Genomic responses to dietary restriction in ageing mice

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Für Oma Mia und Oma Reni.
(...dat hab' ich allet von euch!)

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Summary

Ageing is the process of a more or less continuous functional decline in physiological processes over time, resulting in deteriorating of health and culminating in death. Dietary restriction (DR), i.e. reduced food intake without malnutrition, is one of the most prominent ageing interventions, extending health and life-span in several model organisms. The increasing availability of high-throughput sequencing datasets is enabling researchers to address open questions about how DR can bring about its age-ameliorating effects at the system level.

In the first part of my PhD thesis, I asked how chronic DR and late-life dietary switches from and to DR alter the DNA methylation landscape of the mouse white adipose tissue (WAT). The DNA methylation landscape is known to be altered by ageing, for example in the liver and WAT. Furthermore, the methylome could be a potential source contributing to a recently discovered persistent transcriptional response ("transcriptional memory") of the WAT: late-onset *ad libitum* (AL) feeding following life-long DR altered the transcriptional profile in the WAT, but late-onset DR following life-long AL induced a more persistent AL-mediated transcriptional profile. I found that chronic DR induces differential methylation in the WAT at gene bodies and promoters and these changes are functionally associated with increased lipogenesis, reduced inflammation, and HoxA-mediated tissue differentiation. In response to late-life dietary switches, persistent DNA methylation marks rarely overlapped with the transcriptional memory and were thus insufficient to explain it on a global scale. Instead, late-life dietary switches induced differential methylation that was broadly uncoupled from transcriptional changes. This could be due to different time scales on which DR affects transcription and methylation: DR induces more rapid transcriptional changes associated with metabolic programs, whereas delayed DNA methylation changes are associated with slower WAT re-composition. Persistent DNA methylation marks could thereby store prior feeding information, serving as a "information buffer" to quickly recover transcriptional programs prior to a feeding switch.

In the second part of my PhD thesis, I investigated the effects of ageing and DR on the axis of gene expression correlation. The progressive functional decline of ageing leads to a continuous perturbation of organismal homeostasis, whereas perturbations in one tissue may also affect the functionality in others. For example, chronic inflammation, disruption of circadian rhythms, metabolic diseases or disturbed cellular differentiation processes all are consequences of ageing, but may also contribute to each other and to the ageing process itself. These systemic processes and their perturbations could be reflected at the level of gene expression correlations between tissues. To test this hypothesis, I examined gene-wise expression correlation by comparing the transcriptional profiles of 17 tissues always originating from the same mouse of the *Tabula muris senis* dataset. I found a bias towards positive expression correlation in most tissues, reflecting tight global regulation of circadian rhythm genes, protein folding in metabolically active tissues, and metabolic function in adipose tissues. Strikingly, this expression correlation profile declined with age. Top genes with with frequent loss of correlation are thereby novel targets genes for age-related perturbation of regulation across tissues and included *Btaf1*, *Map3k6*, *Ide*, and *Cirbp*. Both the positive expression correlation bias and its age-related decline were also present in the independent human GTEx dataset. DR improved global expression correlation as compared to AL, and protected against age-related correlation loss in the kidney and liver, especially for circadian genes. Furthermore, DR induced the correlated expression of key transcription factors of the FoxO-mediated insulin signalling regulation, which was absent in AL, but may be related to the systemic age-amending effects of DR in nutrient-sensing tissues.

In conclusion, I shed further light on the complex dynamics of DR and its impact on ageing. DR specifically induced remodelling of the DNA methylation landscape associated with lipogenesis and reduced inflammation in the WAT, and ameliorated the maintenance of tight circadian rhythm regulation throughout life while establishing a novel coordinated expression profile of nutrient sensing key genes in the kidney and the liver.

Zusammenfassung

Altern zeichnet such durch den funktionellen Rückgang physiologischer Prozesse mit der Zeit aus, der zu einer Verschlechterung des Gesundheitszustands und schließlich zum Tod führt. Diätetische Restriktion (DR), die reduzierte Nahrungsaufnahme ohne Mangelernährung, ist eine der bekanntesten Alters-Interventionen, die sowohl Gesundheitsspanne als auch Lebensdauer verlängert. Die zunehmende Verfügbarkeit von Hochdurchsatz-Sequenzierungsdatensätzen ermöglicht eine bessere und detailliertere Charakterisierung der DR und ihres Einflusses auf den Alterunsprozess auf systematischer Ebene.

Im ersten Teil meiner Doktorarbeit untersuche ich, wie chronische DR sowie eine späte Ernährungsumstellung von und auf DR die DNA-Methylierung im weißen Fettgewebe (WAT) der Maus verändern. Es ist bekannt, dass sich die DNA-Methylierung mit zunehmendem Alter verändert, z.B. in der Leber und im WAT. Obwohl sich das WAT auch als Schlüsselgewebe bei der Vermittlung von DR-Reaktionen erwiesen hat, ist weitgehend unklar, wie DR das Methylom des WAT beeinflusst. Zusätzlich könnte das Methylom eine Quelle für das kürzlich entdeckte persistente Transkriptionsmusters ("Transkriptions-Gedächtnis") des WAT sein, bei welchem eine spät angesetzte *ad libitum* (AL)-Ernährung nach lebenslanger DR das Transkriptionsprofil im WAT schnell verändert, während eine spät angesetzte DR nach lebenslanger AL nur wenig Einfluss auf das AL-induzierte Transkriptionsprofil hat. Ich fand heraus, dass chronische DR das Methylierungsmuster des WAT vor allem an Genen und deren Promotern veränderte, die funktionell mit erhöhter Lipogenese, reduzierter Gewebe-Entzündung und HoxA-vermittelter Gewebedifferenzierung assoziiert waren. Eine späte Ernährungsumstellung in alten Tieren konnte das transkriptionelle Gedächtnis im WAT nicht erklären, da nur wenige persistente DNA-Methylierungen mit diesem Gedächtnis assoziiert waren. Darüber hinaus waren differentielle Methylierungen, die durch eine spätere Ernährungsumstellung induziert wurden, generell selten mit transkriptionellen Veränderungen assoziiert. Hypothetisch könnte dies auf unterschiedliche Zeitskalen zurückzuführen sein, auf denen DR wirkt. Transkriptionsänderungen stehen im Zusammenhang mit Stoffwechselprogrammen und könnten deswegen früher und schneller

ablaufen. Spätere, langsamere DNA-Methylierungsänderungen stehen dahingegend im Zusammenhang mit der Umstrukturierung des WAT. Persistente DNA-Methylierung könnten als "regulativer Puffer" Informationen über vergangene Ernährungsweisen speichern, um vergangene regulative Prozesse schnell wieder ansetzen zu können.

Im zweiten Teil meiner Dissertation untersuche ich die Auswirkungen des Alterns und von DR auf die Korrelation der Genexpression. Der kontinuierliche funktionelle Abbau während des Alterns führt zu einer kontinuierlichen Störung der Homöostase eines Organismus, wobei Beeinträchtigungen in einem Gewebe auch die Funktionalität anderer Gewebe beeinträchtigen können. So sind z. B. chronische Entzündungen, Störungen des zirkadianen Rhythmus, Stoffwechselkrankheiten oder fehlerhafte zelluläre Differenzierungsprozesse Folgen des Alterns, können aber auch einander beschleunigen und zum Altern selbst beitragen. Solche systemischen Prozesse und deren altersbedingte Verschlechterung könnten sich auf der Ebene der Korrelation der Genexpression zwischen den Geweben widerspiegeln. Um diese Hypothese zu testen, habe ich die Korrelation der Genexpression untersucht, indem ich die Transkriptionsprofile von 17 Geweben der jeweils selben Maus aus dem *Tabula muris senis*-Datensatz verglichen habe. Ich fand eine überwiegend positiven Expressionskorrelation in den meisten Geweben, die eine globale Regulierung von Genen für den zirkadianen Rhythmus, die Proteinfaltung in stoffwechselaktiven Geweben und die Stoffwechselfunktion in Fettgeweben widerspiegelt. Auffallend ist, dass dieses Expressionskorrelationsprofil mit zunehmendem Alter abnimmt. In diesem Zusammenhang habe ich Gene mit häufigem Korrelationsverlust zwischen Geweben aufgedeckt, die neue Zielgene für altersbedingte Störungen der Regulierung in allen Geweben sind, darunter *Btaf1*, *Map3k6*, *Ide* und *Cirbp*. Sowohl die Präsenz der Expressionskorrelation als auch ihr altersbedingter Rückgang waren auch in dem unabhängigen menschlichen GTEx-Datensatz zu beobachten. DR verbesserte die globale Expressionskorrelation der Niere und Leber im Vergleich zu AL und schützte vor altersbedingtem Korrelationsverlust, insbesondere bei zirkadianen Genen. Darüber hinaus induzierte DR, anders als AL, eine korrelierte Expression von Schlüsseltranskriptionsfaktoren der FoxO-vermittelten Insulinsignalregulation, die mit den systemischen altersverbessernden Effekten von DR in nährstoffsensiblen Geweben in Verbindung stehen könnte.

