varies depending on the direction of media change.

We are limited in our ability to draw meaningful conclusions from the data due to the

small sample size after removal of outliers. The inferences that can be made are discussed in the results section.

Other potential limitations of these experiments require consideration, particularly with regard to the glucose absorption by HEK293T cells from the experimental media.

Glucose, being a large, polar molecule, requires transporter proteins on the plasma membrane for cellular uptake. In HEK293T cells, the primary glucose transporters are members of the SLC2A family. Basal glucose uptake through facilitated diffusion is primarily mediated by GLUT1(74), while GLUT3, which is also expressed on the plasma membrane, has a higher affinity for glucose and plays a more significant role in low glucose conditions(75). The Michaelis constant (K_m) values of GLUT1 and GLUT3 supports their physiological roles with GLUT1 having a K_m of approximately 10-20mM and GLUT3 having a K_m of around 3mM(76).

This suggests that in our physiological LG condition (5mM), GLUT3 would likely be the most efficient transporter for glucose uptake, though it would probably be saturated. Conversely, in the HG condition (22.5mM), GLUT1 would be expected to play a more prominent role. However, since glucose absorption rates were not directly measured during the incubation period, we cannot definitively confirm whether there were differences in glucose uptake between the HG and LG conditions. While direct measurement of glucose uptake would have strengthened the study, the current experimental design effectively distinguishes between the effects of prior glucose exposure and potential confounding factors such as media changes or aspirational

effects. Therefore, the observed chromatin changes are more likely to reflect true metabolic memory rather than spontaneous or mechanical alterations.

Future studies should incorporate direct measurements of glucose uptake kinetics to confirm the extent of cellular glucose exposure under each condition. This would strengthen interpretations about metabolic memory by validating that the observed chromatin accessibility changes are indeed a consequence of altered intracellular glucose metabolism, rather than assumed from extracellular concentrations alone. Such data would provide a more precise link between nutrient sensing and long-term epigenetic remodelling and will strengthen the experimental design of the current study.

Another potential confounding factor that may be influencing the results of the study is the physical stress and pressure exerted on the cells during media aspiration and subsequent media change, despite careful handling. This physical manipulation may be influencing the perceived changes in chromatin accessibility. To address this issue, a more appropriate control condition, or additional control condition, could involve removing the media from the control samples using a pipette and immediately replacing it. The media would remain unchanged, retaining the same metabolites, this approach would still subject the cells to the shear forces associated with media removal and replacement similar to the other experimental conditions. Implementing this method would help isolate the effects of physical stress from those caused by the changes in media composition, providing a clearer understanding of how chromatin accessibility is influenced.

The comparison of results regarding metabolic memory between RAW264.7 macrophage cells and HEK293T cells warrants careful consideration. A major consistent finding between this study and previous experiments is the observation of changes in accessibility of the CTCF transcription factor motif(46), which has been discussed prior as a potential mediator of metabolic memory. However, despite similar experimental conditions, there do not appear to be significant correlations in the analyses between the two cell lines.

Macrophage and kidney cells are functionally differentiated specifically for their physiological roles, which may influence how each cell type utilises environmental glucose. Macrophages exhibit high metabolic flexibility, transitioning between aerobic glycolysis and oxidative phosphorylation depending on their activation state. In contrast, kidney cells are frequently exposed to high glucose concentrations, so may be more primed to handle the HG condition(77).

These differences in metabolic profiles may contribute to the observed variation in chromatin accessibility, potentially reflecting how each cell type modulates glucose utilisation, thus influencing the accessibility of different regions of the genome.

Further complicating the interpretation of these findings is evidence suggesting that HEK293T cells are inefficient at metabolising glucose and primarily rely on glutamate as their energy source(78). This raises important considerations about the extent to which glucose-induced metabolic memory can be observed in HEK293T cells, given their reported preference for glutamate metabolism over glucose. This metabolic bias may

help explain the divergence between our results and those of previous in-house experiments and existing literature

In conclusion, this study demonstrates replacement of media with either high or low glucose concentrations induces significant distinct changes in chromatin accessibility in HEK293T cells. The direction of glucose change appears to trigger a chromatin remodelling response, potentially indicative of metabolic memory. Key transcription factors, such as CTCF, AP-1, P53, and NRF2, are implicated in mediating these changes, suggesting a complex interplay between chromatin reprogramming, metabolic shifts, and stress responses. These findings are critical for advancing our understanding of how epigenetic changes occur in response to metabolic fluctuations, particularly in the context of metabolic memory and its molecular mechanisms. Importantly, they lay the groundwork for future studies aimed at identifying the molecular drivers of metabolic memory, determining the reversibility of these epigenetic marks, and testing whether modifying these pathways can prevent or reverse disease-associated chromatin states. Such insights have significant therapeutic potential, particularly for chronic diseases like diabetes, cancer, and neurodegenerative disorders, where metabolic dysfunction is closely tied to persistent epigenetic alterations.

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