Multi-omics analysis of the ageing liver

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"Douglas Adams once said that 'There is an art to flying, or rather a knack. The knack lies in learning how to throw yourself at the ground and miss.' Immortality requires learning a similar knack: you must first be born and then subsequently avoid dying forever. This is challenging in part because there are many different causes of death to avoid [...] and because our bodies slowly change in ways that make most of these causes of death increasingly probable."

Nicholas Stroustrup
Measuring and modelling interventions in ageing (Current opinion in cell biology, 2018)

LIST OF ABBREVIATIONS

General

AD	Alzheimer's disease
ATAC	Assay for Transposase-Accessible Chromatin using sequencing
ATP	Adenosine triphosphate
C. elegans	Caenorhabditis elegans
CGIs	CpG islands
ChIp-seq	Chromatin Immunoprecipitation Sequencing
CHIP	Clonal haematopoiesis of indeterminate potential
circRNA	Circular RNA
CpG	Cytosine-guanine dinucleotide
CUT&RUN-seq	Cleavage under targets and release using nuclease sequencing
D. melanogaster	Drosophila melanogaster
D. rerio	Danio rerio
DNAm	DNA methylation
FISSEQ	Fluorescent in situ sequencing
FISSEQ	Fluorescent in situ sequencing
HCC	Hepatocellular carcinoma
LD	Lipid droplet
LDA	Latent Dirichlet Allocation
lncRNA	long non-coding RNA
LSI	Latent semantic indexing
M. musculus	Mus musculus
MERFISH	Multiplexed error-robust fluorescence in situ hybridization
miRNA	Micro RNA
mtDNA	Mitochondrial DNA
mTOR	Target of Rapamycin
mTORC1	Target of Rapamycin complex 1
N. furzeri	Nothobranchius furzeri
NAD+	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
ncRNA	Non-coding RNA
NET-seq	Native elongating transcript sequencing
NFR	Nucleosome-free region
PC	Principal component
PCA	Principal component Analysis
PCR	Polymerase chain reaction
PTM	Post-translational modification

RNA	Ribonucleic acid
S. cerevisiae	Saccharomyces cerevisiae
SASP	Senescence-associated secretory phenotype
scATAC-seq	Single cell Assay for Transposase-Accessible Chromatin using sequencing
scRNA-seq	Single cell RNA Sequencing
SeqFISH	Sequential Fluorescence in situ Hybridization
SPACO	Spatial components
STARmap	Spatially-resolved transcript amplicon readout mapping
SVD	Singular value decomposition
SVG	Spatially variable genes
TAD	Topologically associating domain

Gene and protein names

Cdr1as	circular RNA Cdr1as
Cidea	cell death-inducing DNA fragmentation factor
Cidec	cell death-inducing DFFA-like effector c
Cps1	Carbamoyl-Phosphate Synthase 1
CTCF	CCCTC-Binding Factor
Cyp1a2	cytochrome P450 family 1 subfamily A member 2
Cyp2e1	cytochrome P450 family 2 subfamily E member 1
DNMT3B	DNA (cytosine-5)-methyltransferase 3 beta
Elovl	Elongation of very long chain fatty acids protein
FAS	fatty acid synthase
H2A	Histone H2A
H3	Histone H3
H3K14	Histone H3 Lysine 14
H3K14ac	Histone H 3 Lysine 14 acetylation
H3K27	Histone H 3 Lysine 27
H3K27ac	Histone H 3 Lysine 27 acetylation
H3K36	Histone H3 Lysine 36
H3K36me3	Histone H3 Lysine 36 tri-methylation
H3K4	Histone H3 Lysine 4 tri-methylation
H3K4me3	Histone H3 Lysine 4 tri-methylation
H3K9	Histone H3 Lysine 9
H3K9ac	Histone H 3 Lysine 9 acetylation
H4	Histone H4
H4K12ac	Histone H 4 Lysine 12 acetylation
H4K16ac	Histone H4 Lysine 16 acetylation
H4K16ac	Histone H 4 Lysine 16 acetylation
H4K20	Histone H4 Lysine 20

H4K5ac	Histone H 4 Lysine 5 acetylation
H4K8ac	Histone H 4 Lysine 8 acetylation
Hamp	Hepcidin Antimicrobial Peptide
Hamp2	Hepcidin Antimicrobial Peptide 2
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HMT	Histone methyl transferase
IGF-1	Insulin-like growth factor 1
KAT7	Histone acetyltransferase KAT7
miR-122	microRNA-122
miR-29a	MicroRNA 29a
Mnase	Micrococcal nuclease
MYC	MYC proto-oncogene, bHLH transcription factor
Neat1	Nuclear Paraspeckle Assembly Transcript 1
NELF	Negative elongation factor E
Pol II	RNA polymerase II
Rev-erba	nuclear receptor subfamily 1 group D member 1
SIRT family	Sirtuin protein family
SPT4	Transcription elongation factor SPT4
SPT5	Transcription elongation factor SPT5
TET protein family	family of ten-eleven translocation methylcytosine dioxygenases

ABSTRACT

This dissertation presents three manuscripts that originated from my work on the ageing liver. The first two manuscripts concentrate on integrative multi-omics approaches, such as scATAC-seq and scRNA-seq, spatial transcriptomics, CUT&RUN sequencing and lipidomics. They reveal distinct ageing signatures within the murine liver, mainly in hepatocytes. The third manuscript introduces a new methodology for the analysis of spatial sequencing data, which was developed with the ageing liver as an intended application.

The first manuscript, **"Single-cell resolution unravels spatial alterations in metabolism, transcriptome, and epigenome of ageing liver"**, establishes how spatial location and microenvironmental changes impact the ageing trajectories of hepatocytes within liver tissue. Through the integration of spatial transcriptomics, single-cell ATAC- and RNA-seq, lipidomics, and functional assays, the study elucidates zonation-specific and age-related changes in the epigenome, transcriptome, and metabolic states. We identified a zonation-dependent shift in the epigenome and show that changing microenvironments within a tissue exert strong influences on their resident cells that can shape epigenetic, metabolic and phenotypic outputs. From a functional perspective, periportal hepatocytes exhibited diminished mitochondrial fitness, whereas pericentral hepatocytes demonstrated an increased accumulation of large lipid droplets.

The second manuscript, **"Ageing is associated with increased chromatin accessibility and reduced polymerase pausing in liver"**, examines the chromatin landscape of the ageing liver by using CUT&RUN for RNA polymerase mapping, integrated with ATAC-seq, RNA-seq, and NET-seq. The study reveals an increase in chromatin accessibility at promoter regions as a characteristic of ageing, which is not accompanied by a corresponding increase in transcriptional output. Ageing is also found to be associated with a decrease in promoter-proximal pausing of RNA Polymerase II. Our observations suggest that alterations in transcriptional regulation associated with ageing may be due to decreased stability of the pausing complex.

The third manuscript, **"Dimension reduction by spatial components analysis improves pattern detection in multivariate spatial data"**, introduces SPACO, a new statistical approach designed to enhance pattern recognition in multivariate spatial sequencing data. SPACO stands out by focusing on gene co-regulation and maximising local covariance. It provides a more sensitive and accurate test for the identification of genes with a spatial expression pattern. Moreover, the use of spatial components for gene denoising by SPACO boosts the effective linkage of histological observations with gene expression patterns, even in high-noise conditions.

1. INTRODUCTION

This dissertation is structured in a cumulative format and comprises three individual parts, each corresponding to a distinct manuscript. These manuscripts collectively contribute to our understanding of the ageing liver and research methods around it. In the subsequent parts, we will delve into each part and explore the specific research questions they address, their methodologies, findings, and the implications of these findings. The manuscript in part one is currently in the peer review process but already available at bioarxiv (https://doi.org/10.1101/2021.12.14.472593), the manuscript of part two is already published (https://doi.org/10.15252/msb.202211002). The manuscript of part three will be submitted to a journal soon.

1.1. The Biology of Ageing

The World Health Organization has reported a forecasted shift in global demographics, with an ageing population surpassing the younger demographic by 2050. Precisely, it is estimated that the population of individuals aged 60 and older will be approximately 2.1 billion, outnumbering adolescents aged 10–24, who are expected to number around 2.0 billion. Furthermore, the segment of the population aged 80 years and older is projected to triple from its 2020 figure, increasing to 426 million by 2050. Despite improvements in longevity, advancements in prolonging the health span of individuals are relatively moderate, emphasizing the importance of health promotion during the ageing process as this will inevitably pose a great challenge for public health systems around the world (Puth et al., 2017). This approach is endorsed by the World Health Organization as a critical factor in mitigating the adverse effects of an ageing population on a global scale (Suzman and Beard, 2011; Rudnicka et al., 2020).

Elucidating the complex biology of ageing is vital for understanding its effects on the individual level and identifying intervention vectors to increase health span and longevity. Ageing is typically described as a progressive decline in cellular functionality and homeostasis over time (López-Otín et al., 2013). In recent years, several processes that modulate the rate and acceleration of ageing in organisms have been identified. These "hallmarks of ageing" are an ever-growing list of biological phenomena, including genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis. The noted processes could be identified across a wide range of mammalian and non-mammalian model organisms (Figure 1) (López-Otín et al., 2013, 2023).

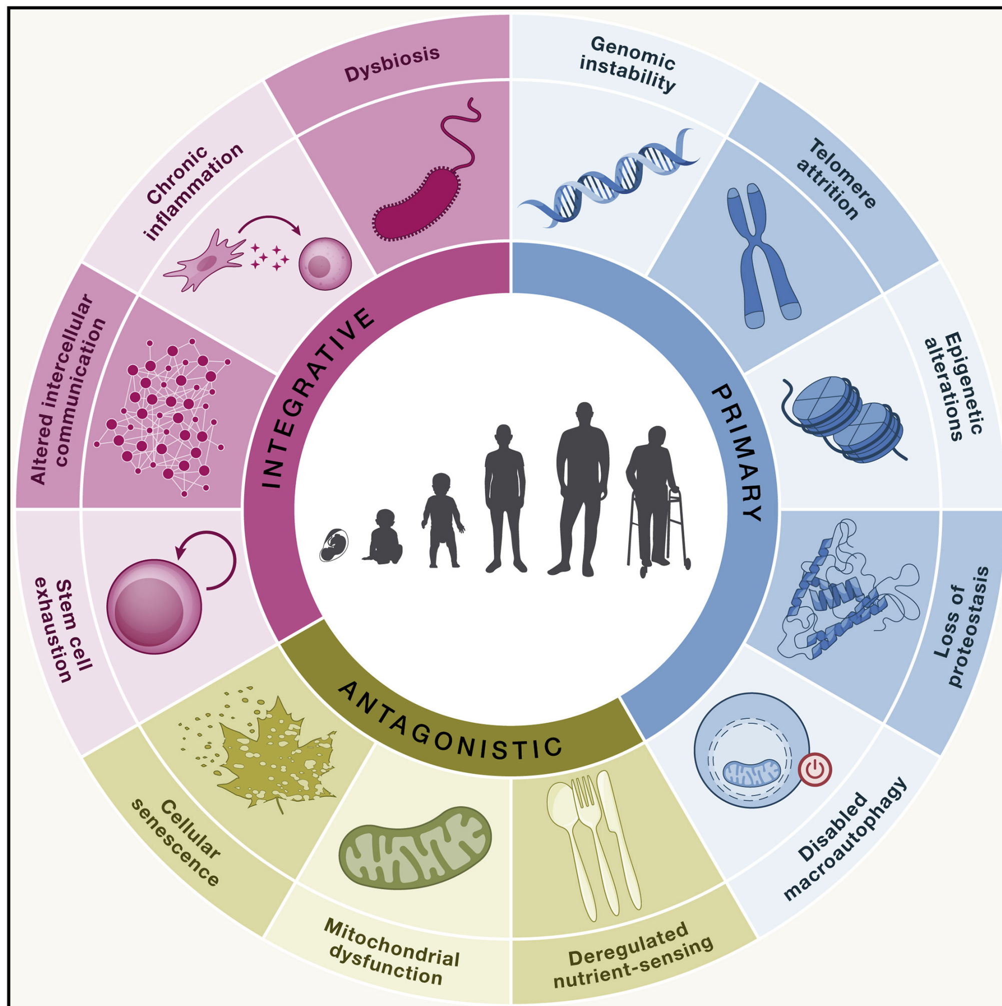

Figure 1: This illustration presents the twelve "hallmarks of ageing": Genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis. These symptoms of ageing are categorized into three groups: primary, antagonistic, and integrative. Obtained from López-Otín et al., (2023).

These hallmarks of ageing can be further grouped into three categories primary, antagonistic and integrative. These categories impose a kind of hierarchy or rather a dependency structure on the process of ageing, but of course, there is no strictly directed relationship but rather a complex interplay of factors and phenomena. The primary category can be subsumed as the sum of molecular damages and errors that accumulate on a cellular level during the life span of an organism due to cellular metabolism or environmental exposition that inevitably lead to the ageing process (Gladyshev et al., 2021).

Of particular relevance to this thesis are the hallmarks mitochondrial dysfunction, cellular senescence, stem cell exhaustion and chronic inflammation.

Mitochondrial dysfunction

The functionality of mitochondria, particularly their ability to provide energy-rich molecules, is critical for maintaining the health and vitality of cells. This is achieved primarily through the process of oxidative phosphorylation, a metabolic pathway that uses

energy released by the oxidation of nutrients to produce ATP, the primary energy carrier in cells.
As cells age and accumulate mtDNA mutations, the mitochondria's capability to efficiently produce ATP can be severely compromised. This is because these mutations result in defective proteins of the respiratory chain complexes, which are essential for oxidative phosphorylation. In essence, a defective respiratory chain can drastically impede the cell's ability to produce ATP. Compromised mitochondrial energy production can have numerous consequences, as ATP is required for a vast array of cellular processes (Druzhyna et al., 2008).
Emerging evidence suggests that certain mtDNA mutations might lead to an adaptive metabolic shift towards glycolysis, an ATP-producing process that does not rely on mitochondria. However, while this shift might help to compensate for reduced ATP production in the short term, it is generally less efficient than oxidative phosphorylation and can contribute to cellular ageing and pathology in the long run (Chung et al., 2022).