Zusammenfassend habe ich zu der Beantwortung offener Fragen bezüglich der komplexen Dynamik der DR und ihrer Auswirkungen auf das Altern beigetragen. Ich habe gezeigt, dass DR im WAT zur Veränderung der DNA-Methylierungslandschaft beiträgt, die mit der Lipogenese und der Verringerung von Entzündungen verbunden ist. Außerdem verbessert DR die Aufrechterhaltung einer stabilen Regulierung des zirkadianen Rhythmusregulierung und aktiviert neue, zwischen Leber und Niere koordinierte Expressionsprofile, welche mit bekannten altersassoziierten Stoffwechselwegen in Verbindung stehen.

List of Abbreviations

General terms

Abbreviation	Full name
AL	*Ad libitum*
ALDR	*Ad libitum* to dietary restriction (a feeding switch)
BAT	Brown adipose tissue
BH	Benjamini-Hochberg
BP	Biological Process
bp	Base pair
CDF	Central density function
CDS	Coding sequence
CGI	CpG island
CpG	Cytosin-guanin dinucleotide
DEG	Differentially expressed gene
DMR	Differentially methylated region
DNAm	DNA methylation (in the context of epigenetic clocks)
DR	Dietary restriction
DRAL	Dietary restriction to *ad libitum* (a feeding switch)
ECM	Extracellular matrix
FACS	Fluorescence activated cell sorting
FC	Fold change
GO	Gene ontology
GTEx	Genotype tissue expression (a dataset)
KEGG	Kyoto Enzyclopedia for Genes and Genomes
MSCs	mesenchymal stem cells
PCA	Principal component analysis
SD	Standard deviation
TMS	*Tabula muris senis*
TSS	Transcription start site
WAT	White adipose tissue
WGBS	Whole genome bisulfite sequencing

Genes, proteins, and their families

Due to different nomenclatures applied throughout the thesis (e.g. due to different model organisms, or the mentioning of both protein and gene), the following are listed only in upper-case for each gene, protein, and/or their families.

Abbreviation	**Full name**
AACS	Acetoacetyl-CoA synthetase
ACACA/ACC	Acetyl coenzyme-A carboxylase
ACACB	Acetyl-CoA carboxylase beta
ACLY	ATP-citrate lyase
ACOT9	Acyl-coenzyme A thioesterase 9
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs *(Representative for ADAMTS1, ADAMTSL2)*
AHSA	Activator of HSP90 ATPase activity *(Representing a co-chaperone family)*
AMPK	AMP activated protein kinase
ARNTL	Aryl hydrocarbon receptor nuclear translocator-like 1
BTAF1	B-TFIID TATA-box binding protein associated factor 1
CIRBP	Cold-inducible RNA-binding protein
CLOCK	Circadian locomotor output cycles kaput
CRY	Cryptochrome *(Representative for CRY1 and CRY2)*
CTCF	CCCTC-binding Factor
DBP	D-Box binding PAR BZIP transcription factor
DNAJ	DnaJ heat shock protein *(Representing a co-chaperone family)*
DNMT	DNA methyltransferase *(Representative for DNMT1, DNMT3A, and DNMT3B)*
FABP4	Fatty acid binding protein 4
FASN	Fatty acid synthase
FOXO	Forkhead-box-protein O *(Representative for FOXO1, FOXO3)*

GBP	Guanylate binding protein 4 *(Representative for GBP1, GBP2, GBP3, GBP4, and GBP7)*
GPAM	Glycerol-3-phosphate acyltransferase 1
HSP	Heat shock protein *(Representative for various heat shock family proteins)*
HOX	Homeobox (clusters A-D and their genes) *(Representative for HOX clusters A to D and their genes)*
IDE	Insulin degrading enzyme
IGF2	Insulin-like growth factor 2
IL6	Interleukin-6
IRF	Interferon regulatory factor *(Representative for IRF8/IRF9)*
IRGM2	Immunity-regulated GTPase family M member 2
KLF	Krüppel-like factor *(Representative for KLF9, KLF11, KLF13, and KLF15)*
LPIN	Lipin *(Representative for LPIN1 and LPIN2)*
LYL1	Lymphoblastic leukemia-derived sequence 1
MAP3K6	Mitogen-activated protein kinase kinase kinase 6
ME1	Malic enzyme 1
MRAP	Melanocortin 2 receptor accessory protein
MTOR	Mammalian target of rapamycin
NR1D	Nuclear receptor subfamily 1 group D *(Representative for NR1D1 and NR1D2)*
PCK1	Phosphoenolpyruvate carboxykinase 1
PER	Period circadian clock *(Representative for PER1, PER2, and PER3)*
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1α
PPAR	Proliferator-activated receptor *(Representative for PPARA, PPARD, and PPARG)*
SIRT1	Sirtuin-1
SREBF	Sterol regulatory element binding factor

SREBP	Sterol regulatory element-binding protein *(Representative for SREBP1 and SREBP-1c)*
TAOK3	TAO kinase 3
TAP1	Transporter 1, ATP binding cassette subfamily B member 1
TBP	TATA-box binding protein
TET	Ten-eleven translocatoin *(Representative for TET1, TET2, and TET3)*
TGTP1	T-cell specific GTPase 1
TIMP4	Tissue inhibitor of metalloproteinase 4
TNFα	Tumour necrosis factor alpha
UCP1	Uncoupled ptoein 1
VEGF	Vascular endothelial growth factor

1 General Introduction

1.1 Ageing is a process of progressive decline in physiological function

Ageing is commonly defined as the progressive loss of physiological function and homeostasis over time, culminating in death (López-Otín et al., 2013). While ageing affects most organisms, its effects are not uniform across species. Parameters such as maximum lifespan vary widely even between closely related genera (A. A. Cohen, 2018; Flatt & Partridge, 2018). In mammals, for example, the difference in maximum lifespan between short-lived species such as *Mus musculus* (∼4 years, R. A. Miller et al., 2002 and long-lived species such as the bowhead whale (>200 years, George et al., 1999) can vary by two orders of magnitude.

Even within a species, ageing is highly individualistic. In humans, for example, each individual's ageing trajectory is strongly influenced by a combination of the environmental factors (Lv et al., 2011; Remoundou & Koundouri, 2009) and lifestyle choices (Andersen & Fernandez, 2013; Bosnes et al., 2019; Daskalopoulou et al., 2019). Furthermore, each individual has a genetic predisposition to longevity, which is predominantly defined by the additive effects of multiple loci (Kaplanis et al., 2018). The heritability of longevity at the whole-population level is low, with estimates typically ranging from 10 to 25% (Kaplanis et al., 2018; Melzer et al., 2019). Interestingly, long-lived individuals have been found to have specific longevity-promoting loci (Joshi et al., 2017; Pilling et al., 2017), which increase the heritability of longevity as they cluster in their respective families (van den Berg et al., 2019). All of above factors, their interactions, and further stochastic effects (Gladyshev, 2016) contribute in a protective or detrimental way to the health and lifespan of each individual.