The integrity of the mitochondrial membrane, which is largely determined by its lipid composition, is vital for the proper functioning of the mitochondria.
Mitochondrial membranes are predominantly composed of phospholipids, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and cardiolipin. Among these, cardiolipin, a unique dimeric phospholipid, is of particular importance as it is crucial for the stability and function of many proteins that are involved in mitochondrial energy metabolism.
With advancing age, however, the lipid composition of the mitochondrial membrane can change significantly. There is often a decrease in cardiolipin and an increase in other lipids like cholesterol. This altered lipid profile can affect the membrane's fluidity and permeability, which in turn, can disrupt the function of the respiratory chain complexes. Consequently, energy production via oxidative phosphorylation can be compromised, and the production of reactive oxygen species can be increased, further accelerating cellular damage.
Age-related changes in lipid metabolism can also contribute to the accumulation of lipid peroxides, which can cause oxidative damage to both mtDNA and proteins, leading to mitochondrial dysfunction. Moreover, changes in the lipid composition can disrupt the dynamics of mitochondrial fusion and fission, essential processes for maintaining a healthy mitochondrial network within the cell (Pollard et al., 2017).

Cellular senescence

One response to genomic instability and other cell damages accumulated over time is cellular senescence. In this state, cells become insensitive to growth-promoting stimuli and enter cell cycle arrest. They also undergo a fundamental rewiring of key characteristics such as morphology, metabolism, chromatin architecture, and gene expression, leading to the adoption of very heterogeneous, but often pro-inflammatory phenotypes, denoted as the senescence-associated secretory phenotype. Ideally, this shift in cell status has as a consequence that immune cells are recruited and thereby, the senescent cells are removed, but if this response is insufficient, or the immune answer is absent, the

result on the tissue microenvironment ultimately can lead to fibrosis (Gorgoulis et al., 2019; Tuttle et al., 2020).

Stem cell exhaustion

The immune clearance of senescent cells and of injury is usually followed by a tissue-specific repair process via stem cells, progenitor cells or dedifferentiated cells. Here should be noted that the exact repair process can differ vastly between tissues. During ageing, tissue renewal at a steady state is at a progressive loss as stem cells and progenitor cells are subjected to the hallmarks of ageing to the same extent as cells with no stemness potential (Clevers and Watt, 2018). Further excessive proliferation of stem cells can lead to stem cell exhaustion and, thereby, premature ageing (Rera et al., 2011).
Stem cell exhaustion, dysregulated nutrient sensing, and increased cellular senescent are closely connected to altered intercellular communication. During ageing, a progressive increase in noise in cell-to-cell signalling and, thereby, compromise of homeostatic as well as hermetic regulation can be observed. Often the most health span and longevity-increasing effects in that context can be linked to insulin-like signalling or stress related responses (Miller et al., 2020).

Chronic inflammation

Chronic inflammation or "inflammaging" is a result of ageing, manifesting systemically as well as in specific localised pathological phenotypes. This ageing-related inflammatory state is particularly pronounced in the liver, contributing to the development of diseases like Non-Alcoholic Fatty Liver Disease (NAFLD), Non-Alcoholic Steatohepatitis (NASH), Alcoholic Steatohepatitis (ASH), and Hepatocellular Carcinoma (HCC) (Loeser, 2011; Nilsson, 2015).

Inflammaging arises from various hallmarks of ageing. Genomic instability, or the increasing frequency of DNA mutations and epigenetic alterations with age, contributes to this. The phenomenon called clonal haematopoiesis of indeterminate potential (CHIP) is particularly noteworthy here. In CHIP, certain myeloid cells that carry pro-inflammatory properties expand, potentially driving the ageing process in the liver (Wong et al., 2023).
Mutations that lead to CHIP often affect epigenetic modifiers like DNMT3 and TET2, which play a crucial role in DNA methylation - a vital process for regulating gene expression, which will be discussed later in Chapter 1.2. These mutations can foster an environment that promotes inflammation, potentially accelerating the development of liver diseases (Cobo et al., 2022; Wang et al., 2023).
Additionally, the immune system's functionality declines with age, further promoting inflammaging. Age-associated shifts in T cell populations favour pro-inflammatory responses while reducing the efficiency of immune surveillance - a balance crucial for maintaining liver health (Robinson et al., 2016).

All 12 hallmarks of ageing show a high grade of interdependency and interconnectivity. This opens the possibility for a wide vector of interventions in reducing or even reversing ageing. For all hallmarks of ageing, several interventions were proposed or even shown

and are subject of multidisciplinary research. These can coarsely be grouped into four different categories: Genetic interventions, pharmacological, behavioural and dietary interventions and through measuring and modelling of ageing biomarkers (Bernardes de Jesus et al., 2012; Lee and Longo, 2016; Novelle et al., 2016; Rebelo-Marques et al., 2018).

The hallmarks of ageing model by López-Otín et al. (2013, 2023), though highly accepted in the field, have drawn criticisms. Chief among them is the disputed role of accumulated cellular damage in driving ageing (Gems and de Magalhães, 2021). Alternative theories suggest that biological processes optimised for early life stages could induce physiological dysfunction in later life due to weak influences from natural selection (Maklakov and Chapman, 2019).
Critics question whether these hallmarks cause or result from ageing, emphasising a lack of clarity in distinguishing causative factors from consequential changes, and claim that the causation between primary, antagonistic, and integrative effects is not always proven and that mechanistic links to age-associated diseases are as well not always given (Gladyshev and Gladyshev, 2016).
The selection of hallmarks has been labelled arbitrary, potentially limiting research scope and interventions. The recent extension from 9 to 13 hallmarks could support this claim or reflect the rapid maturation of ageing research (López-Otín et al., 2013, 2023; Gems and de Magalhães, 2021).
Finally, ageing research's heavy reliance on non-human data raises concerns about its applicability to human ageing, given inter-species differences in ageing mechanisms (De Magalhães, 2014).

While the hallmarks of ageing paradigm has significantly contributed to the understanding of ageing, the criticisms presented underscore the intricate complexities of ageing and raise important questions about causality, model selection, and the translation of findings from non-human models to humans. Given these points of contention and the ongoing extension and refinement of the theories, it is clear that the ultimate understanding of the ageing process remains an open question. Therefore, further comprehensive and integrative research is paramount to unveil the true nature of ageing and its associated diseases, which will, in turn, allow us to develop more effective interventions for healthy ageing.

1.2. Epigenetic Alterations of Ageing

Epigenetic alterations are one primary hallmark of ageing and are also widely acknowledged by its critics for their significance during ageing. I will discuss the topic in more detail as it is a central focus of this thesis. Epigenetics is nowadays defined as alterations in gene expression that are mitotically (and potentially meiotically) heritable, which do not involve alterations in the DNA sequence but modulate chromatin organization by various chemical modifications. The major mechanisms of epigenetic regulation are DNA methylation, histone modifications, chromatin remodelling and non-coding RNA molecules (Retis-Resendiz et al., 2021). The involvement of epigenetic changes in ageing

and longevity could be shown across a wide range of model organisms. From unicellular eukaryotes like *S. cerevisiae* (Kaeberlein et al., 1999) over invertebrates (*C. elegans* (González-Aguilera et al., 2014) and *D. melanogaster* (Rose and Charlesworth, 1980)) to vertebrates (*D. rerio* (Mayne et al., 2020), *N. furzeri* (Zupkovitz et al., 2021), *M. musculus* (Wagner, 2017) and more). Further, it should be noted that also information encoded in the DNA could be identified as a factor for ageing and longevity even in the plant kingdom (Popov et al., 2022). The epigenetic alterations of ageing are summarized in Figure 2 and will be described in detail in the following chapter.

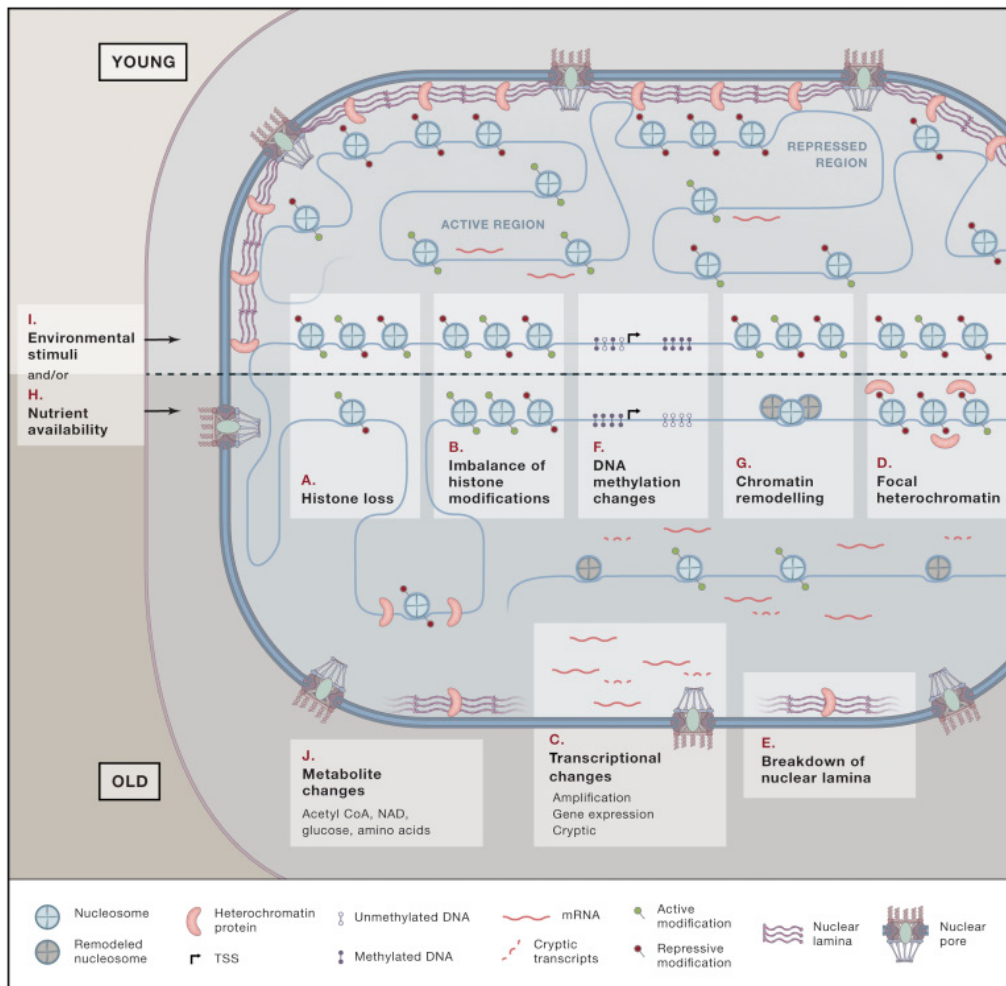

Figure 2: Epigenetic alterations of ageing are characterized by (A) loss of histones, (B) imbalance of activating and repressive modifications, (C) transcriptional changes, (D) losses and gains in heterochromatin, (E) breakdown of nuclear lamina, (F) global hypomethylation and focal hypermethylation, and (G) chromatin remodelling. These changes are heavily dictated by (H) environmental stimuli and (I) nutrient availability that in turn (J) alter intracellular metabolite concentrations. Modified from Sen et al. (2016).

1.2.1 The DNA Methylome

DNA methylation (DNAm), a critical epigenetic mechanism primarily characterized by adding a methyl group to the carbon five position of the cytosine ring within a CpG dinucleotide, usually represses gene expression. DNAm is found within about 70-80% of the mammalian genome, with CpG islands (CGIs), localized regions rich in G + C bases,

being associated with promoter regions. Methylation rates differ depending on tissue and cell type (Ben-Hattar and Jiricny, 1988; Deaton and Bird, 2011).
The ageing process is accompanied by a global loss of methylation marks, or hypomethylation, particularly in repetitive genomic regions, which can lead to genomic instability. Conversely, certain gene promoter regions, usually sparsely methylated under normal conditions, tend to become aberrantly hypermethylated with age. This phenomenon can lead to inappropriate gene silencing and contribute to age-associated gene dysfunction (Jones et al., 2015).

DNA hypo- or hyper-methylation has been linked to age-related diseases like cancer and Alzheimer's disease. DNAm can be influenced by factors like smoking, diet, and exercise, among others and can result in differential methylation patterns, which are then copied during DNA replication, leading to long-lasting effects on the genome. Moreover, inherent stochastic errors and a decrease in the capacity of DNA damage repair mechanisms with age can further accentuate these changes (Perna et al., 2016; Seale et al., 2022). Age-related alterations can occur similarly across tissues as well as being tissue-specific (Christensen et al., 2009; Day et al., 2013; Hannum et al., 2013).

DNA methylation patterns, due to their relative stability, dynamic range, and ease of assessment, make them an attractive biomarker for estimating biological age. Global DNAm can be measured using bisulfite sequencing, which offers single-nucleotide resolution, or array-based techniques (Kurdyukov and Bullock, 2016).

The DNAm status' generalizability has led to the creation of "epigenetic clocks", predictive models of biological age, such as the Horvath clock, based on specific DNAm sites. Since then, various other ageing clocks designed for multiple tissues and organisms and even multispecies and multi-specious clocks have been published (Horvath, 2013; Lu et al., 2022). Other examples include Hannum's Clock, PhenoAge, and GrimAge (Hannum et al., 2013; Levine et al., 2018; Lu et al., 2019).
In addition to methylation-based clocks, "transcriptomic clocks" have been introduced recently, leveraging transcriptomics data for organisms without a methylome or in settings where DNA methylation cannot be readily assessed (Meyer and Schumacher, 2021).

1.2.2. Histones and Histone Modifications

Genomic DNA, if linearised, would extend to about two meters. To fit into the cell nucleus, it is condensed into a chromosomal structure that can be de-condensed as needed. This complex, dynamic spatial chromatin organisation allows genome regions to interact and be accessible for various cell functions (Millán-Zambrano et al., 2022).

Nucleosomes, composed of two copies of each core histone H2A, H2B, H3 and H4, play a crucial role in this organisation. They can alter their form and position and partially and fully disassemble. Their protruding N-terminals and their globular core domains are subject to various post-translational modifications (PTMs), including methylation,

acetylation and more (Strahl and Allis, 2000). Histone variants can further increase this variety (Weber and Henikoff, 2014).

These PTMs can be written, read, and erased, controlling diverse transcriptional outcomes and linking to metabolic states (Dai et al., 2020). Histone "writers" are enzymes that add PTMs to a histone tail. Histone "readers" are proteins that identify specific histone modifications and often trigger cellular responses. These "readers" commonly interact with other proteins for DNA repair, replication, and transcription (Yun et al., 2011). "eraser" proteins contribute to the dynamic regulation of histone PTMs by removing histone modifications (Hyun et al., 2017).

PTMs are categorised into two types: indirect, which involve the binding of effector proteins, and active, which directly cause chromatin alterations. Additionally, PTMs can result from DNA-templated processes such as transcribing polymerase activity, which means that transcribing polymerases can promote histone PTMs, which in turn could itself cause a downstream event. One example of this is Histone 3 lysine 36 tri-methylation (H3K36me3) by SETD2 (Millán-Zambrano et al., 2022; Molenaar and van Leeuwen, 2022).

Histone modifications ("marks") significantly influence chromatin structure and function, with distinct modifications often correlating with specific genomic regions and their respective functionalities:

Acetylation and Deacetylation:
Histone acetyltransferases and histone deacetylases add and remove acetyl groups to histones, resulting in gene activation or repression, respectively (Yun et al., 2011; Seto and Yoshida, 2014).

Histone Methylation and Demethylation
Histone methyltransferases and histone demethylases respectively add and remove methyl groups to histones, respectively, modulating gene expression levels based on the specific context and the methylation level (mono-, di-, or tri-methylation) (Rice et al., 2003; Hyun et al., 2017)

Promoters
Promoters function as an assembly point for the transcription machinery. Active promoters are frequently marked by histone acetylations, such as at H3 lysine 9, 14 and 27 (H3K9ac, H3K14ac and H3K27ac) and H4 lysines 5, 8, 12, and 16 (H4K5ac, H4K8ac, H4K12ac, H4K16ac). These acetylation marks neutralise the positive charge of histones, leading to a weakened interaction between DNA and histones, thus opening the chromatin structure for transcription initiation (Akhtar and Becker, 2001; Robinson et al., 2008). Furthermore, promoters often bear methylation marks of histone H3 on lysines 4, 36, and 79 (H3K4, H3K36, H3K27). These marks are associated with active transcription (Black et al., 2012; Hyun et al., 2017).

Enhancers
Specifically for enhancers, histone H3 lysine 27 acetylation (H3K27ac) is a defining mark of active enhancers. These regions can be located far from the transcription start site of the gene they regulate and yet can loop towards the TSS to mediate transcriptional activation. The presence of H3K27ac at these enhancers denotes an open chromatin configuration that enables the binding of transcription factors and coactivators, ultimately leading to increased gene expression. Another histone 3 acetylation to mention here is lysine 14 acetylation (H3K14ac). Additionally, mono methylation of histone H3 on lysine 4 (H3K4me) is associated with both active and poised enhancers (Beacon et al., 2021).

Gene Bodies
As mentioned above, actively transcribed genes are often marked by tri-methylation of histone H3 on lysine 36 (H3K36me3) and lysine 79 (H3K79me3). These modifications play crucial roles in the prevention of spurious transcription initiation within the gene body and in the elongation phase of transcription, respectively (Millán-Zambrano et al., 2022).

Centromeres and Telomeres
These specialised chromosomal regions often exhibit unique histone modification patterns. For instance, pericentric heterochromatin is often marked by methylation of histone H3 on lysine 9 (H3K9me3), while subtelomeric regions are characterised by H3K27 (H3K27me) methylation (Wong et al., 2011).

Repressed regions
Genomic regions undergoing transcriptional silencing often bear tri-methylation of histone H3 on lysine 27 (H3K27me3) or lysine 9 (H3K9me3). These marks correlate with heterochromatin (Wong et al., 2011).

These diverse modifications, including acetylation and methylation, collectively establish unique chromatin states at distinct genomic regions, pivotal in gene regulation and other DNA-dependent processes. However, the relationships between these histone modifications and genomic functions are multifaceted and often context-dependent.

Cis- and trans-acting histone modifications
Histone post-translational modifications can influence chromatin state locally or at distant loci, shaping the chromatin landscape. Local or cis-modifications include acetylation of histone lysine residues, which relax local chromatin structure, while H3K9me3 and H3K27me3 induce chromatin condensation and transcription repression (Ninova et al., 2019; Strahl and Briggs, 2021). Conversely, trans-modifications exert effects at a distance by creating binding sites for effector proteins or altering higher chromatin order. For example, H3K9me and H3K27me generate binding sites for polycomb proteins, which can enforce chromatin condensation at neighbouring or distant nucleosomes (Bian et al., 2020).

Histone modifications and nucleosome occupancy in the context of ageing

In the context of ageing, global loss of histones, histone modifications and histone modifiers emerge as contributors and regulators of gene expression changes and patterns associated with ageing. As stated, H4K16ac impedes chromatin condensation and, with that, promotes gene activation. Changes in H4K16ac levels during ageing could be shown in various organisms. In mice, a global increase in H4K16ac has been reported with ageing, which most likely contributes to the ageing phenotype by loosening chromatin structure and function. (Nativio et al., 2018; Oh and Petronis, 2021)

Ageing has detectable effects on histone methylation as well. H3K4me3 reduction at gene promoters has been observed in general in ageing mice. H3K4me3 is described as promoting gene expression. On the other hand, H3K9me3 and H3K27me3 levels, known for gene expression repression, seem to increase and suggest increased transcriptional repression. However, these effects seem very tissue and DNA context-specific and more research is needed for a clearer picture (McCauley and Dang, 2014).

As already mentioned, the Insulin/IGF-1 signalling pathway is a known regulator of lifespan, and lower activity of it is associated with increased longevity across several organisms. Notably, the SIRT protein family is controlled via the Insulin/IGF-1 signalling pathway and Sirtuins act as NAD^+-dependent deacetylases, including H4K16. Reduced Insulin/IGF-1 signalling pathway activity shifts the NAD^+/NADH ratio and thereby activates Sirtuins, which can, in turn, affect the chromatin architecture and gene expression (Dang et al., 2009).

1.2.3. Chromatin Remodelling

Post-translational modifications of histones and chromatin remodelling often work synergistically and represent interconnected aspects of epigenetic regulation, determining chromatin structure and function. Chromatin remodelling is a key component of DNA accessibility, thereby shaping gene regulation and cellular function.

Nucleosome occupancy and spacing

Nucleosome occupancy refers to the average number of nucleosomes within a specified genomic region. For instance, the mouse genome studied in this thesis contains several million nucleosomes, while the budding yeast genome has about 60,000 nucleosomes (Jiang and Pugh, 2009; Lai and Pugh, 2017). Nucleosome-free regions (NFRs) are typically found around active promoters, rendering the DNA accessible to various proteins and transcriptional machineries. The +1 and -1 nucleosomes define the downstream and upstream borders of an NFR, respectively, with subsequent nucleosomes sequentially numbered (Rando and Ahmad, 2007).

Nucleosome spacing, the distance between adjacent nucleosomes, is highly dynamic and influenced by many factors critical for transcription initiation by modulating RNA Pol II access and transcription factor binding potential and vice-versa. The intricate regulation of nucleosome placement and displacement or eviction of nucleosomes across the DNA is mainly performed by ATP-dependent chromatin remodelling complexes.

DNA-histone interactions are agitated via ATP-hydrolysis, enabling the sliding or removal of nucleosomes.
As mentioned above, histone modifications change the charge of histones, impacting their interaction with the negatively charged DNA and thereby influencing nucleosome positioning.
DNA methylation, conversely, can influence the preference for nucleosome formation, with higher methylation usually correlating with tighter nucleosome spacing (Wong et al., 2011; Black et al., 2012).
Histone variants, which differ in their structural and chemical properties, can also play a role by altering the stability of nucleosomes and, thus, their spacing. As an example, the histone 2 variant H2A.Z is known to facilitate RNA Pol II passing and transcription initiation (Weber et al., 2014)
Non-coding RNAs (ncRNAs) can mediate the recruitment of chromatin remodelling complexes to specific genomic sites, influencing local nucleosome positioning (Patty and Hainer, 2020). Additionally, the DNA sequence itself can impact nucleosome formation and positioning, given that specific sequences exhibit a higher or lower affinity for histone octamers (Travers et al., 2010).

Chromatin remodelling and its spatiotemporal structuring are vital for maintaining cellular identity and proper cellular function. Alterations in the chromatin architecture can lead to cancer, neurodevelopmental disorders and ageing syndromes (López-Otín et al., 2023).
Studies focusing on lifespan regulation in mice show that if certain subunits of the chromatin remodelling BAF complexes become dysfunctional, they can induce premature ageing phenotypes in mouse neural stem cells (Sokpor et al., 2017). This again corroborates the significance of chromatin remodelling during ageing and highlights that chromatin architecture may be a prime modulator for age-associated changes in the gene expression landscape.

1.2.4. Non-coding RNAs

Non-coding RNAs like microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs) significantly influence epigenetics and ageing. miRNAs such as *miR-29a* alter gene expression impacting protein levels and ageing-related phenotypes, including roles in Alzheimer's disease (AD) (Rusu-Nastase et al., 2022). lncRNAs, like *Neat1*, guide transcriptional machinery and interact with mRNAs and miRNAs, with evidence of their expression changes in ageing tissues (Marttila et al., 2020). circRNAs, such as *Cdr1as*, regulate genes through various mechanisms, and their misexpression can lead to age-related neurodegenerative disorders. NcRNAs' interaction with chromatin modifiers further emphasises their potential as health span and longevity intervention targets (D’Ambra et al., 2019).

1.2.5. Three-Dimensional Chromatin Organisation

The complex of DNA, chromatin and its associated proteins is not randomly distributed inside the nucleus like a plate of spaghetti Bolognese. Since spatial configuration impacts gene regulation significantly, it is intricately organised.

A key element of 3D chromatin organisation are chromatin loops. Looping allows to bring genomic regions into close spatial proximity and facilitates interactions between promoters and often distant regulatory elements like enhancers and silencers. Loops are not static and can dynamically form and fall apart. The guidance of these formations is regulated by the Cohesin complex that plays a role in loop extrusion and is bound by CTCF proteins (Evans et al., 2019).

The concept of topologically associating domains (TADs) introduces another layer of 3D chromatin organisation. They can range from hundreds of kilobases to a few megabases and describe genomic regions in which DNA sequences interact to a higher range than with regions outside (if at all). Often, they are seen as functional units of chromatin organisation wherein regulatory elements (e.g., enhancers) interact with their target genes. As mentioned above, they are bound by CTCF insulator proteins and Cohesin (Bastiaan Holwerda and de Laat, 2013).

Changes in chromatin loop dynamics, unregulated compartment switching and the disruption of TAD boundaries have been reported during ageing in various cell types. As these are substantial 3D chromatin alterations, they can affect gene expression changes and can contribute to an ageing phenotype (Sun et al., 2018; Evans et al., 2019).

1.3. The Ageing Liver

One of the primary metabolic organs of mammals is the liver. Its functions are pivotal for organisms, including energy metabolism, xenobiotic and endobiotic clearance, and synthesis and signalling of essential biomolecules like insulin and albumin. This versatility of tasks can only be facilitated with a specialised organ architecture and unique specialisation. The liver is structured into functional zones, but in practice, these units are not strictly segregated but rather form a functional continuum. Generally, the liver is organised in hepatic lobules. Each lobule is hexagonal, and a portal triad (portal vein, hepatic artery, bile duct) is located at each corner of the hexagon (Kalra and Tuma, 2018). Each lobule is subdivided into three further main zones: Pericentral, mid-lobular, and periportal zone. These liver zones, while morphologically similar, serve distinct functional roles. Their spatial arrangement corresponds to the axis of oxygen tension in the liver, distributing metabolic functions accordingly. The oxygen availability is highest in the periportal region, decreases towards the mid-lobular zone, and is lowest in the pericentral region. This oxygen gradient corresponds with variations on the protein and genetic level, facilitating the identification of signature genes for spatial source determination of liver cells. Notably, although hepatocytes constitute the majority of liver cells, non-parenchymal cell types also demonstrate a spatial pattern that aligns with their micro-environment (see also: Figure 3) (Cunningham and Porat-Shliom, 2021).

The periportal zone, also called zone one, is adjacent to the portal triad, consisting of the hepatic artery and the bile duct. As mentioned above, the periportal zone shows the highest oxygen tension and is primarily orchestrating processes that involve high levels of ATP, including gluconeogenesis and cholesterol synthesis (Katz and Jungermann, 1976). Key marker genes in mice for this zone are, among others, *Cyp2f2* and *Cps1,* which is involved in the urea cycle (Ben-Moshe et al., 2019).