Ultimately, ageing disrupts the complex homeostasis of the organism, and it is inherently difficult to disentangle all aspects of the network of contributors and effects involved. Therefore, to better understand the ageing process, it is essential to identify common molecular mechanisms and pathways across different organisms that contribute to the ageing process (Guarente & Kenyon, 2000; Taormina et al., 2019; Valenzano et al., 2017). Following key work in the last decades, 12 hallmarks of ageing have been proposed. These

hallmarks can be grouped according to their impact on ageing and their relationship to each other: Primary hallmarks are those that largely reflect accumulative damage to the genome (e.g. genomic instability, epigenetic changes, or loss of proteostasis). Antagonistic hallmarks are responsive processes that may be beneficial in young individuals or when mildly dosed, but may become detrimental later in life (e.g. cellular senescence or deregulated nutrient-sensing). Finally, integrative hallmarks broadly summarise the consequences of accumulated deterioration with major implications for health and lifespan (e.g. altered intercellular communication and chronic inflammation) (López-Otín et al., 2023).
Deterioration of a hallmark shortens life or impairs health, whereas improvement of a hallmark prolongs it. Longevity intervention studies target this aspect, for example in genetic models such as the long-lived snell dwarf mouse (Flurkey et al., 2001), or in pharmacological studies such as one showing the lifespan-extending effect of supplemental spermidine in mice (Eisenberg et al., 2016). Dietary modifications such as diet composition, food intake, timing of feeding, and the initiation of defined dietary regimens are among the most widely studied interventions for ageing (Gonzalez-Freire et al., 2020). Dietary restriction (DR), which is defined as food restriction without malnutrition, thereby is probably the most prominent longevity intervention (Fontana et al., 2010)

1.2 Dietary restriction improves health and extends lifespan across species

A link between diet and ageing was first suggested in 1935, when reduced food intake was found to significantly extend the lifespan of rats (McCay et al., 1935). Since then, the beneficial effects of DR on ageing have been extensively studied in several species including short-lived model organisms such as yeast (Kaeberlein et al., 2005; Smith et al., 2007), *Caenorhabditis elegans* (Greer & Brunet, 2009; Lakowski & Hekimi, 1998), and *Drosophila melanogaster* (Giannakou et al., 2008; Mair et al., 2003; Pletcher et al., 2002), and longer lived species such as rodents (Simons et al., 2013; Weindruch & Walford, 1982; Weindruch et al., 1986) and non-human primates (Colman et al., 2014; Mattison et al., 2017; Pifferi et al., 2018).
It is difficult to study the long-term effects of DR in humans because a prolonged DR treatment may restrict an individual's lifestyle. However, the influence of diet on human ageing can already be inferred from studies that

correlate health parameters of a population with its characteristic dietary pattern. For example, a Mediterranean diet has been associated with a reduced risk of several age-associated diseases as compared to modern Western diets (Shannon et al., 2021). Specifically for DR, the CALERIE study is the largest to date to investigate effects of DR in humans, with more than 200 patients aged 21-50 years undergoing a moderate calorie reduction over two years (Dorling et al., 2021). DR is thereby represented by an average caloric restriction of 11.9% that was achieved in the study population. This reduction induced significant weight loss (Ravussin et al., 2015), a reduction in circulating cardiometabolic risk factors, increased insulin sensitivity (Kraus et al., 2019), and was associated with improved immune response and reduced tissue inflammation in adipose tissues (Spadaro et al., 2022). Furthermore, while it had no rejuvenating effects on the epigenetic age of some DNAm age models, it introduced a signal of slowed ageing in the DunedinPACE biomarker of pace of ageing (Belsky et al., 2022; Waziry et al., 2023).

Many hallmarks of ageing have been shown to be ameliorated by DR or comparable dietary interventions (V. D. Longo & Anderson, 2022), including deregulated nutrient sensing (Green et al., 2022), age-related inflammation (Hotamisligil & Erbay, 2008; Morgan et al., 2007), mitochondrial dysfunction (Castello et al., 2011; Ruetenik & Barrientos, 2015), or impaired proteostasis (Matai et al., 2019). Furthermore, DR has been found to alter the composition of the gut microbiome (Rinninella et al., 2020; von Schwartzenberg et al., 2021) and is associated with epigenetic changes at the level of histone modifications (Hu et al., 2011; Molina-Serrano et al., 2019) and DNA methylation (Hahn et al., 2017; C. H. Kim et al., 2016). Importantly, these nodes can be interconnected in many ways. For example, inflammation in tissues such as the liver or kidney can be caused by exposure to a high-fat diet, which increases the risk for metabolic disease formation by affecting nutrient-sensing pathways. The impairment of tissue function can in turn be detrimental to the maintenance of whole-organism homeostasis (Lian et al., 2020).

DR-mediated responses are driven in part by transcriptional changes, especially in nutrient-sensing tissues (Swindell, 2009). The white adipose tissue (WAT), the brown adipose tissue (BAT) and the liver, show altered transcriptional profiles in response to DR as compared to *ad libitum* (AL) in 27 old mice, with over 3,200 significantly regulated genes in the WAT and liver, and 2,400 regulated genes in the BAT (Hahn et al., 2019). Thus, their transcriptional changes include a variety of downstream effects that can

only be partially described here. In the WAT, DR regulates the expression of genes associated with the energy- and fatty acid metabolism. *Sirtuin-1* (*Sirt1*), a key regulator of the cellular stress response and metabolic maintenance in the WAT, is up-regulated at the transcriptional level in the adipose tissue in response to DR (H. Y. Cohen et al., 2004; K. N. Miller et al., 2017), but this regulation was exclusive to young WAT (Hahn et al., 2019). SIRT1 regulates the expression of *sterol regulatory element-binding protein 1c* (*Srebp-1c*) (Majeed et al., 2021). Consistent with this, DR in rats increased protein levels of SREBP-1c, and expression of several of its downstream targets including *fatty acid synthase* (*Fasn*), *acetyl coenzyme-A carboxylase* (*Acc*), *ATP-citrate lyase* (*Acly*), and *malic enzyme 1* (*Me1*), effectively remodelling fatty acid synthesis processes and increasing lipogenesis (Kobayashi et al., 2018; Okita et al., 2012). Furthermore, up-regulation of SREBP-1c increases the expression of *peroxisome proliferator-activated receptor gamma coactivator-1α* (*Pgc-1α*), a key regulator of mitochondrial biogenesis (R. M. Anderson et al., 2008; Fujii et al., 2017; K. N. Miller et al., 2019) and an important modulator of DR responses (R. M. Anderson et al., 2009). Short-term DR of 1-5 weeks starting in 8-week-old mice has also been shown to cause WAT browning (Fabbiano et al., 2016), a process by which the WAT acquires functional properties of the BAT, including its main function of uncoupled protein 1 (UCP1)-mediated thermogenesis via uncoupled lipid respiration (Fenzl & Kiefer, 2014). In old mice (27 months) treated with chronic DR, this WAT browning was absent, suggesting that it is a response exclusive to young mice. Furthermore, in the BAT specifically, chronic DR induced an increased lipid transporter activity but decreased the expression of mitochondrial genes including *Ucp1*, suggesting a reduced thermogenic response of the BAT in lifelong DR animals (Hahn et al., 2019). In the liver, similar to the WAT, DR was also found to alter lipid metabolism to a large extent. However, in contrast to the WAT, *Srebf1* and many of its direct downstream targets, including *Acly*, *Me1*, and *Acc*, are down-regulated, reducing hepatic lipogenesis (Hahn et al., 2017). DR has also been found to consistently increase the expression of *Lipin-1* (*Lpin1*) and *Lpin2*, both of which are important in fat metabolism (Swindell, 2009), with *Lpin1* itself being an upstream regulator of hepatic *Pgc-1α* signalling (Finck et al., 2006). Similar to its function in the WAT, *Pgc-1α* is an essential modulator of hepatic mitochondrial content, function, and metabolic processing (Matthew Morris et al., 2012). As mentioned above, the DR-induced transcriptional changes described here only summarise a part of the effects of DR on the WAT, BAT, and liver transcriptome. However, these changes were associated with its

age-ameliorating effects, for example in the regulation of lipogenesis and mitochondrial activity.

Most of the DR-mediated effects discussed so far have been consequences of the onset of DR in early to middle-aged individuals and the subsequent maintenance of DR. However, with the predicted increase in global life expectancy (Raftery et al., 2014) and the resulting increase in the number of elderly individuals, it would be beneficial if DR-mediated effects could also be achieved with an onset later in life. Importantly, it has been shown, for example in the CALERIE study, that even two years of treatment of mild DR in humans is difficult to maintain (Ravussin et al., 2015). The late-onset of DR may increase the overall feasibility of treatment, as the motivation to adhere to DR may be increased in older people who are already exposed to the consequences of age-related functional decline. Furthermore, DR establishment and maintenance at an appropriate level could be done under clinical supervision, which could reduce the potential risks of prolonged and more severe DR at severe or unhealthy levels, leading, for example, to reduced wound healing, loss of strength and endurance, and long-term bone thinning (Dirks & Leeuwenburgh, 2006).