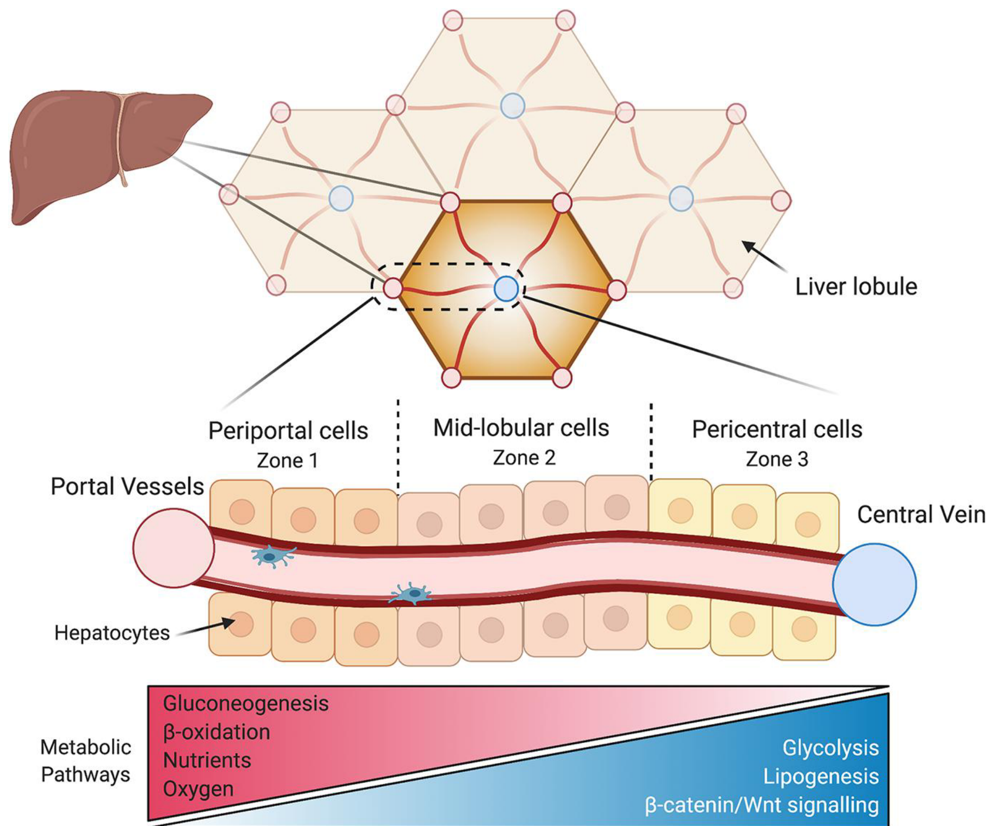

Figure 3: Anatomy of the liver and its fundamental unit, the hepatic lobule. The liver is built from hexagonal structures referred to as lobules. Blood, abundant in oxygen and nutrients, flows in a specific direction starting from the hepatic vessels (depicted in red) located at the lobule's corners, towards a central vein situated in the middle (illustrated in blue). Hepatocytes found near the portal area (zone 1) are at the lobule's exterior, followed by hepatocytes in the middle of the lobule (zone 2), and finally, hepatocytes adjacent to the central vein (zone 3) surround it. This diverse microenvironment along the axis from the periportal to the pericentral region results in distinct areas. Modified from Cunningham and Porat-Shliom (2021).

The mid-lobular zone, or zone two, is considered an intermediate between the periportal and pericentral zone, comprising features of both. Hepatocytes in the mid-lobular zone have been shown to adapt dynamically to the body's metabolic needs. One described marker for the mid-lobular zone is Hepcidin Antimicrobial Peptide (Hamp) and Hepcidin Antimicrobial Peptide two (Hamp2), which regulate hepatic iron homeostasis in mice (Cunningham and Porat-Shliom, 2021).

The pericentral zone, or zone three, is around the central vein and characteristically has a low oxygen tension. Therefore, as expected, primarily anaerobic processes are localised in this area, including glycolysis (Matsumura and Thurman, 1984), lipogenesis (Hijmans et al., 2014), glutamine synthesis (Gebhardt et al., 2007) and bile synthesis (Cunningham and Porat-Shliom, 2021).
Similarly, cytochrome P450 enzymes like *Cyp2e1* and *Cyp1a2* are described as marker genes for the pericentral region in mice. They are involved in a variety of mono-oxygenation and hydroxylation of many endogenous and exogenous compounds like steroids, prostaglandins, drugs and alcohol (Oinonen and Lindros, 1998).

It should be noted that mRNA and protein levels measured in the different liver zones also show conflicting expression between protein and mRNA levels, hinting towards additional regulation via post translational modification (Ben-Moshe et al., 2019).

The unravelling of the spatial zonation and their genetic and epigenetic organisation has significantly advanced the understanding of metabolism and general liver function. This will not only help to understand liver pathophysiology in liver diseases but also liver ageing.

As the liver is orchestrating a plethora of energy metabolism processes like glucose and lipid homeostasis, balancing of steroid levels, and insulin signalling, all are connected to the hallmarks of ageing, it is quite obvious that alterations in the liver during ageing have implications on an organism-wide level.

The structural ageing phenotype of the liver is described with a gradual decline in size from decreased hepatic cell mass, leading to reduced liver volume. This is accompanied by vascular modifications, including a decreased number of fenestrations in the endothelial lining and thickening of the vascular walls, leading to decreased hepatic blood flow and impeded exchange of molecules between the liver parenchyma and the blood. Simultaneously, irregular hepatic lobule contours start to extent, as well as fibrotic septa (Ayala-Peña and Torres-Ramos, 2014).

At the cellular level, hepatocytes, the principal cell type of the liver, progressively undergo senescence. This growth arrest, in consequence, leads to hepatocytes adopting a senescence-associated secretory phenotype resulting, as discussed in Chapter 1, in a release of a myriad of factors like pro-inflammatory cytokines, chemokines and proteases. This pro-inflammatory environment, together with an increasing number of senescent cells, perturbates the changes in the liver architecture and the decline in liver function (Radonjić et al., 2022). Hepatocyte polyploidy is also increasing during ageing, but if this mechanism is compensatory or detrimental to liver function is not fully understood (Matsumoto et al., 2021)

These declines are also having notable compromising effects on the regenerative capacity of the liver, which is a defining characteristic of it. This means that the aged liver becomes more vulnerable to injuries and has a reduced capability to recover from insults such as surgical procedures, toxic metabolites and environmental toxins or viral infections. This happens in combination with a decline in the capacity to facilitate detoxification and metabolic processes (Guicciardi et al., 2013).

Clinically liver ageing is a significant factor. With age, the probability of several age-related diseases is increasing. This includes non-alcoholic fatty liver disease, hepatocellular carcinoma, and liver cirrhosis. Lastly, it is also reported that aged livers show a notable decline in drug metabolism with a reduced drug clearance that elevates the risks of adverse drug reactions (Anantharaju et al., 2002).

Energy metabolism in the ageing liver

The liver performs an extensive variety of indispensable roles in maintaining the body's energy metabolism. Among others are the regulation of blood glucose levels, lipogenesis, beta-oxidation, processing of dietary nutrients, and coordination of the responses to feeding and fasting. Therefore, alterations in the livers' metabolomic capacity have profound impacts on a systemic level (Anantharaju et al., 2002).

The livers' glucose metabolism is central to the homeostasis of blood glucose metabolism. Gluconeogenesis, the process of converting non-carbohydrate precursors like pyruvate and others into glucose, becomes compromised during ageing. To mention specifically here is the reduced activity of key enzymes in gluconeogenesis, including phosphoenolpyruvate carboxykinase and glucose-6-phosphatase that is hampering the livers' capability to produce glucose in fasting states (Wimonwatwatee et al., 1994). In tandem goes a decline in the capability to synthesize hepatic glycogen and its subsequent storage, mainly due to the reduced activity of glycogen synthetase. Consequently, glycogenolysis, as the primary source of glucose, is increased during fasting events. This leads to the depletion of the hepatic glycogen storage in order to maintain glucose levels, thereby impeding the ability to maintain euglycemia during prolonged fasting events (Khandelwal et al., 1984).

Lipid metabolism in the ageing liver

A substantial alteration in lipid metabolism is also observed during the age-related decline in liver function. The liver has an outstanding role in lipid metabolism and is the main actor in lipid synthesis, degradation, and lipoprotein assembly and secretion.
The *de novo* synthesis of fatty acids (lipogenesis) is particularly affected by ageing via a decline in enzymatic activity but also through regulatory mechanisms (Gong et al., 2017).

Lipogenesis is facilitated by several key enzymes that transform carbohydrates into their storage form fatty acids. The ATP citrate lyase first converts citrate into acetyl-CoA. Acetyl-CoA is subsequently carboxylated by the acetyl-CoA carboxylase to form malonyl-CoA, which is after that covalently bond to two further carbon units by the fatty acid synthase to form the 16-carbon saturated fatty acid palmitate which then is further modified to form a variety of classes of other fatty acids.

A significant change in liver fatty acid composition can be detected during the ageing of mice. This is covered in part one of this thesis, where the concepts, methods, and results are detailed. In short, an increase in intrahepatic triacylglycerides, several diacylglycerides, and cardiolipins could be found. In contrast, phosphatidylcholines and sphingomyelins were decreased, indicating an extensive remodelling of liver lipogenesis during ageing.
This might be connected to recent studies indicating a change also in the activity of other factors like liver X receptors, carbohydrate-responsive element-binding protein and the insulin-mediated phosphatidylinositol 3-kinase/Akt pathway (Huang et al., 2018).

On the opposite of maintaining lipid homeostasis in the liver, there is hepatic lipid degradation via beta-oxidation. During beta-oxidation excess fatty acids are converted into energy. During ageing, the capacity of the liver to oxidise fatty acids is altered, like lipogenesis.
The Peroxisome proliferator-activated receptor alpha regulates numerous genes involved in fatty acid transport and beta-oxidation. Its reduced expression has been correlated with the reduced expression of its target genes in aged mouse livers, thereby increasing oxidative stress (Erol, 2007).
Furthermore, Rev-erba, a transcriptional repressor involved in upregulating fatty acid oxidation and down-regulating lipogenesis, has also been reported to show reduced expression during ageing. Hence, the observed imbalances in lipid homeostasis are likely caused by a complex web of malfunctioning genes and proteins during ageing (Griffett et al., 2020).

Overall, the characterisation of alterations in liver homeostasis is a crucial point of interest for understanding the factors involved during liver ageing. This thesis aims to further elucidate the effects on the epigenetic, transcriptional, and intercellular levels to provide new insights into the pathogenesis of age-related metabolic disorders in the ageing liver

Epigenetic changes in the ageing liver

So far, it is known that the liver epigenome undergoes profound changes during ageing in almost all aspects described before. DNA methylation patterns, histone modifications and non-coding RNA profiles contribute to the age-related liver ageing phenotype. Changes have been reported for hepatocytes, Kupffer cells, Hepatic stellate cells and other cell populations within the liver. This further corroborates the complexity of liver ageing.

Hepatocytes, facilitating the majority of metabolic tasks, show a broad spectrum of epigenetic changes during ageing. DNA methylation and hypermethylation events have been observed around promoter regions of key genes like Elongation of very long chain fatty acids protein (*Elovl*) involved in lipogenesis and PPARA, as mentioned, involved in beta-oxidation regulation. Additionally, histone marks, including H3K4me3 and H3K36me3, show a decline. These marks are known for transcription activation and indicate a possible decrease in transcriptional activity or transcription initialisation (Wang et al., 2022). Also, in the branch of non-coding RNAs are observable for hepatocyte-specific miRNAs, such as *miR-122*, recorded to be of critical importance in lipid and glucose metabolism (Willeit et al., 2017). All other liver cell types also undergo various epigenetic alterations, but these are out of the scope of this thesis and shall not be discussed here.
This shows that cell-specific epigenetic changes shape not only the ageing-liver phenotype but also the chromatin landscape of the liver in general. A comprehensive understanding of these strongly interconnected processes demands further exploration. They hold the potential for a better understanding of liver ageing and new intervention targets.

1.4. Understanding Liver Ageing: Harnessing the Power of (Single-Cell) Omics-Methods

The advent of omics-methodologies – epigenomics, genomics, transcriptomics, proteomics, and metabolomics, among others - has revolutionized biological research by allowing comprehensive examination of biological systems. In the field of ageing research, especially concerning liver ageing, these approaches hold immense promise. Applying omics-technologies to liver ageing research permits a deep and nuanced understanding of these complex changes at multiple biological layers. Epigenomics, which explores changes in gene activity regulation that don't involve alterations to the genetic code itself, is of particular interest in ageing research. It is now widely acknowledged that ageing correlates with significant alterations in the epigenome. The highly dynamic nature of the epigenome enables it to respond to a multitude of environmental signals, making it a critical regulator of age-related changes (Sen et al., 2016)

High-throughput omics-technologies allow simultaneous assessment of these multiple dimensions, uncovering the interactions and regulatory feedback loops that exist between them. Cross-referencing these diverse data types can yield significant insights. For instance, integrating epigenomic data with transcriptomic or proteomic data can help to delineate how changes in the epigenetic landscape influence gene expression and protein abundance during liver ageing (Figure 4) (Lorusso et al., 2018).

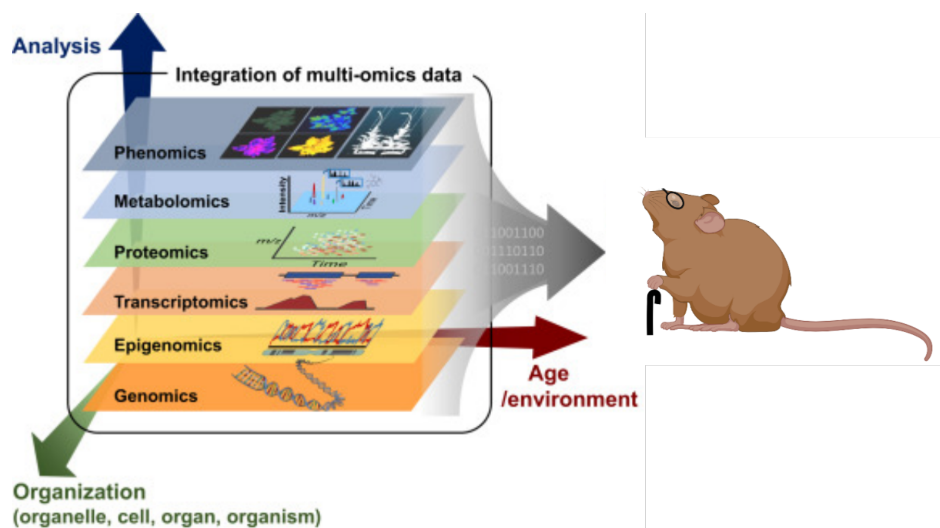

Figure 4: Understanding ageing and liver Ageing through a multidimensional lens: Ageing and liver ageing are complex processes. The emergence of omics-technologies empowers us to examine these processes from a multifaceted perspective. Each layer of omics data corresponds to a vast set of parameters specific to that layer, enabling the acquisition of high-resolution insights into various biological states and abstraction levels. These insights can then be integrated to decode the multi-layered mechanisms of ageing, with specific emphasis on liver ageing in this thesis. The 'Age/environment' axis encompasses both internal factors (such as age) and external factors (including chemical, physical, and biological agents, along with overall environmental aspects). The organization axis refers to various levels of biological structure, spanning from organisms to organs, cells, and organelles. The analysis axis corresponds to a range of high-throughput

omics-technologies and their integrative applications across diverse layers including genomics, epigenomics, transcriptomics, proteomics, metabolomics, and phenomics. Modified from Kim et al., (2016) Made with BioRender.com.

Moreover, integrated analysis can guide the unveiling of the causal relationships and the sequence of events during the ageing process. It can determine whether a specific epigenetic modification results in altered gene expression or whether it is a downstream consequence of other molecular changes. Therefore, integrative analyses are invaluable in pinpointing critical ageing drivers and potential therapeutic targets.

However, the integration of multiple omics data types poses significant challenges, including the need for robust statistical methods, appropriate computational tools, and strategies to deal with the high dimensionality of omics data. These hurdles, while substantial, are not insurmountable, and advancements in bioinformatics are continually providing improved methods for multi-omics data integration.
In conclusion, the application of omics methods, especially with an epigenomic perspective, to liver ageing research presents an expansive view of the ageing process. By cross-referencing the results of these methods, researchers can gain a more profound and holistic understanding of ageing, opening the door to novel interventions for age-related liver disorders (Singh and Benayoun, 2023).

1.4.1. scATAC-seq

Since the advent of Transposase-Accessible Chromatin sequencing (ATAC-seq) in the Greenleaf lab at Stanford in 2013, the method had a triumphal procession into epigenetic labs worldwide (Buenrostro et al., 2013). Since then, the number of studies and data published superseded the number of studies published with comparable methods for detecting chromatin accessibility like DNase I hypersensitive sites sequencing, Formaldehyde-Assisted Isolation of Regulatory Elements sequencing, micrococcal nuclease digestion with deep sequencing and others by far (Yan et al., 2020). This corroborates its value in answering biological questions and the lower amounts of input material needed than for other methods, and less time-consuming sample preparation times allow for a broader application (Buenrostro et al., 2013). The advances in single-cell technologies as well as in microfluidics, allow today to profile the chromatin accessibility for up to millions of cells via microfluidic and nano-well-based approaches (Buenrostro et al., 2015; Cusanovich et al., 2015; Mezger et al., 2018). The details for the library preparation can be found in chapter 5.1.

Bioinformatic Analysis of Single-Cell Assay for Transposase-Accessible Chromatin Sequencing

This project is based on the 10x Genomics single-cell ecosystem. After preliminary analysis of the data in Part one, we observed that biological findings can depend on the choice of data processing method – which made us question the reproducibility of the results. Therefore, two different analysis approaches were performed to ensure that the obtained results offer the best biological interpretability and statistical robustness. Approach one is based on the CisTopic R package (Bravo González-Blas et al., 2019) and

approach two on the Signac R package (Stuart et al., 2021). The general concepts are introduced here, and the mathematical details of these two approaches are discussed in Chapter 1.5.1. and Chapter 1.5.2.

Both methods follow the approach to model the scATAC-seq data as commonly applied in natural language processing. CisTopic applies a method known as Latent Dirichlet Allocation (LDA) for dimensionality reduction and downstream analysis. It is a generative probabilistic model originally designed to identify “topics” in a corpus of documents.
In the scATAC-seq data, individual cells represent a document and the “words” are the genomic regions which were detected as accessible in a cell. The inference tries to identify a set of “topics” represented by distinct genomic regions that can, among others, define a transcription factor binding site, an enhancer region or a cis-regulatory element which are co-accessible across a subset of cells (Bravo González-Blas et al., 2019; Stuart et al., 2021).

LDA offers some potential advantages over other approaches: It does not necessarily require peak calling or binarization, hence retains the complete quantitative structure of the data. Additionally, it is designed explicitly for data sparsity, as it is very common for scATAC-seq data. Finally, the topic probability distributions are a very easy-to-interpret way to identify cells with regions of co-accessibility and common regulatory elements. All usual downstream processing, including non-linear dimensionality reduction, clustering, the computation of gene activity scores, differential accessibility analysis, transcription factor motif analysis of the topic-defining regions, and integration with single-cell RNA Sequencing (scRNA-seq) data, can be carried out based on the cell to topic probability matrix.

Seurat is a single-cell analysis tool for various single cell technologies and applications that also has a specialised analysis pipeline for scATAC-seq data called Signac (Hao et al., 2021; Stuart et al., 2021).
The standard Seurat pipeline for transcriptomics and epigenomics analysis can be divided into the following steps: Initial quality control, assessment of highly variable features, scaling and normalisation and dimensionality reduction, and differential expression/accessibility testing.

For scATAC-seq data, the analysis pipeline is tailored and adjusted for the peculiarities of ATAC data, as discussed before. Input is here the via 10x Genomics chromium analysis pipeline software generated fragment file that contains the peak coordinates and the polymerase chain reaction (PCR) duplicate count for the cells detected in the dataset.
Initial quality control is the starting point of the Signac analysis. In contrast to normalisation, scaling and Principal component Analysis (PCA), Latent semantic indexing (LSI) (Deerwester et al., 1990) and truncated single value decomposition are used for dimensionality reduction (Stuart et al., 2021).

The LSI approach might come with some limitations as it is based on the peak-level analysis. This might allow a detailed exploration of chromatin accessibility as well but may

be more susceptible to data sparsity. It is even noted by the authors that usually, the first dimension of the LSI is strongly correlated with the sequencing depth of the cells and should be discarded. This makes the choice of parameters for downstream processing more tedious and needs thorough testing for every dataset, requiring a high level of user-based parameter choices that could potentially skew the results of the analysis and might make it less reproducible for others (Stuart et al., 2021).

Taken together, both approaches facilitate scATAC-seq data analysis. They follow different strategies coming with their unique focuses and strengths. The downstream processing of both approaches remains the same, whereas the initial dimensionality reduction approach is very different. It should be noted that both methods allow for the integration of the dimensionality reduction results in one of the other pipelines allowing for easy comparison between the two approaches. The choice between one method or the other relies on the level of user expertise, the expected complexity of the dataset and the specific research question. However, for very sparse datasets, the LDA will likely prove superior for exploratory analysis, while for more hypothesis-driven investigations, Signac might present a higher value.

In conclusion, scATAC-seq is a technology that most likely revolutionised the field of studying chromatin dynamics of singular cells. A variety of bioinformatic approaches exist to unravel these dynamics, each coming with its unique strengths and limitations. Despite their differences, both methods allow for highly valuable contributions to the knowledge in the field. Nonetheless, the field of bioinformatic methods to analyse scATAC-seq data is far from being saturated and further improvements, benchmarkings, and novel tools will be needed and should be the subject of ongoing research in order to unravel the intricacies of chromatin dynamics and accessibility.

Employing scATAC-seq as part of the multi-omics analysis, we investigated age-dependent, zonated alterations in chromatin accessibility in murine liver hepatocytes. We correlated them with spatial transcriptomics data and could associate specific chromatin alterations with metabolic and transcriptional regulation changes. Details can be found in part one of this thesis.

1.4.2. CUT & RUN Sequencing

Protein-DNA interactions, studied through numerous localization and genome-wide analysis methods like Electrophoretic Mobility Shift Assay, Yeast One Hybrid, and Chromatin Immunoprecipitation Sequencing (ChIP-seq), hold significant research value (Dey et al., 2012; Ferraz et al., 2021). However, ChIP-seq has inherent limitations, including its requirement for large amounts of input material, making it challenging for certain animal tissues or organs, and its reliance on formaldehyde crosslinking which may introduce false positives due to non-specific or indirect DNA-protein interactions (Teytelman et al., 2013; Meers et al., 2019)
cleavage under targets and release using nuclease sequencing (CUT&RUN-seq), proposed by Skene and Henikoff (2017), offers an alternative, circumventing these issues by in-situ generation of DNA-protein complexes. Its key component is a micrococcal nuclease (MNase) tailored to target specific interactions. The process comprises four stages: immobilizing unfixed nuclei, adding specific antibodies with protein A-MNase fusion protein, activating MNase via calcium to facilitate DNA cutting, and retrieving DNA-protein complexes through cell lysis and centrifugation. The purified DNA fragments are prepared for next-generation sequencing. Fragmented spike-in DNA is added to each sample to enable quantitative comparison.

The bioinformatics analysis involves quality control, adapter trimming, PCR duplicate removal, read sorting and indexing, alignment to the reference genome, normalization, and peak calling, particularly using SEACR (Meers et al., 2019). The called peaks contribute to downstream analysis, visualization, motif discovery, and functional annotation.

CUT&RUN-seq, a cost-effective, low-input method with higher precision than ChIP-seq, is crucial for chromatin research and ageing context, aiding our understanding of age-associated chromatin and transcriptional landscape changes.

In part two, we demonstrated that CUT&RUN-seq is an invaluable tool for understanding age-regulated changes during transcriptional regulation. It could be shown that during ageing, the liver exhibits an increase in the accessibility at promoter regions, but this does not correspond to an increase in transcriptional output. Further, we could show that the promoter proximal pausing of RNA polymerase II (Pol II) decreases during ageing and cannot be attributed to reduced transcription initiation. This important finding was only possible due to the precision of CUT&RUN-seq.

1.4.3. Spatial transcriptomics

Spatial sequencing, the 2020 method of the year, has revolutionised genomics by adding a spatial dimension to RNA expression studies. It illuminates the microenvironment and spatial organisation within a tissue, providing an unparalleled view of the transcriptional landscape and enhancing tissue understanding (Marx, 2021)

In contrast to RNA-sequencing techniques like bulk-seq and scRNA-seq that eliminate cellular positional context, spatial transcriptomics preserves the original mRNA location within tissue sections, allowing multi-layered gene expression analysis.

Spatial transcriptomics broadly falls into three categories: in situ hybridisation (e.g., Multiplexed error-robust fluorescence *in situ* hybridisation (MERFISH), Sequential fluorescence *in situ* Hybridisation (SeqFISH)), in situ sequencing (e.g., Fluorescent in situ sequencing (FISSEQ), Spatially-resolved transcript amplicon readout mapping (STAR-map)), and in situ capturing. The latter captures mRNA molecules for next-generation sequencing using positional barcodes, reverse transcription primers, and poly-T mRNA capturing sequences (Lee et al., 2015; Wang et al., 2018; Eng et al., 2019; Xia et al., 2019; Piñeiro et al., 2022).

The spatial gene expression platform Visium from 10x Genomics uses the library preparation method similar to scRNA-seq but with modifications for spatial context preservation. Tissue sections are placed on a designed glass slide with capture spots carrying unique barcodes and oligo(dT) sequences. The mRNA bound to these sequences is reverse transcribed to cDNA, allowing origin identification and gene expression quantification.

Despite the capturing of transcriptional patterns, the technology is limited by the need for high-quality tissue samples and its inability to capture mRNAs in single-cell resolution. Yet, in ageing research, it holds great promise for discovering location-specific ageing signatures and new biomarkers, as shown by a recent study on spatiotemporal mapping of the ageing mouse brain (Hahn et al., 2022)

Bioinformatic analysis of spatial sequencing data

To harness the full potential of the multidimensional data generated with spatial sequencing, statistically robust and reliable bioinformatic tools are indispensable. The R package Seurat, originally developed for scRNA-seq, offers a comprehensive toolbox that enables advanced analysis and interpretation of spatial sequencing data (Hao et al., 2021).

The Seurat pipeline includes data pre-processing, quality assessment, dimensionality reduction, spot clustering and detection of spatially variable genes. All these are the key steps for deciphering the complex multidimensional gene expression data.
A standard workflow for spatial data analysis starts with the pre-processing. Raw gene expression matrices can be imported, assessed for quality, and technical variability can be addressed via normalisation.

The normalised genes × spots matrix serves as a basis for all further analysis steps. As this matrix still contains unwanted noise and is high-dimensional, dimensionality reduction is strongly advised. Commonly this is done with principal component analysis (For more details, see chapter 1.5.1 Principal Component Analysis)
As spatial sequencing data can also be interpreted as scRNA-seq like data, it is also possible to apply non-linear dimensionality reduction methods like t-Distributed Stochastic Neighbour Embedding or Uniform Manifold Approximation and Projection for 2D visualisation neglecting the spatial context of the data. This step allows to visualise structures and patterns in the data.
As common in scRNA-seq clustering, methods like K-nearest neighbour clustering are often used to group the spots based on the linear dimensionality reduction. Visualising the clusterings on the image of the tissue slide can help to identify biological structures and cell populations represented by the clusters. The other way around, clusters in histologically interesting areas can be tracked back into the data and can be used to further characterise these areas on a genetic level (For example, the central area of a cancer sample or the periportal and pericentral zones of liver tissue). Commonly at the end of the pipeline, differential gene expression testing is performed between the clusters, followed by a biological annotation of the significant genes via, for example, GO-Term analysis. However, there are several caveats to this procedure. Firstly, the unsupervised clustering of the spots can be severely biased by the user. The number of principal components and the parameters used for the clustering can highly influence the cluster assignment and, thereby, the result of the differential gene testing. Secondly, in contrast to scRNA-seq, the spots from spatial sequencing do not represent individual cells but are instead a mixture of the expression of cells in the specific area of the spot. It has to be thoroughly evaluated if all the mathematical assumptions for the test of choice still hold. Lastly, as the clustering and differential gene testing completely ignore the spatial context of the spots, it is very likely that this approach overlooks spatial trends and patterns in the data and thereby, important differences in the tissues' gene expressional landscape are non-detectable or overlooked.