In flies, DR in young and old individuals almost completely rescued an AL-specific increase in mortality over the course of two days, and conversely, switching from DR to AL at any investigated time point acutely increased mortality (Mair et al., 2003). Thus, the effects of DR on mortality were independent of the age of onset. However, this independence of age on DR-mediated effects does not translate to studies in mice. Importantly, while late onset of DR in mice has been consistently reported to be beneficial for health span, its effects on lifespan have been conflicting in many studies, arguably because they depend on factors such as genotype, sex and experimental conditions (Forster et al., 2003; Liao et al., 2010; Mitchell et al., 2016). Nevertheless, it has been reported that the onset of DR at 19 months of age increases median lifespan (Weindruch & Walford, 1982) and reduces age-related mortality (Dhahbi et al., 2004). To further elucidate the effects of late-life onset of AL or DR in mice, a previous study by our group performed dietary switches from AL to DR (ALDR) and from DR to AL (DRAL) at 24 months of age (Figure 1.1A) (Hahn et al., 2019). DRAL reduced mortality, but to a lesser extent than chronic DR would. In contrast, the reciprocal ALDR switch acutely increased mortality. This suggests that in mammals, DR needs to be initiated earlier in life and then maintained in order to promote longevity. The imbalance in the mortality shift of ALDR and DRAL was associated with persistent transcriptional profiles (Figure 1.1B)

particularly in the aged WAT, but also in the BAT. In the WAT following ALDR, 1,609 genes (~50% of all differentially expressed genes (DEGs) in AL vs. DR) were persistently differentially expressed (Figure 1.1C). This highlights them as candidates for a transcriptional 'AL memory', which summarizes genes that remain at their level of expression in AL after late-life DR onset, and thus are unresponsive to late-life DR. Interestingly, the WAT also showed a weak 'DR memory' of 423 genes (~13%), but remained responsive in young mice, suggesting an age-related loss of transcriptional flexibility in the WAT that could be accentuated by chronic AL feeding. AL memory was also present in the BAT, but less pronounced than in the WAT with only 866 persistent genes (~35%), while the liver generally remained responsive to both ALDR and DRAL dietary switches in late life. However, the molecular origin of the dietary memory of AL feeding in WAT (and BAT) is currently elusive. (Hahn et al., 2019)

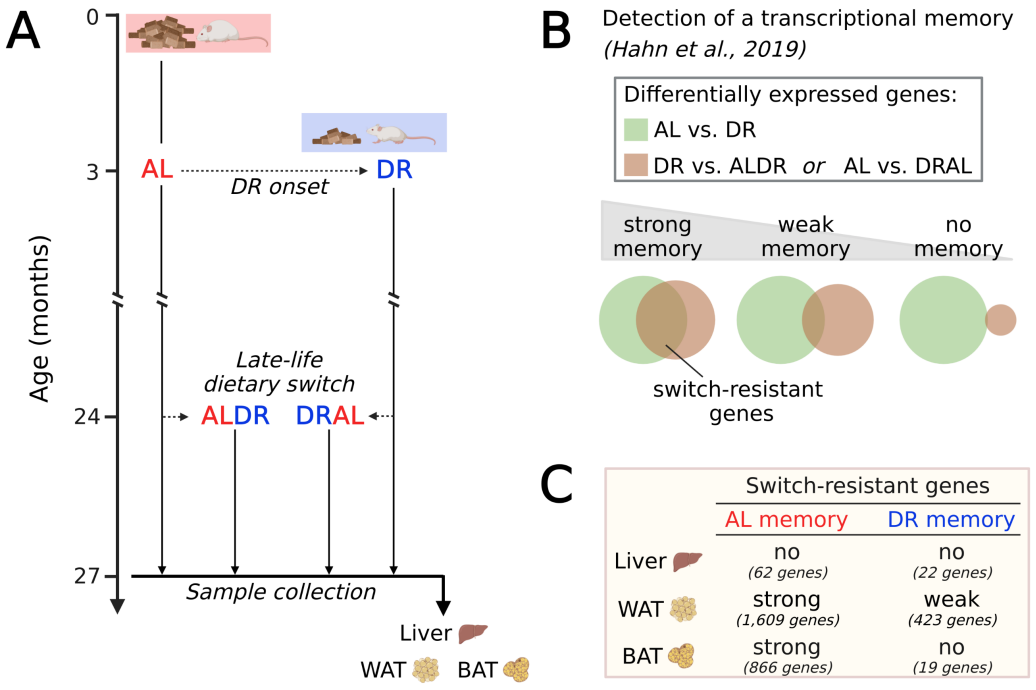


Figure 1.1 | **Detection of an adipose-tissue specific transcriptional feeding memory.** (A) Overview of the experimental setup used to collect liver, WAT and BAT samples from 27 months old mice in (Hahn et al., 2017) and (Hahn et al., 2019). (B) Schematic representation of how the transcriptional memory is defined. The strength of the transcriptional memory is defined by the number of switch-resistant genes. Switch-resistant genes are defined as genes that are differentially expressed in AL vs. DR, and also differentially expressed in DR vs. ALDR (= AL feeding memory), or in AL vs. DRAL (= DR feeding memory). (C) Overview of the number of switch-resistant genes in the liver, WAT, and BAT detected in (Hahn et al., 2019) that represent an AL or DR feeding memory.

In summary, chronic DR treatment increases lifespan in a wide range of organisms, and health in humans, and positively affects several hallmarks of ageing. Its effect is also associated with the remodelling of metabolic and transcriptional profiles of lipid- and energy metabolism in the WAT, BAT, and the liver. In mice, contrasting flies, DR-mediated benefits are dependent on the age of onset. Late-life DR effects are attenuated as compared to chronic DR, and can only partially rescue the effects of chronic AL feeding, potentially due to a refractory transcriptional pattern in adipose tissues. Arguably, the strong "AL memory" in the WAT could be encoded on the epigenetic level.

1.3 DNA methylation is a predictor for age and affected by dietary restriction

The epigenome is commonly defined as reversible modifications to the DNA and its associated histones that do not alter the genetic information. It acts as a dynamically modifiable 'code layer' over the DNA sequence that controls gene expression in response to environmental cues (Szyf, 2007). The epigenome plays an essential role in many developmental processes, such as controlling critical stages in early organismal development (Skinner, 2011), but also influences cell differentiation processes later in life (Suelves et al., 2016). The epigenome changes with age, and these changes include a general loss of histones, site-specific alteration of heterochromatin, and changes in histone marks as well as CpG methylation (hereafter referred to as DNA methylation) patterns (Booth & Brunet, 2016; Sen et al., 2016). The DNA methylation landscape is dynamically shaped by proteins of the DNA methyltransferase (DNMT) family which are responsible for DNA methylation maintenance (DNMT1) and *de novo* methylation (DNMT3A, DNMT3B) (Lyko, 2017), and Ten-eleven translocation (TET1, TET2, TET3) proteins which play a role in DNA demethylation (D. Li et al., 2015).
In recent years, DNA methylation has been established as a potent biomarker of ageing through the development of so termed epigenetic clocks, which (Bell et al., 2019; Horvath, 2013; Jylhävä et al., 2017). Briefly, epigenetic clocks are based on longitudinal datasets, usually covering (at least) the DNA methylation state of single CpGs, and use regression algorithms to express site-specific methylation changes as a function of age (Field et al., 2018). By integrating phenotypic and clinical parameters with DNA methylation changes, these clocks have also been extended to predict the

healthspan of an organism (Levine et al., 2018; Lu et al., 2019). Importantly, ageing interventions and lifestyle choices can alter the DNA methylation landscape, which is also reflected in the 'speed' of epigenetic clocks. For example, it has been reported that age-related changes in human DNA methylation patterns can be 'slowed down' by an isocaloric dietary intervention that targeted food composition, or by increased physical exercise over two years (Fiorito et al., 2021). In contrast, lifestyle parameters such as smoking, but also mortality in general are associated with an accelerated epigenetic clock (Oblak et al., 2021). Another accelerating factor is diet-induced obesity, which alters methylation patterns associated with an increased cancer risk in mouse colon tissue (R. Li et al., 2018).