Genes varying in a spatially dependent manner are often called spatially variable genes (SVG) and typically show significant variation across different spatial locations. In contrast to differentially expressed genes, they sometimes do not show a big expression difference between user-defined clusters but rather a gradient-like distribution across a specific tissue area and are, therefore, harder to detect with traditional methods.
Since the advent of spatial-omics methods, have been proposed to address this problem specifically. Among these are spatialDE (Svensson et al., 2018), SPARKX (Zhu et al., 2021) and Trendsceek (Edsgärd et al., 2018).

SPARKX (spatial pattern recognition via kernels) is an R package designed specifically for identifying spatially variable genes. SPARKX models spatial gene expression patterns through a generalised linear mixed model that models non-Gaussian spatial data. The random effect in the model is the underlying stationary process. The observed expression is modelled by a Negative Binomial distribution. The model accounts for spatial autocorrelation and technical noise via a zero-mean stationary Gaussian process. Several

kernels are available to approximate the true gene expression pattern as best as possible via a Bayesian approach based on a penalised quasi-likelihood algorithm. SPARKX is proposed to work for all three types of spatial transcriptomics categories (Sun et al., 2020; Zhu et al., 2021).
Trendsceek identifies spatially variable genes via a marked point process. It ranks genes with dependencies between the spatial distribution of the cells and the gene expression in those cells. The approach is non-parametric and is suited to identify non-linear expression patterns. P-values for each gene are calculated for each gene by comparing the observed data to a random permutation-generated null distribution (Edsgärd et al., 2018).
Like SPARKX, SpatialDE uses a Gaussian process model to identify spatially variable genes. The expression of a gene across all cells is decomposed into spatial and non-spatial variance effect terms that are then tested against a model without the spatial variance component (Svensson et al., 2018).

The choice of a specific method depends on the research question and the nature of the dataset, as every method has its strengths and limitations. New methods are published frequently, but so far, no gold standard approach has emerged. In part three of this thesis, a novel approach for the detection and identification of spatially variable genes is displayed. Spatial Components (SPACO) is designed for 10x Genomics Visium spatial data and outperforms existing methods in terms of SVG detection. Further, it proposes a flexible framework for data denoising and feature selection.

Further, in part one, we leverage the potential of spatial transcriptomics to elucidate diverse ageing trajectories within liver tissue. This vital tool uncovered the zonation of age-related alterations, providing a comprehensive view of spatial and molecular shifts that occur during ageing.
Spatial transcriptomics illuminates the previously undisclosed zonation of lipid deposition in aged livers, associating this phenomenon with DNA accessibility and gene expression changes in *Cidea* and *Cidec*. These genes, integral to lipid droplet dynamics, were upregulated specifically in old pericentral hepatocytes, providing potential insights into the distinct lipid accumulation patterns seen in aged liver tissue.

In conclusion, spatial transcriptomics proves indispensable in this study, driving vital insights into the spatially resolved molecular alterations during liver ageing. By linking zonation of lipid deposition to specific gene expressions, it provides a robust foundation for future ageing research

1.5. Dimensionality Reduction Methods

1.5.1. Latent Dirichlet Allocation

Latent Dirichlet allocation is originally a method developed for natural language processing that aims to explain a set of observations through unobserved groups. On the other hand, each group explains why some parts of the data are similar. It is a topic model that uses words in a corpus of documents. Each word in a document is attributable to a topic in a document, and every document contains a small number of topics individually. On a mathematical level, the core assumption for the LDA is that the observed accessibility across cells is based on a mixture of underlying "topics", which can be a cell type, a metabolic state or virtually any kind of state causing co-occurring DNA accessibility. The "topics" are assumed to be a probability distribution over all genomic regions, and every cell is represented as a probability distribution over these topics. Biologically, that means that several processes may influence accessibility in a particular region: the ones with higher probability are more likely to do so, while the processes with lower or zero probability are unlikely to cause a change in accessibility in that region. Let us assume the following notation: If Z is the topic assignments, Θ_i the topic proportions for each cell ranging from 1 to K and φ_k the chromatin region distribution for every topic ranging from 1 to K. The generative process for the LDA takes the following form (For plate notation, see also Figure 5):

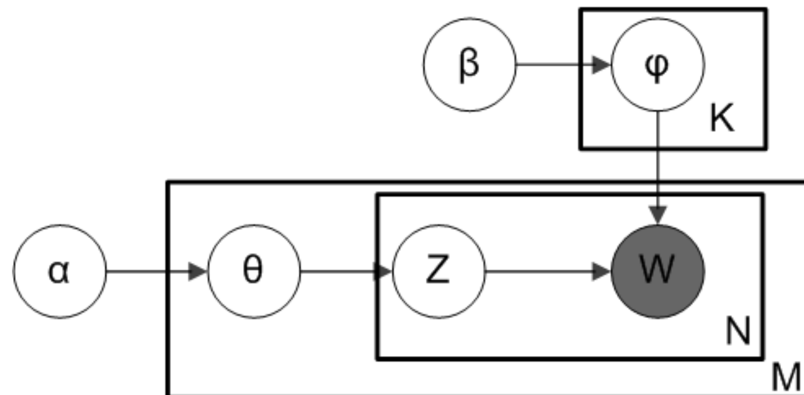

Figure 5: Latent Dirichlet Allocation plate notation with Dirichlet-distributed topic-word distributions. Figure obtained from Wikimedia commons (Commons Wikimedia, 2009). *Explained below.*

- M denotes the number of documents
- N is number of words in a given document (document i has N_i words)
- V number of words in the vocabular
- K number of topics
- Z identity of topic of word w in document d
- W topic origin of all words in all documents (non observable)
- α is the parameter of the Dirichlet prior on the per-document topic distributions (<<1)
- β is the parameter of the Dirichlet prior on the per-topic word distribution (<<1)

- Θ_i is the topic distribution for document i
- φ_k is the word distribution for topic k
- z_{ij} is the topic for the j-th word in document i
- w_{ij} is the specific word.

The probability distribution over the topic-word distribution is intuitively sparse as it is most likely skewed towards only a few words occurring with high probability in a specific topic. Therefore, a sparse Dirichlet prior can be used to model the topic-word distribution. In plate notation in figure 5 K is the number of topics, which in cisTopic are determined by the maximum of the likelihood function for the user defined number of topics 1 to K. $\varphi_1 \ldots. \varphi_k$ are V-dimensional vectors for the number of words V with the Dirichlet distributed topic-word distributions (Deerwester et al., 1990; Bravo González-Blas et al., 2019).

For the actual inference it is assumed that the generative process for every document is as described in the following:

- $\Theta_i \sim Dirichlet(\alpha)$, where$i \in 1, \ldots. M$. The α parameter is usually small (<<1). For cisTopic organism specific parameters are suggested.
- $\varphi_k \sim Dirichlet(\beta)$, where$k \in 1, \ldots. K$. The β parameter is usually small (<<1). For cisTopic organism specific parameters are suggested.
- For each word positions i, j, where $i \in 1, \ldots. M$ and $j \in 1, \ldots. N_i$.
    - Choose a topic $z_{i,j} \sim Categorical(\Theta_i)$
    - Choose a word $w_{ij} \sim Categorical\left(\varphi_{z_{i,j}}\right)$

Inferential algorithms such as variational Bayes or Gibbs sampling can be used to estimate the latent variables from the observed data. CisTopic uses a collapsed Gibbs sample for variable inference. Initial parameter settings are user defined. However, the authors give organism specific recommendations (Bravo González-Blas et al., 2019).

1.5.2. Latent Semantic Indexing and Single Value Decomposition

Latent semantic indexing was originally, like LDA, developed for Natural Language Processing. Again, comparable to LDA for LSI, “documents” represent the individual cells and “terms” to genomic regions (peaks). The entirety of the whole dataset is considered the “corpus”.

LSI is a two-step procedure. First, a term frequency-inverse document frequency (TF-IDF) matrix is computed and can be seen as an analogue to data scaling and normalisation. The Term frequency in Signac is defined as follows:
$TF = C_{ij}/F_j$, where $C_{ij}$is the total number of counts for peak i in cell j and F_j is the total number of counts for cell j.

The inverse document frequency is defined as:
$IDF = \frac{N}{n_i}$, where N is the total number of cells in the dataset and n_i is the total number of counts for peak i across all cells (Stuart et al., 2021).

The final TF-IDF matrix is then computed with a modification adjusted to work for scATAC-seq data and not text documents:

$$\mathrm{TF} - \mathrm{IDF} = \log(1 + (\mathrm{TF} \times \mathrm{IDF}) \times 10^4).$$

According to the authors, the TF-IDF allows the down-weighting of highly accessible loci that are commonly shared across cells (high TF, low IDF) and the up-weighting of loci that are distinctively accessible (low TF, high IDF). The resulting matrix of weighted genomic regions in each cell favours cell-specific chromatin accessibility, as compared to a less informative accessibility landscapes shared among multiple cells (Stuart et al., 2021).

It is commonly assumed that most of the signal (variability in the data) can be explained by only a few (latent/ unknown) variables. Hence, it is further necessary to reduce the dimensionality of the data to capture latent variations. This is done by singular value decomposition (SVD) of the TF-IDF matrix. The singular value decomposition (Martin and Porter, 2012) is represented as follows:

Given a $m \times n$ matrix A, the SVD is a factorisation of $A = U\Sigma V^T$
Where:

- U is an $m \times m$ real or complex unitary matrix
- Σ is an $m \times n$ rectangular diagonal matrix with non-negative real numbers on the diagonal and are known as the singular values of A. The number of non-zero singular values is equal to the rank of M.
- V is an $n \times n$ real or complex unitary matrix.
- V^T (the conjugate transpose of V, or simply the transpose if V is real) is an $n \times n$ real or complex unitary matrix.

For any real-valued matrix A, there exists an SVD with the orthogonal matrices U and V.

Geometric Interpretation
Geometrically, the SVD can be understood as three linear transformations performed sequentially on the space of the original matrix A.

- The first transformation is a rotation applied in the input space (n-dimensional). This is achieved by the matrix V^T. The result aligns the basis vectors with the principal axes of the original space. Or in other terms, the axes of the basis vectors are rotated to line up with the direction that captures the most variability in the data, akin to principal component analysis, which is discussed in the next chapter.

- Second, a scaling transformation is applied by the diagonal matrix Σ. By the transformation, each basis vector is scaled by the corresponding singular value of Σ. The singular value can be seen as the magnitude of stretching along each axis.
- Third and final is another rotation in the m-dimensional space defined by U. Every data point is now projected on coordinates in this rotated space that corresponds to the left singular vectors (Raghavendar et al., 2017).

The diagonal elements of Σ are the singular values of A. They can be used as a summary of the structure for dimensionality reduction (for instance, by truncating A to a subset of right and left singular vectors with non-zero singular values etc.). The dimension-reduced cells × singular values matrix puts cells in "semantic" space, where the axes are related to the patterns of chromatin accessibility that differentiate cells from each other.

For scATAC-seq data, a truncated SVD is applied as often the full SVD is impractical to compute as the number r of the non-zero singular values can be large (Stuart et al., 2021). The smallest singular values are therefore truncated to compute only r non-singular values. It should be noted that the truncated SVD is no longer an exact decomposition of the A matrix but rather a low-rank approximation $\tilde{A}$ by the matrix of rank t. The truncated SVD then takes the following form:

$$\tilde{A} = U_t \Sigma_t V_t^T,$$

Where:

- U_t is a $m \times t$ matrix
- Σ_t is $t \times t$ diagonal
- V_t^T is $t \times n$

Hence only the t column vectors of U and t row vectors V^T corresponding to the largest singular values of $\Sigma_t$are calculated. The whole operation can be solved in less time than computing the full SVD (Chicco and Masseroli, 2015).

1.5.1 Principal Component Analysis

Introduced by Karl Pearson in 1901, Principal component Analysis has most likely become one of the most common dimensionality reduction approaches in computational biology (Pearson, 1901). Its main purposes are to extract meaningful information from high-dimensional datasets and data visualisation. PCA transforms the original set of possibly correlated variables into a new set of uncorrelated variables. These new variables are called principal components (PCs). These are derived in a decreasing order of variance.

Mathematically, PCA can be divided into several key steps. Considering a dataset with n samples and p samples represented by an $n \times p$ matrix X. With each column centred to have mean of zero.

Covariance matrix calculation
The initial step is to calculate the covariance matrix of x. It is a square matrix c that is a square $p \times p$ matrix where the element in the i-th row and j-th column is the covariance between the i-th and j-th variables of X. The covariance matrix for the centred data $X = (x_1 .... x_n)$ Is defined as

$$C = \left(\frac{1}{n-1}\right) \cdot X^T X$$

Eigendecomposition
The next step is to compute the eigenvalues and eigenvectors of the covariance matrix. An eigenvector v of C and its corresponding eigenvalue λ are defined by the following equation:

$$Cv = \lambda v$$

When sorted from the largest eigenvalue λ to the lowest, the corresponding eigenvectors represent the orthogonal directions in the feature space in which the data shows maximum variance. The eigenvalues represent the amount of variance in that direction (Pearson, 1901; Ma and Dai, 2011).

Projection
Usually during the last step, the original data is projected onto the eigenvectors to obtain the principal components. The i-th coordinate in the principal component space of a data point x in X can be calculated as the dot product between x and the i-th eigenvector.

Geometrically, PCA can be seen as a rotation and translation of the coordinate system of the data. The principal components represent new axes and are oriented into the directions where the data spreads the most, meaning where the variance of the data is highest in decreasing order. Meaning, the first PC corresponds to the direction with the highest variance, the second corresponds to the direction with the second highest variance orthogonal to the first and so on.