In addition to its role as a biomarker of ageing, DNA methylation also plays a role in the regulation of gene expression (Moore et al., 2013). Methylation of upstream features of genes, including promoters, first exons, and first introns, is inversely correlated with gene expression (Anastasiadi et al., 2018). DNA methylation can alter the binding affinity of transcription factors to respective elements and thereby affect transcription (Kaluscha et al., 2022). In contrast, gene body methylation has been found to be positively correlated with gene expression (Ball et al., 2009; Jjingo et al., 2012; P. A. Jones, 1999). Two possible explanations for this are that hypermethylation increases overall transcriptional efficiency by silencing gene-body spanning features such as alternative promoters or splicing sites (Yang et al., 2014), or that a highly expressed gene has a more open chromatin landscape that is also more accessible for DNMTs to actively methylate the region (Jjingo et al., 2012).

Thus, DNA methylation not only has regulatory potential, but could also be the consequence of transcriptional processes themselves. This dynamic view of the DNA methylation landscape is consistent with the results of a recent study (Broche et al., 2021) in which hypermethylation was introduced across CpG islands (CGIs), mostly located on promoter regions, via the inducible expression of a DNMT3A-zinc-finger fusion protein (ZnF-3AC). After cessation of ZnF-3AC expression, only 10% of all hypermethylated sites remained stably methylated over time, most of which were enriched for the gene silencing-associated histone modification H3K27me3 (Cai et al., 2021). This suggests that the DNA methylome is dynamically demethylated, and maintenance of methylation marks is an actively counteracting process that could be associated with histone modifications. Notably, the induced hypermethylation also negatively correlated with gene expression, consistent with the inhibitory role of DNA methylation on upstream gene features.

The findings that DNA methylation changes with age and in response to environmental cues, and its potential role as a regulator of gene expression makes it a potential mediator of longevity interventions. Consistent with this notion, DR was shown to delay age-associated DNA methylation drift in multiple tissues of rhesus monkeys, which also correlated with the gene expression of selected metabolic target genes in the liver (Maegawa et al., 2017). In mouse liver, age-related DNA methylation changes were slowed down in long-lived Ames dwarf mice and in mice treated with dietary restriction or rapamycin (Cole et al., 2017; Hahn et al., 2017).
Furthermore, DR-induced DNA methylation changes in the liver were found to correlate with changes in the expression of lipogenesis genes, for example including *Srebp-1c* and its downstream target genes such as *Acly*, *Fasn*, *Acc*, and *Me1*, which suggests a role for DNA methylation in DR-dependent reprogramming of lipid metabolism (Hahn et al., 2017). In addition to the liver, WAT function is also regulated by DNA methylation (X. Ma & Kang, 2019). DNA methylation has been reported to play a role in adipocyte differentiation (Pinnick & Karpe, 2011; Sakamoto et al., 2008) and adipose tissue browning (Xiao & Kang, 2019). DNA methylation in the WAT has also been associated with the regulation of insulin signalling and lipogenesis genes (Barajas-Olmos et al., 2018) and it changes in response to a high-fat diet (Zwamborn et al., 2017), and in metabolic diseases (Castellano-Castillo et al., 2019). As outlined above, DR alters WAT gene expression and DNA methylation has been implicated in WAT function. However, whether DR affects the methylome of the WAT and whether the methylation changes correlate with gene expression is currently unknown.
DNA methylation can have long-lasting effects on the expression of metabolic genes. As an example, early life diet alters the DNA methylation landscape of the maternally imprinted gene *insulin-like growth factor 2* (*Igf2*) (DeChiara et al., 1991; Giannoukakis et al., 1993) to permanently regulate *Igf2* expression (Waterland et al., 2006). In addition, a maternal high fat diet was sufficient to affect the DNA methylation profile of the brown fat in the offspring (Q. Zhang et al., 2021). Thus, DNA methylation patterns can be persistent over prolonged periods and affect gene expression. However, whether DNA methylation pattern in the WAT contribute to the observed transcriptional memory upon late-life onset of DR is currently unknown (Hahn et al., 2019).
In conclusion, dietary restriction delays age-dependent changes in DNA methylation patterns in mammals, with DNA methylation itself being a highly potent biomarker of ageing. Elucidating the interplay between

changes in the DNA methylation landscape and transcriptional adaptations in response to DR will shed further light on the complex link between DNA methylation and ageing responses. While integrative studies have already revealed an epigenetic reprogramming of the liver, the impact of DR in mediating the DNA methylation landscape of the WAT remains elusive. In the first part of my PhD thesis, I therefore addressed the following questions:

1. What is the impact of DR on the methylome of the WAT in old mice?
2. Are DR-mediated DNA methylation changes associated with corresponding changes in gene expression?
3. Does DNA methylation contribute to the transcriptional memory of the WAT in response to late-onset DR?

1.4 Ageing deteriorates organismal homeostasis

The health of an organism depends on the proper functioning of all its compartments, including individual cells, tissues, or organs (López-Otín & Kroemer, 2021). These compartments are thereby not separate entities, but parts of a highly interconnected network. The edges of this network are the axes of communication between individual compartments. At the inter-tissue level, for example in mammals, the edges connecting tissues could be circulating factors such as metabolites, hormones of the endocrine system, neuronal signals, or the intrinsic circadian expression of genes. Importantly, these edges that carry information between tissues are not separate from each other. For example, the circadian system is influenced by endocrine feedback (Leliavski et al., 2014) and vice-versa (Gnocchi & Bruscalupi, 2017), and hormonal regulation is influenced by metabolic processes and vice-versa (Galsgaard et al., 2019; Koyama et al., 2021; Matthews et al., 1985; Mullur et al., 2014). Together, all nodes and edges of this framework form a functioning system that maintains a homeostasis that is robust to external changes (Billman, 2020).
Because of the dense interconnectedness of this organismal framework, dysfunction in one part also affect other compartments, and this can cascade into diseases that disrupt the homeostasis of an organism. For example, mutations in a single cell can cause it to become tumourigenic, leading to various downstream pathologies throughout the body (Hanahan & Weinberg, 2011). Another example is the metabolic syndrome, which

describes a cluster of metabolic diseases resulting from perturbations in the network of metabolically active tissues, with ultimately fatal consequences (Castillo-Armengol et al., 2019; Priest & Tontonoz, 2019). Ageing is a prominent source that constantly challenges the functioning of the organismal framework, introducing dysfunction with implications for organismal homeostasis, health and lifespan (López-Otín et al., 2023) (Figure 1.2).

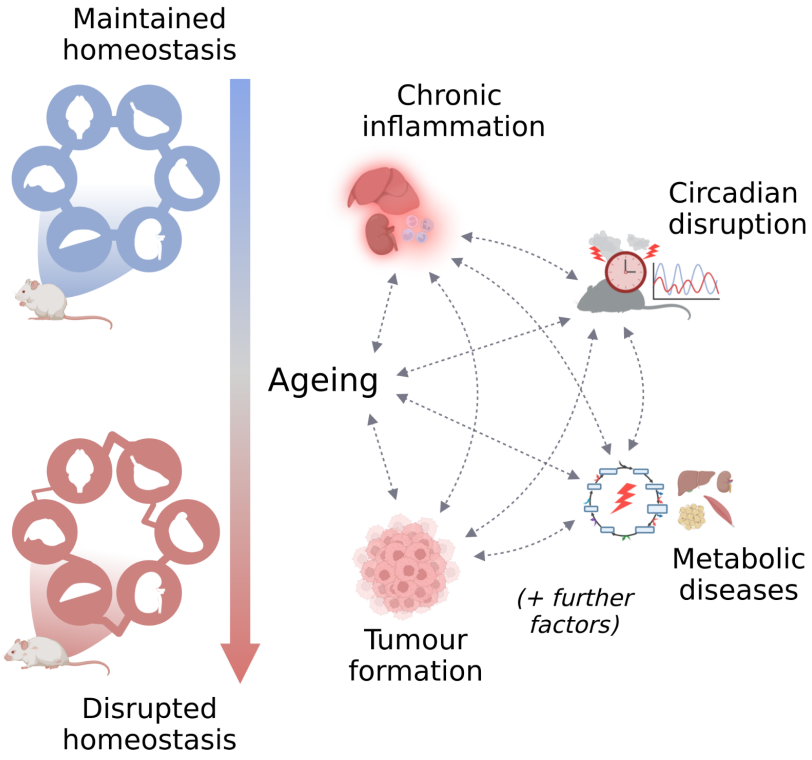


Figure 1.2 | **Factors contributing to (age-related) loss of inter-tissue homeostasis.** The maintenance of an organism's homeostasis is constantly challenged by multiple systemic perturbations, many of which are associated with ageing. Systemic perturbations, such as chronic inflammation, circadian disruption, metabolic disease onsets or tumour formation, are often interrelated, with the onset of one perturbation often contributing to the onset of others. Ultimately, the sum of all individual systemic perturbations leads to an ever-increasing disruption of an organism's inter-tissue homeostasis.

One factor of systemic dysfunction induced by ageing is chronic tissue inflammation, or inflammageing. As an integrative hallmark of ageing (López-Otín et al., 2023), inflammageing is both established as a consequence of accumulated age-associated damage, but can also induce further age-associated deterioration (Franceschi et al., 2018) including the development of cardiovascular and metabolic diseases (Freund et al., 2010; Furman et al., 2019). In contrast to acute inflammatory responses, which help to regenerate a tissue after exposure to stress (L. Chen et al., 2018),

chronic inflammation is usually established in the absence of infection or acute tissue damage. Chronic inflammation is characterized by higher expression levels of inflammatory genes and over time can impair tissue function (Furman et al., 2019). For example, inflammation contributes to the formation of tissue fibrosis (Mata et al., 2021), increases oxidative stress (Jurk et al., 2014), and promotes cellular senescence (J. L. Ren et al., 2009; Shang et al., 2020). One indicator of chronic inflammation is an elevated level of circulating factors (Moreau et al., 2020) such as interleukin-6 (IL6) and tumour necrosis factor alpha (TNFα) (Alberro et al., 2021), which can both have systemic effects in other tissues. For example, overexpression of IL6 in mouse skeletal muscle has been shown to promote increased levels of circulating IL6, which then induced liver inflammation and impaired insulin signalling (Franckhauser et al., 2008). In contrast, anti-TNFα therapy in humans significantly reduces insulin resistance and counteracts the development of metabolic disease (Burska et al., 2015). In summary, circulating factors secreted by inflamed tissues contribute to systemic inflammation and tissue dysfunction, and their inhibition, for example via reduced secretion or inhibition of their target receptors could be a therapeutic starting point (Dinarello, 2010).

The circadian system is another important regulator of systemic homeostasis that is impaired with ageing. For example, ageing was shown to impair the effectiveness of circadian entrainment in some tissues in response to environmental cues (Tahara et al., 2017), and induced a large-scale reprogramming of circadian gene expression with a general dampening of oscillation amplitudes under AL which could be rescued by DR and timed feeding (Acosta-Rodríguez et al., 2022). The circadian system consists of a central clock located in the suprachiasmatic nucleus of the hypothalamus in the brain (Ibuka & Kawamura, 1975), and tissue-specific peripheral clocks (Acosta-Rodríguez et al., 2021; Bonaconsa et al., 2014; Mohawk et al., 2012) which are maintained by a complex, cell-autonomous transcriptional loop that induces an oscillating expression of circadian genes (Rijo-Ferreira & Takahashi, 2019). The endogenous clock drives the daily expression of essential body functions including feeding, body temperature, and secretion of hormones (Gilette & Tischkau, 1999). The rhythm of the clock is constantly resynchronized by environmental cues such as light (Rimmer et al., 2000) and food intake (Escobar et al., 2020; Manella et al., 2021). Rhythmic expression of circadian proteins regulates downstream transcriptional programmes with systemic effects on hormonal (T. W. Kim et al., 2015; Lamia et al., 2011) and metabolic processes (Manella et al., 2021; Pickel

& Sung, 2020). It has been estimated that the circadian clock directly affects around 10% of expressed genes in a tissue, and indirectly influences roughly half of the transcriptome (Patel et al., 2015). Due to this large regulatory potential and the tight interconnection of the circadian clock with further systemic processes, it is not surprising that its perturbation can have various health implications (Chattu et al., 2019). Lack of expression of circadian locomotor output cycles kaput (CLOCK), a central protein of the circadian clock, significantly reduced lifespan in mice (Dubrovsky et al., 2010), demonstrating the importance of a functional clock for long-term survival. Other effects of circadian disruption include the increased risk of metabolic disease (Gale et al., 2011; Gan et al., 2015) or cardiovascular disease formation (Martino et al., 2008). In consequence, maintenance of circadian integrity is essential to maintain tissue homeostasis also with ageing.

Tumourigenesis, metabolic diseases, inflammageing, and disruption of the circadian clock are examples of interrelated systemic perturbations that can be caused by ageing and contribute to age-related functional decline. DR has been shown to be an effective intervention against the development of such systemic impairments, probably because its beneficial effects are affecting multiple tissues of an organism: DR rewires general metabolic processes at the organismic level (R. Anderson & Weindruch, 2007; Fontana & Partridge, 2015) and ameliorates the effects of ageing on key metabolic tissues such as the kidney (Afsar et al., 2021), adipose tissue, and the liver (Bruss et al., 2010; Cole et al., 2017; Hahn et al., 2019). It also can attenuate chronic inflammation (I. S. Lee et al., 2010) and can help to align circadian expression of genes to improve lifespan (Acosta-Rodríguez et al., 2022).

It becomes obvious that the processes that promote organismic homeostasis are inherently complex. However, the increasing availability of new methodologies and large data sets offers great potential for continuously unravelling this complexity. A novel and still under-explored approach to study inter-tissue coordination is to compare gene expression patterns between tissues. Conceptually, systemic changes will almost always affect gene expression of individual tissues in some way. If two tissues are receiving the same upstream signal (for example because they are recipients of the same circulating factors), both may induce similar signalling cascades which is then reflected in shared expression of downstream genes. This ultimately increases expression profile similarity. This concept was already used by some studies, and, for example, highlighted gene co-expression networks

that were dysregulated across tissues with ageing (Ferreira et al., 2021), or revealed a convergence of transcriptional profiles of tissues with ageing, likely reflecting a continuous loss of tissue identity (Izgi et al., 2022).
In the second part of my thesis, I will use the concept of gene expression correlation, and specifically explore gene expression correlation of tissues originating from the same individual and how it changes with ageing. The axis of gene expression correlation is still under-explored, probably because a correlation-based approach requires a high number of samples to provide sufficient statistical power, but longitudinal RNA-seq datasets with sequenced tissues that originate from the same individual are still rare. One suitable dataset is the publicly available Tabula muris senis bulk RNA-seq dataset (Schaum et al., 2020), which I will explore to ask the following questions:

1. Are gene expression profiles of tissues of the same individual correlated?
2. Are correlated genes indicators for upstream regulatory processes, or suitable to infer downstream tissue functionality?
3. Does does the expression correlation dynamic change with ageing?
4. Using the liver and kidney as an example, how does DR impact the age-related change in expression correlation?

1.5 Aims of this thesis

My PhD thesis consists of two projects, both focusing on the *in silico* analysis of the age-ameliorating effects of DR in the mouse.

In the first project (project A), I am focusing on the potential of DR and late-life dietary switches to alter the DNA methylation landscape and transcriptome of the WAT and the liver. The WAT was recently found to exert a nutritional metabolic memory of chronic AL feeding and a milder memory of chronic DR feeding, both of which were absent in the liver (Hahn et al., 2019). The DNA methylome is a promising target for storing information and could therefore be a hard drive for such a dietary memory. I will analyse how dietary treatments, specifically lifelong DR and late-life dietary switches at 24 months from AL to DR or DR to AL, affect the DNA methylation landscape of the WAT, and how they are associated

with transcriptional changes. In this way, I will investigate how chronic DR treatment, as opposed to AL, alters the DNA methylation landscape of the WAT to potentially have functional consequences for its transcriptome. Furthermore, I will answer whether the DNA methylation landscape can explain the observed feeding memory, or has potential other roles in the context of late-life dietary switches.

In the second project (project B), I will focus on the inter-tissue expression correlations between tissues in the mouse as an axis to investigate organismic homeostasis and ageing. To answer whether transcriptional profiles are correlated across tissues, and what functional roles these correlated genes have, I will compare the expression of individual genes and gene clusters across tissues of the same mouse from the publicly available Tabula muris senis bulk RNA-seq dataset (Schaum et al., 2020). Next, I will investigate if and how gene expression correlation is affected by ageing and validate findings in the independent GTEx dataset (Lonsdale et al., 2013). Finally, by generating and examining a new RNA-seq dataset of kidney and liver from AL and DR-fed mice, I will ask whether these ageing effects can be altered by DR.