Principal component analysis and singular value decomposition
From a theoretical perspective, PCA and SVD are closely related. In short, PCA for a (centred) data matrix X $c$an be performed by calculating the SVD of the corresponding covariance matrix $\sigma = X^T X$.

As described in Chapter 1.5., SVD decomposes a matrix X into the product of three separate matrices:

$$X = U \Sigma V^T$$

Here, U and V are not only orthogonal but identical matrices, and hence left and right singular vectors are identical and called 'eigenvectors'. Moreover, eigenvalues λ are non-negative which allows us to talk about the fraction of explained variance, captured by each individual eigenvector.

The key relationship between PCA and SVD is that the principal components of X are equivalent to the right singular vectors in the SVD of X, and the singular values in Σ are related to the square roots of the eigenvalues of the covariance matrix of X.

PART 1: SINGLE-CELL RESOLUTION UNRAVELS SPATIAL ALTERATIONS IN METABOLISM, TRANSCRIPTOME AND EPIGENOME OF AGEING LIVER

1.1. Contribution Statement

My contributions were primarily in the realm of bioinformatics. I performed the bioinformatic analysis of the spatial transcriptomics data following the fastq-file generation from the sequencing facility. I also played a practical role in the extraction of nuclei from liver tissues in the wet lab for both scATAC-seq datasets. Upon the generation of the fastq files from the sequencing facility, I handled the bioinformatic analysis of both scATAC-seq datasets.

In the writing phase, I was responsible for the respective method sections and made contributions to the main text of the manuscript. Throughout the project, I was actively engaged in conceptualizing and planning the methodology of the research.

DOI of the Publication: https://doi.org/10.1038/s43587-023-00513-y

PART 2: AGEING IS ASSOCIATED WITH INCREASED CHROMATIN ACCESSIBILITY AND REDUCED POLYMERASE PAUSING IN LIVER

2.1. Contribution statement

My contributions were primarily in the realm of bioinformatics for the CUT & RUN experiment. I carried out the complete bioinformatic analysis of the experiment after fastq file generation.

For the manuscript I authored the respective method section. Moreover, I reviewed and edited the main text.

DOI of the Publication: https://doi.org/10.15252/msb.202211002

PART 3: DIMENSION REDUCTION BY SPATIAL COMPONENTS ANALYSIS IMPROVES PATTERN DETECTION IN MULTIVARIATE SPATIAL DATA

3.1. Contribution statement

My contribution to this project was the implementation and evaluation of the method. Throughout the project, I was actively engaged in conceptualizing and planning the methodology of the research.

The manuscript was written by me with Achim Tresch.

DOI of the Publication: https://doi.org/10.1101/2023.10.12.562016

4. CONCLUSION AND DISCUSSION

Ageing, defined as the irreversible decrease in cellular and physiological functions with compromised genetic integrity, increases mortality risk. This phenomenon, impacting all biological processes across all living organisms, is of great scientific attention due to its health and lifespan implications. The "hallmarks of ageing" represent the core biological effects constituting the ageing process (López-Otín et al., 2023). Epigenetics – the study of mitotically and/or meiotically heritable changes in gene function without sequence alteration (Dupont et al., 2009) - is a crucial aspect of these hallmarks. Epigenetic mechanisms identified so far (DNA methylation, histone modification, 3D chromatin architecture, non-coding RNAs) undergo changes over time, leading to "epigenetic drift", which influences organism health and lifespan through significant alterations in gene expression and cell integrity.

While these hallmarks and the concept of 'epigenetic drift' form a general framework for understanding ageing across different organisms, it is important to recognise that the ageing process can be highly context-dependent. This variability becomes particularly evident when we look at different organs within the same organism. Each organ's ageing process is distinct, influenced by its specific physiological role, environmental exposures, cellular makeup, and regenerative capacity. The liver, central to xenobiotic detoxification, key plasma protein synthesis, and metabolism, undergoes significant ageing-induced changes, impacting overall health. Ageing-related liver alterations include impaired regeneration, inflammation, fibrosis, hepatocyte senescence, and reduced blood flow, all of which enhance the risk of liver diseases like hepatocellular carcinoma, cirrhosis, and non-alcoholic fatty liver disease (Anantharaju et al., 2002; Guicciardi et al., 2013; Radonjić et al., 2022).

In the liver, these ageing-related changes have profound implications not just for the organ itself but also for overall health. One of the key players mediating these changes and contributing to the age-related decline in liver function is the realm of epigenetics. These epigenetic modifications have a profound influence on the expression of genes and, ultimately, the functioning of hepatocytes, the primary cells of the liver. Epigenetic changes, integral to liver ageing, can disrupt the delicate balance of gene expression in ageing cells, leading to aberrant gene expression and cellular dysfunction. This thesis globally analyses DNA accessibility and transcriptional regulation changes in hepatocytes, particularly focusing on the impact of ageing on these parameters in relation to hepatocytes' spatial location and microenvironment within the liver lobule.

In the first part of this thesis, we identified regions of DNA with altered accessibility due to ageing-associated chromatin structural changes, profoundly impacting transcription regulation, as demonstrated by the Cide gene loci. Predominantly upregulated in the liver lobule's central area, Cide gene family members (*Cidea*, *Cideb*, *Cidec*) regulate LD dynamics and growth. Given prior research (Gong et al., 2011) suggests that the increase in *Cidea* and *Cidec* expression might be one underlying reason for the increase in LD size with age.

Part two of this thesis revealed that liver ageing affects more than just increased DNA accessibility in the liver. Transcription initiation and promoter-proximal pausing are also impacted, evidenced by decreased pausing complex stability. These findings were demonstrated through techniques such as CUT&RUN-seq and NET-seq.

Based on our previous observations that the spatial organisation and microenvironment of tissues play a critical role in the physiological functions and disease states of organs, we turned our focus on spatial transcriptomics methodologies to harness the full potential of this technology.
In part three, I presented our contribution to the fast-growing field of spatial transcriptomics data analysis. We propose a multivariate approach for pattern recognition, data denoising and SVG detection in spatial transcriptomics data. Our method stands out in its ability to mitigate non-spatial variation while demonstrating robustness and specificity.

In summary, multi-omics methodologies (including scRNA-seq, scATAC-seq, CUT&RUN sequencing, NET-seq and spatial transcriptomics) offer a unique chance to probe the intricate landscape of the ageing liver. By cross-referencing diverse cellular components, epigenetic alterations, gene expression changes, and spatial interactions, they promise to illuminate the complex factors driving liver ageing. These insights could substantially improve our comprehension of liver physiology, age-associated liver diseases, and overall ageing.

In the following, all three parts of this thesis will be discussed in detail separately to weave together the findings and the significance of the results of this thesis. I will emphasise the parts of the individual projects which were my contribution.

4.1. Part 1: Single-cell resolution unravels spatial alterations in metabolism, transcriptome and epigenome of ageing liver

Cells are not isolated solitary in tissues, and their location can influence function and, as we showed in this study, ageing. Constraints on technology and cost have limited research into this. Traditional methods of studying cell zonation involved cell sorting or location inference, but these were biased or limited and required precise location markers (Halpern et al., 2018; Ben-Moshe et al., 2019). Spatial transcriptomics presents an unbiased approach, detecting genes expressed in specific regions.

The impact of the microenvironment, particularly on tissue-resident stem cells' fate, has been a topic of interest. Age-related perturbations of certain features, such as vascular niches, are implicated in the functional decline of hematopoietic stem cells and osteoprogenitors (Kusumbe et al., 2016). Recent reports demonstrate widespread attrition of vascularisation across various organs, including the liver (Chen et al., 2021), underpinning that tissue microenvironments undergo considerable modifications with age. This notion aligns with age-related liver blood flow reduction (Wynne et al., 1989). Given the vascular system's role in establishing hepatocytes' functional segregation, the liver is an optimal tissue to investigate tissue organisation and location impacts on a specific cell type (Cast et al., 2015).

In liver ageing, spatial transcriptomics, corroborated by scATAC-seq, revealed that ageing is associated with zonated changes in metabolic processes, particularly those related to amino acid and lipid metabolism, as well as mitochondrial energy generation. These results, confirmed by Seahorse assays, microscopy and lipidomic quantification, reveal the interplay across cellular function layers. This underscores the value of integrating molecular cell biology with modern omics-based approaches.

Hepatic ageing includes LD accumulation, particularly around the liver lobule's central vein. Spatial transcriptomics showed increased expression of Cide genes, *Cidea*, *Cideb*, and *Cidec*, controlling LD growth (Gong et al., 2011). An increase in *Cidea* and *Cidec* expression, reflected in the epigenome as observed by scATAC-seq and in independent H3K27ac (enhancer) data, may explain LD size increase with age. An increase in *Cidea* and *Cidec* expression is also linked to hepatic steatosis development (Zhou et al., 2012; Sans et al., 2019) and prolonged hepatic lipid storage potentially results in liver metabolic dysfunction, associated with increased cellular senescence and inflammation (Wang et al., 2009; Ogrodnik et al., 2017)

Distinct ageing signatures were observed in hepatocytes, but transcriptional changes did not always align with epigenetic alterations, suggesting a decoupling of chromatin and RNA. Genes involved in post-transcriptional processing were among the top-dispersed genes in the scRNA-seq experiment, hinting at deregulation with age. One aspect of this layer involves mRNA splicing. Recent studies have indicated that splicing is strongly influenced by age and might contribute to ageing (Lee et al., 2016; Heintz et al., 2017; Lai and Pugh, 2017). Notably, the role of mRNA stability and storage with ageing remains unexplored and highlights again how the integration of more analysis methods could close the knowledge gap between these layers. The decoupling mirrors the age-related decoupling of mRNA and protein levels (Kelmer Sacramento et al., 2020). The recent publication of the tissue-wide atlas of the ageing effects on the murine proteome could be a valuable resource for result validation and cross-referencing our results

(Barao et al., 2022). In conclusion, these findings imply a progressive loss of gene expression cohesion and initiation, contributing to ageing (Kelmer Sacramento et al., 2020; Bozukova et al., 2022).

Recent research interest in the reproducibility of omics methods in ageing studies, emphasised by Singh and Benayoun (2023), necessitates a comprehensive discussion. Omics- approaches, given their complexity, demand sophisticated designs and analysis to ensure robust and reproducible results. Notably, we exclusively studied male mice, which given sex-related ageing differences and in relation to drug metabolism—a primary liver function, this is a potential limitation (Uno et al., 2017; Austad, 2019). We controlled the genetic background, known to significantly impact study outcomes, by using a uniform mouse strain, C57BL/6M, bred under identical conditions. This approach aligns with the findings of Liao et al. (2010), which underscored the profound influence of the genetic background of mice strains on results from caloric restriction studies. However, despite our control measures, the same study also suggests that certain aspects of the genetic constitution may still influence our results, pointing to potential limitations of our study

Age group selection is critical; hence, our young (4 months) and old (18 months) cohorts were chosen based on their reproductive stability and ageing phase, respectively, in concordance with established guidelines for animal use in gerontological research, mice in this age bracket (18 months) are considered to be in the "ageing" phase, rather than being fully aged (Miller and Nadon, 2000). To ensure reproducibility, we employed multiple replicates in our study designs. For example, our spatial transcriptomic analysis included two individuals per group, and our scATAC-seq experiment pooled four individuals per age group for both scATAC-seq experiments.

Library preparation quality can significantly affect experimental outcomes. Using different versions of the 10x Genomics Chromium reagent kit revealed reductions in detected nuclei and library complexity. Usage of the 10x Genomics Chromium reagent kit version 1.1 (10x Genomics, 2019) resulted in a high-quality library. However, upon switching to the Chromium version 2 reagent kit for the second scATAC-seq experiment (10x Genomics, 2021), we noted a reduction in the number of detected nuclei and decreased library complexity. Nevertheless, both datasets met quality standards and supported initial findings.

As also the suggested, the pre-processing pipeline (Cellranger ATAC from Version 1.2 vs 2.0) was changed together with changes in the chemistry. We also completely reprocessed our first dataset to ensure result consistency. This underscores that even minor alterations in the library preparation and pre-processing protocol can substantially influence experimental outcomes and, thus, require thorough evaluation. We could verify our results with both pre-processing methods.

Additionally, we leveraged two disparate methodologies to model and identify changes in DNA accessibility, namely Latent Dirichlet Allocation and Latent Semantic Indexing. This approach was necessitated by the absence of a universally accepted best practice for the analysis of scATAC-seq data at the time of our study. The validity of our findings was corroborated via both methodological approaches.

Post-processing challenges, such as the lack of existing methods to address batch effects in spatial transcriptomics datasets, were mitigated using best practice methods from scRNA-seq data. Further, we performed a principal component analysis on the integrated data and intersected the loadings of the first

principal component with published liver ageing marker genes. Transcriptional changes in signature genes for ageing hepatocytes, taken from public resources (Almanzar et al., 2020; Zhang et al., 2021), accounted for majority of the top 50 genes that contributed to the first principal component (35/50).

Result validation, critical for reproducibility, included experimental verification through low-throughput methods, analysing other omics-layers to verify the crosstalk of these layers and cross-referencing with other studies like Tabula Muris Senis (Almanzar et al., 2020). For instance, we used RNAscope to verify our periportal and pericentral markers. To validate our findings regarding the zonated expressional changes of the Cide gene family, we compared this with the age-dependent changes of these loci in co-accessibility in our scATAC-seq data. Additionally, we used publicly available data for active enhancer marks (H3K27ac) and could detect an increase in acetylation marks upstream of *Cidea* in aged mice liver tissue.
Further, we used microscopy to confirm the changes in pericentral lipid droplet sizes and by cross-referencing these findings with our lipidomics experiment results we discovered a remodelling of the livers lipidome. The zonated changes in mitochondrial energy generation potential were verified by functional Seahorse assays.