2 Project A:

Short- and long-term responses of DNA methylation and gene expression to diet in mouse white adipose tissue and liver

(Unpublished manuscript)

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Author contributions: AB, LP, OH, and SG conceived the initial idea for the project. DNA methylation data was generated together with the group of WR. OH and DSG performed pre-processing of the DNA methylation data. DSG developed algorithms and performed all further steps of the dataset processing, analysis, and visualization of public and in-house data. OH contributed to the project by continuous helpful discussions. AB, LP, and SG contributed to the project by critical supervision. DSG wrote the manuscript. AB, LP, and SG critically reviewed the manuscript. Generated raw and processed data is stored on the server of the Max Planck Institute for Biology of Ageing, Cologne, Germany.

2.1 Abstract

Dietary restriction (DR) can extend lifespan and health-span in multiple species when applied throughout adulthood. White adipose tissue (WAT) plays a key role in mediating transcriptional responses to DR. As DNA methylation is a persistent epigenetic mark that can be altered by diet, we asked if DNA methylation can explain a previously reported transcriptional memory of earlier life feeding, present in mouse WAT but not in the liver. We integrated RNA-seq data with Whole Genome Bisulfite Sequencing (WGBS) data from WAT of 27-month-old female mice subjected to long-term DR or ad libitum (AL) feeding, and from both the WAT and the liver of mice subjected to late-life dietary switches at 24 months of age, either from AL to DR or DR to AL. In the WAT, life-long DR caused differential methylation of gene bodies, promoters, exons, CpG islands, and Hox clusters. Functionally, these differentially methylated regions (DMRs) were associated with increased lipogenesis and reduced tissue inflammation, suggesting a role of DNA methylation in mediating these effects of DR. However, in late-life dietary switches, the DNA methylation landscape did not globally explain the transcriptional memory. While switch-resistant DMRs were present in both tissues, the majority were not associated with switch-resistant transcriptional patterns. Moreover, we found that late-life switch-induced DNA methylation changes were generally not associated with transcriptional changes in either tissue. In the WAT, the DNA methylation patterns were consistent with remnants of transcriptional programs steering WAT tissue recomposition. In summary, our findings suggest a functional regulatory role of DNA methylation in promoting the effects of life-long DR in the WAT, whereas its role in transcriptional response to late-life dietary switches is doubtful.

2.2 Introduction

Dietary restriction (DR), reduced nutritional intake without malnutrition, is currently the most prominent intervention against the consequences of ageing (Liang et al., 2018; V. D. Longo & Anderson, 2022; Piper et al., 2011). Life-long DR extends health- and lifespan in a wide array of organisms (Fontana & Partridge, 2015; C. Lee & Longo, 2016) including rodents (McCay et al., 1935), and delays the onset of age-related diseases such as cancer, metabolic diseases, and cardiovascular diseases (V. D. Longo & Fontana, 2010; Omodei & Fontana, 2011). White Adipose Tissue (WAT) plays a key role in the responses to DR (R. Anderson & Weindruch, 2007). DR-induced transcriptional changes in mice include enhanced fatty acid synthesis (Bruss et al., 2010; Kobayashi et al., 2018), increased sensitivity to insulin metabolite stimuli (Corrales et al., 2019; Valle et al., 2010), and formation of functional beige adipose tissue (Fabbiano et al., 2016; Sheng et al., 2021).

Short term DR treatment can increase health-span and life-span (Forster et al., 2003; Liao et al., 2010; Mitchell et al., 2016). For example, onset of DR at 12 or 19-months in male mice in three different long-lived strains induced an increase in median lifespan accompanied by rapidly reduced body weight (Weindruch & Walford, 1982). Temporary DR in 19-month-old mice also reduced age-related mortality and induced alterations of hepatic transcriptional profiles that could be reverted by re-introducing Ad Libitum (AL) feeding (Dhahbi et al., 2004). Finally, a late-in-life dietary switch in female mice at 24 months from AL to DR (ALDR) and inversely from DR to AL (DRAL) revealed an acute mortality increase in the DRAL switch, but only a milder effect in the ALDR switch. While the hepatic transcriptome remained responsive to both dietary switches, the WAT transcriptome response was partially refractory, suggesting an "AL feeding memory", and a milder "DR feeding memory" (Hahn et al., 2019).

One potential source for this feeding memory is CpG methylation, henceforth referred to as DNA methylation. DNA methylation is an epigenetic mark associated with transcriptional regulation in a tissue- and cell-type-specific manner (Moore et al., 2013). DNA methylation can have persistent effects, for example via parental imprinting and during development (Frantz et al., 2001) through the establishment and maintenance of a cellular memory (Bird, 2002; Hemberger et al., 2009; M. Kim & Costello, 2017). Many studies have reported an inverse association of DNA methylation and transcription, where hypermethylation is associated with repression of transcription

(and vice versa) for example on promoters, CpG islands (CGIs), and CGI-associated regions (Anastasiadi et al., 2018; Yang et al., 2014), and also intergenic regions (Schulz et al., 2006). However, more recent findings suggest a dynamic, tissue-specific, and context-dependent regulation process, with DNA methylation better maintained, or more frequently de-methylated, at varying rates across the genome (Broche et al., 2021), and hypermethylation of gene bodies (Aran et al., 2011), or promoter and CpG islands frequently promoting gene expression (de Mendoza et al., 2022).
DNA methylation can store information about early-life feeding to permanently affect metabolism (Vickers, 2014), for example through the altered expression of *insulin-like growth factor 2* (*Igf2*) (Waterland et al., 2006). Furthermore, DR effectively and persistently delayed age-related DNA methylation changes in rhesus monkey whole blood (Maegawa et al., 2017) and mouse hepatic tissue (Cole et al., 2017; Hahn et al., 2017; Keleher et al., 2018; T. Wang et al., 2017). DNA methylation in the WAT can also be altered by diet (Benton et al., 2015; Perfilyev et al., 2017), with DNA methylation marks affecting adipogenesis, adipocyte differentiation and function, and its dysregulation inducing metabolic diseases (Barajas-Olmos et al., 2018; Castellano-Castillo et al., 2019; X. Ma & Kang, 2019).
The impact of DR on the DNA methylation landscape and any functional consequences in WAT are not known, but these findings highlight DNA methylation as a putative information storage of past feeding. In consequence, we asked if the DNA methylation landscape mediated by life-long feeding can explain the previously reported ALDR and DRAL memory in the WAT. We profiled DNA methylation of the WAT of 27-month-old female mice from the 4 feeding conditions (long-term AL, long-term DR, ALDR switch at 24 months, and DRAL switch at 24 months), and of the liver of the ALDR and DRAL mice using Whole genome bisulphite sequencing (WGBS) and integrated our findings with transcriptional data from the corresponding mice (Hahn et al., 2019; Hahn et al., 2017). We thus profiled the DNA methylation landscape of WAT induced by life-long AL or DR feeding and its association with transcriptional profiles, and assessed switch-resistant DNA methylation patterns in response to the late-life ALDR and DRAL switches, in both the WAT and liver to determine if DNA methylation patterns could explain the WAT-exclusive transcriptional memory. Finally, we asked for specific diet-switch-induced DNA methylation changes to assess its potential role in transcriptional regulation in the WAT and the liver.