Ensuring long-term usability and reproducibility, all raw and processed data, annotated objects, metadata, and the analysis pipeline will be publicly available. Despite certain limitations like using only male mice, this study underscores the meticulous approach necessary in ageing research using omics methods and highlights the significant influence of biological, technical, and post-processing variables.

4.2. Part 2: Ageing is associated with increased chromatin accessibility and reduced polymerase pausing in liver

Omics-technologies have advanced our understanding of age-related epigenomic transcriptional regulation during ageing. Using genome-wide sequencing techniques like NET-seq, ATAC-seq, RNA-seq, ChIP-seq and CUT&RUN-seq on mouse liver tissue, we studied the role of chromatin accessibility in transcriptional regulation during ageing through a multi-omics approach.

Earlier studies show variable findings in age-related chromatin landscape change, likely due to tissue- and cell-type specific alterations. In this study, we could show specific alterations in aged murine liver tissue. Despite increased global promoter accessibility, histone gene expression and nucleosome occupancy levels outside of promoter regions remained practically unchanged, as described in other studies (Chen et al., 2020), underscoring the value of cross-referencing datasets for a nuanced view of genome-epigenome interactions.

Minor effects were seen on both nascent and steady-state transcriptomes, implying gene expression preservation in ageing liver tissue, as also observed in other studies (Zhang et al., 2021). Minimal overlap between differentially expressed and transcribed genes indicates possible post-transcriptional buffering mechanisms which is consistent with the results of part one of this thesis (Nikopoulou et al., 2021).

Despite slight transcriptional changes, promoter-proximal Pol II pausing significantly decreased with age, potentially due to reduced DSIF subunit SPT4 recruitment. By integrating Net-seq, RNA-seq, and CUT&RUN-seq data, we ruled out RNA Pol II loading as a causative factor for this decrease. Notably, with the analyses employed in this study it is not possible to distinguish between reduced Pol II transcription elongation machinery recruitment or increased dissociation from chromatin.

Our model proposes that ageing correlates with increased global chromatin accessibility at gene promoters, stable polymerase recruitment, and decreased promoter-proximal pausing. A change in the stability of the pausing complex with ageing might cause the decrease. However, this hypothesis needs further research. Speculatively, decreasing pausing complex stability during increased transcription initiation could buffer transcriptional output.

Consistent with the project delineated in part one, the planning and execution of this study placed paramount importance on robustness and reproducibility. The investigative approach was restricted to male C57BL/6N mice, acknowledged as a constraint of the study, while it also ensures a fixed genetic background. Given that variations in transcriptional and regulatory network functionalities between sexes have been documented in humans, albeit not in mice (Lopes-Ramos et al., 2020), it is plausible that the observed age-associated effects may present differently in female mice or in mice with different genetic background.
Our samples comprised of mice aged 3, 12, and 18 months were in line with guidelines for ageing mouse studies (Miller and Nadon, 2000). All mice were bred under constant conditions and were physiologically healthy.

In the CUT&RUN-seq experiment, which constituted my area of contribution to this project, we accounted for biological variability and age-related stochastic variance using liver tissue from four (4

months old) young and (18 months old) old mice. By incorporating yeast DNA 'spike-in' control, we achieved normalization across samples, mitigating potential batch effects. The synchronous generation of these samples ensured a consistent experimental context, aiding the reduction of extraneous variables.

We conducted a comprehensive evaluation of data integrity and library complexity post-sequencing. The assessment scrutinized fragment size distribution, read duplication rate, library size, adapter content percentage, and alignment rate, ensuring the robustness of the primary data.

We employed recognized CUT&RUN-seq data analysis methods for post-processing, notably the SEACR package (Meers et al., 2019). We further validated our findings by incorporating three different antibodies for RNA polymerase II in three distinct phosphorylation states (S2, S5 and CTD phosphorylation).

Our research, combining various omics technologies, offers a comprehensive understanding of age-related epigenomic alterations, despite focusing only on male mice. We uphold transparency and reproducibility by making all data publicly accessible, fostering further exploration of our findings.

4.3. Part 3: Dimension Reduction by Spatial Components Analysis improves Pattern Detection in multivariate spatial Data

Through the introduction of SPACO, we propose a pioneering approach for the elucidation of spatial gene expression patterns for in situ spatial transcriptomics datasets. Its capabilities surpass competing methods in robustness and specificity. We offer a comprehensive framework to mitigate variation due to technical limitations of in situ capture techniques. This includes a meticulously designed suite for dimensionality reduction, denoising and SVG testing. We validated SPACO's effectiveness using synthetic data and three different biological spatial transcriptomics datasets.

Rather than focus on individual genes, SPACO accounts for gene co-regulation within distinct regulatory networks, integrating established principles of dimensionality reduction and feature extraction from PCA into spatial transcriptomics analysis. By maximising local covariance (through Moran's I) instead of global variance optimisation, SPACO accurately discerns spatial patterns from spatially independent noise.

In our study, we performed a rigorous benchmarking of SPACO's SVG testing procedure against SPARKX. SPARKX can be regarded as the reference method with superior statistical power and robustness compared to other SVG analysis methods, as corroborated in both self-reported studies as well as independent benchmarking reviews (Zhu et al., 2021; Chen et al., 2022). SPACO outperformed SPARKX in sensitivity and specificity. We found SPACOs SVG test results consistent with known spatially organised processes via GO-term analysis, supporting our premise that genes within a common regulatory network exhibit similar spatial trends. We also confirmed that SPACO can uncover known SVG and suggest potential new ones.

While Testing SVG detection methods on real data, we noted that random permutation or unrestricted bootstrap methodologies could create anticorrelated patterns in the dataset. This observation and the high association between per-spot read coverage and gene expression levels underscore the issue of false discovery rates in spatial transcriptomics methodologies (Chen et al., 2022). As a consequence, we established the coverage-adjusted local bootstrap method to circumvent these artefacts.
Testing SVG detection methods on real data is challenging due to the lack of "gold standard" datasets. Coverage-adjusted local bootstrapping utilises existing knowledge of tissue-specific marker genes. Our benchmarking circumvented the lack of standard datasets, using these genes as bona fide true positives while mitigating the per-spot coverage artefacts.

With our coverage-adjusted bootstrapping approach, we have shown that the projection of SVG onto the relevant spatial components is a reliable denoising method. This capability is particularly advantageous when one wants to link histological observations with gene patterns, also possibly for genes with high noise levels and dropout rates.

For future applications and implementations, it appears plausible to utilise these denoised profiles to calculate local gradient lengths and curvature, vital metrics for edge detection and regional expression delineation. These metrics could subsequently be leveraged for the clustering of SVG, thereby facilitating

the detection of spatial patterns alongside their corresponding groups of genes. This refinement could ultimately enhance the utility of SPACO in dissecting complex spatial gene expression landscapes via grouping (trough clustering) of SVG.

By design, SPACO merely relies on a neighbourhood matrix and a count matrix. It assumes that each locus represents a full vector of measurements (one count/abundance value for each gene). One obvious improvement of the current implementation of SPACO is the extension of SPACO to also be applicable to other spatial sequencing data such as MERFISH, SeqFISH or STARmap (Wang et al., 2018; Eng et al., 2019; Xia et al., 2019). Currently, there is no implementation to construct a neighbourhood matrix of irregular or 3-dimensional coordinate matrices. With an adequate transformation of the coordinates obtained from these experiments, SPACO could also be applied to datasets generated with these methods.

As spatial transcriptomics experiments are becoming increasingly affordable, we anticipate a surge in projects incorporating numerous consecutive tissue slices from identical tissues or divergent individuals. SPACO, in its existing form, is prepared to accommodate these expanding datasets, necessitating only the block-diagonal concatenation of neighbourhood matrices for successful implementation. Thus, SPACO offers a robust and adaptable framework suitable for addressing the evolving complexity of spatial transcriptomics investigations.

Additionally, we envisage the extension of SPACO's SVG test to standard scRNA-seq data as a viable undertaking. A predominant hurdle in differentially expressed gene testing for scRNA-seq data is the subjective nature of population assignments via clustering, a process notably influenced by user parameter choices and complicated by the typically indeterminate number of groups or cell types present in a given dataset (Zappia and Oshlack, 2018). Prevalent scRNA-seq workflows routinely employ the Louvain or Leiden algorithms for clustering, both of which utilise k-nearest neighbour graphs for scRNA-seq applications (Stoeckius et al., 2017; Traag et al., 2019). Should such a neighbourhood graph replace the spatial neighbourhood matrix in SPACO, it is conceivable that differential expression testing could be performed devoid of bias, effectively repurposing SVG as genes that delineate or segregate cell communities.
This principle can be equivalently applied employing a (weighted) k-nearest neighbour graph as, e.g., constructed by Uniform Manifold Approximation and Projection or the similarity graph constructed by t-Distributed Stochastic Neighbour Embedding. These considerations not only demonstrate the versatility of the SPACO approach but also highlight its potential applicability to a wider range of bioinformatics contexts.

Outside the realm of transcriptomics, the SPACO algorithm could be applied to any kind of spatial data for which a neighbourhood or adjacency matrix can be constructed. Examples include ecology, where it can assist in the analysis of spatial patterns in plant or animal distribution, public health for studying patterns of disease spread, or social sciences and economics for understanding spatial distribution of income levels, unemployment rates, voting patterns, and more.

In conclusion, the development of SPACO offers a novel approach for spatial gene expression pattern analysis, enhancing both robustness and specificity. SPACO's focus on gene co-regulation and maximisation of local covariance sets it apart to facilitate accurate discernment of spatial patterns. Its superiority to SPARKX in SVG testing is validated through rigorous benchmarking and supported by its consistency with known spatially organised processes known for the tissues analysed. The establishment of

a coverage-adjusted local bootstrapping method mitigates the lack of “gold standard” datasets in real data SVG testing benchmarking. Importantly, SPACO's ability to project SVG onto spatial components for denoised gene projections holds significant potential for linking histological observations with gene expression patterns, even in high-noise conditions. Future extensions of the SPACO library will likely extend its applicability towards other established spatial sequencing methods, scRNA-seq and even possibly beyond the analysis of biological spatial data.

5. SUPPLEMENTAL INFORMATION

5.1 Library Preparation for Single-Cell Assay for Transposase-Accessible Chromatin Sequencing

Library quality is crucial for meaningful results in single-cell Assay for Transposase-Accessible Chromatin using sequencing (scATAC-seq) experiments. Following tissue extraction, the process begins with tissue disaggregation to form a single-cell suspension, typically involving optimised mechanical or enzymatic methods. Subsequently, cell lysis and nuclei isolation occur. The critical transposition reaction then employs a hyperactive Tn5 transposase to cut and ligate sequencing adapters to the DNA at accessible regions. Sorted into individual oil droplet reaction volumes, nuclei associate with a micro gel bead carrying unique barcode sequences for tracking post-sequencing. After droplet-based barcoding, the DNA fragments undergo amplification for next-generation sequencing. Final library validation, such as TapeStation analysis for fragment size distribution, confirms quality before sequencing. Each stage requires careful optimisation to secure high-quality data and prepare for bioinformatic analysis (10X Genomics, 2019; Yan et al., 2020).

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7. TABLE OF FIGURES

Figure 1: This illustration presents the twelve "hallmarks of ageing": Genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis. These symptoms of ageing are categorized into three groups: primary, antagonistic, and integrative. Obtained from López-Otín et al., (2023). 2

Figure 2: Epigenetic alterations of ageing are characterized by (A) loss of histones, (B) imbalance of activating and repressive modifications, (C) transcriptional changes, (D) losses and gains in heterochromatin, (E) breakdown of nuclear lamina, (F) global hypomethylation and focal hypermethylation, and (G) chromatin remodelling. These changes are heavily dictated by (H) environmental stimuli and (I) nutrient availability that in turn (J) alter intracellular metabolite concentrations. Modified from Sen et al. (2016). 6

Figure 3: Anatomy of the liver and its fundamental unit, the hepatic lobule. The liver is built from hexagonal structures referred to as lobules. Blood, abundant in oxygen and nutrients, flows in a specific direction starting from the hepatic vessels (depicted in red) located at the lobule's corners, towards a central vein situated in the middle (illustrated in blue). Hepatocytes found near the portal area (zone 1) are at the lobule's exterior, followed by hepatocytes in the middle of the lobule (zone 2), and finally, hepatocytes adjacent to the central vein (zone 3) surround it. This diverse microenvironment along the axis from the periportal to the pericentral region results in distinct areas. Modified from Cunningham and Porat-Shliom (2021). 14

Figure 4: Understanding Ageing and Liver Ageing through a Multidimensional Lens: Ageing and liver ageing are complex processes. The emergence of omics-technologies empowers us to examine these processes from a multifaceted perspective. Each layer of omics data corresponds to a vast set of parameters specific to that layer, enabling the acquisition of high-resolution insights into various biological states and abstraction levels. These insights can then be integrated to decode the multi-layered mechanisms of ageing, with specific emphasis on liver ageing in this thesis. The 'Age/environment' axis encompasses both internal factors (such as age) and external factors (including chemical, physical, and biological agents, along with overall environmental aspects). The organization axis refers to various levels of biological structure, spanning from organisms to organs, cells, and organelles. The analysis axis corresponds to a range of high-throughput omics-technologies and their integrative applications across diverse layers including genomics, epigenomics, transcriptomics, proteomics, metabolomics, and phenomics modified from Kim et al., (2016). 18

Figure 5: Latent Dirichlet Allocation plate notation with Dirichlet-distributed topic-word distributions. Figure obtained from Wikimedia commons (Commons Wikimedia, 2009). Explained below. 26

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9. ERKLÄRUNG ZUR DISSERTATION

„Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht."

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Teilpubliaktionen:

Nikopoulou, C., Kleinenkuhnen, N., Parekh, S., Sandoval, T., Schneider, F., Giavalisco, P., et al. (2021). Single-cell resolution unravels spatial alterations in metabolism, transcriptome and epigenome of ageing liver. bioRxiv, 2021.12.14.472593. doi: 10.1101/2021.12.14.472593.

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