2.3 Results

2.3.1 DR alters the DNA methylation landscape of the WAT

We initiated our analysis by evaluating the effects of life-long DR on the DNA methylome of the WAT. We performed WGBS on WAT isolated from 27-month-old C3B6 F1 hybrid female wild-type mice. 31.8 million CpGs passed our filtering criterion of three observations in all conditions. To avoid biasing our analysis towards CpG-rich regions, we used a sliding binning approach with each bin covering 50 CpGs and overlapping adjacent bins by 25 CpG according to (Hahn et al., 2017; von Meyenn et al., 2016). This generated 1,273,220 bins with a median bin size of 3336 bp, and a generally high methylation level of 77.29% in AL and 77.58% in DR conditions (Figure 2.1A) comparable to methylation levels in human whole blood and adipose tissue samples (Busche et al., 2015), and mouse liver (Cole et al., 2017; Hahn et al., 2017). Of all bins, 10,676 were differentially methylated upon DR, 6211 hypomethylated and 4465 hypermethylated (Figure 2.1B). Therefore, DR-induced DNA methylation changes in the WAT are larger as compared to changes reported in the liver (Hahn et al., 2017). The global hypomethylation in the WAT could depict a mild increase in global accessibility of the DNA, which is in line with the down-regulation of the two *DNA methyltransferases Dnmt1* and *Dnmt3a* (Figure 2.1C) (Hahn et al., 2019), which are involved in the maintenance and *de novo* methylation of DNA, respectively (Fatemi et al., 2002; Hsieh, 2005).
We next investigated the localization of DMRs on functional genomic elements such as promoters, gene bodies, exons, or introns (Figure 2.1D), to classify their regulatory potential. We considered a DMR and a genomic element to be associated when they overlapped by at least 30% of the DMR length. DMRs were significantly (Fisher's exact test, $p < 0.05$ in all cases) under-represented in intergenic regions (fold change < 0.89) but enriched over CGIs (fold change > 1.27), promoter regions (fold change > 1.13), and gene bodies (fold change > 1.11), suggesting that they might play a role in the regulation of gene expression. In total, DMRs overlapped with 3178 gene bodies with a significant overlap of 979 genes that were both differentially expressed (= differentially expressed genes, DEGs) and differentially methylated in their gene body in response to DR (Figure 2.1E). In summary, we find that DR induced DNA methylation changes, potentially by the regulation of *Dnmt1* and *Dnmt3a*, which in consequence could alter

the accessibility of the DNA. As DMRs are enriched on functional genomic elements and associated with differential expression, the methylome changes are thereby potentially associated with transcriptional regulation.

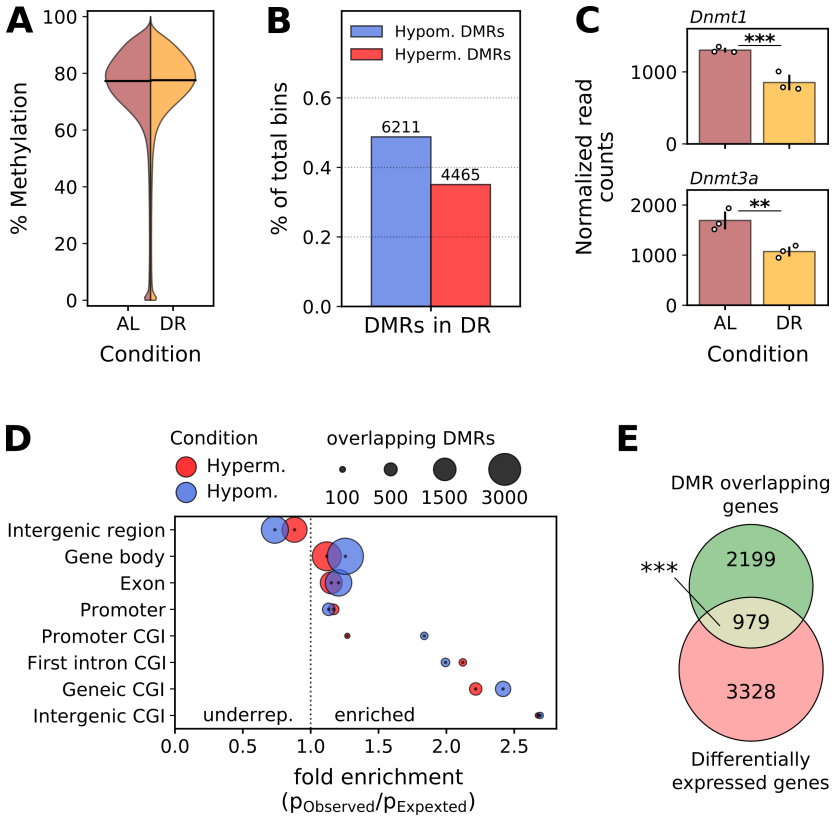


Figure 2.1 | **DNA methylation differences in aged white adipose tissue of long-term DR-fed and AL-fed mice.** (A) Global bin DNA methylation level distribution (n = 1,273,220). Horizontal lines indicate the median. (B) Number of DMRs between DR versus AL feeding (χ^2-test, $p_{adj} < 0.05$; minimum region DNA methylation difference > 10%) normalized on total bin number. (C) Transcription levels of *Dnmt1* and *Dnmt3a* (normalized read counts; **: $p_{adj} < 0.01$, ***: $p_{adj} < 0.001$). (D) Enrichment of hyper- and hypomethylated DMRs overlapping genomic features (overlapping base pairs >30% of bin or genomic feature length) against all bins as control background. The enrichment was significant for all displayed features (two-sided Fisher's exact test, $p < 0.05$). (E) Venn diagram of differentially expressed genes ($p_{adj} < 0.05$) and genes that overlap with DMRs (***: two-sided Fisher's exact test, $p < 0.001$).

2.3.2 DR induces differential methylation and transcription of key genes of lipogenesis

To further investigate the functional association of genes whose expression change was associated with differential methylation, we grouped the 979 DMR overlapping DEGs genes into hyper-/hypomethlated and up-/down-regulated (Figure 2.2A). Globally, there was only a weak but significant

correlation of expression and methylation (r = -0.16, p < 0.001). However, a functional gene ontology (GO) term analysis revealed a significant enrichment of GO terms only in the inversely regulated, i.e. 298 hypomethylated and up-regulated genes, and 264 hypermethylated and down-regulated genes. Genes that were significantly hypomethylated and up-regulated were enriched for functional GO terms related to metabolic processes such as "organic acid metabolic process", and "lipid metabolic process" (Figure 2.2B) and included lipogenesis genes such as *ATP citrate lyase* (*Acly*), *Malic enzyme 1* (*Me1*), *Fatty acid synthase* (*Fasn*), *Acetoacetyl-CoA synthetase* (*Aacs*), *Acetyl-CoA carboxylase beta* (*Acacb*), and *Glycerol-3-phosphate acyltransferase 1* (*Gpam*). All of them are targets of the *de novo* lipogenesis master regulator and transcription factor *Sterol regulatory element binding protein 1* (*Srebf1*) (Kuriyama et al., 2005) and displayed a significant whole gene body hypomethylation (Figure 2.2D, 2.2E, $p_{adj} < 0.001$ for *Me1*, *Fasn*, *Acly*, *Gpam*; $p_{adj} < 0.01$ for *Aacs*, $p_{adj} < 0.05$ for *Acacb*). *Srebf1* itself was also transcriptionally up-regulated and its gene body hypomethylated ($p_{adj} < 0.05$), but with no associated significant DMRs (Figure S2.1A, S2.1B). The inverse association of whole-gene-body DNA methylation and transcriptional change of these genes in response to DR was also previously reported in the liver (Hahn et al., 2017), but it was inverse, as DR induced hypermethylation of lipogenesis-related gene bodies which was associated with their down-regulation. This strongly suggests a regulative role of DNA methylation in the regulation of the lipogenesis in both tissues, potentially based on whole-gene-body methylation, but regulating lipogenesis gene expression in opposite directions in the liver and WAT.

The 264 genes significantly down-regulated and hypermethylated upon long-term DR were enriched for immunity-related terms such as "regulation of immune response" and signalling terms including "regulation of signaling" and "cell surface receptor signaling pathway" (Figure 2.2C), a pattern also exhibited by down-regulated genes with hypermethylated promoters (Figure S2.1C), that could indicate a DR-associated reduced tissue inflammation of the WAT partly mediated by DNA methylation marks. As before, some down-regulated and hypermethylated genes exhibited a DNA methylation pattern spanning most of their gene bodies (Figure 2.2D, 2.2E). Among these genes were the related transcription factors *Interferon regulatory factor 9* (*Irf9*) and *Irf8*, and *Lymphoblastic leukemia-derived sequence 1* (*Lyl1*, $p_{adj} < 0.001$). *Irf9* is also associated with metabolic control via interaction with proliferator-activated receptor alpha (PPARA) in the liver (L. Wang et al., 2013), whereas *Lyl1* deficiency was recently linked to adiposity

and accelerated adipocyte differentiation (Hussain et al., 2021). Thus, DR-mediated DNA methylation changes were associated with the regulation of some target genes with tissue-specific functionality.

The 173 DEGs with DMRs in their promoter region (Figure 2.2F) showed a mild negative correlation between mean DMR methylation change and transcriptional levels (Pearson correlation = 0.37, $p < 0.001$), in line with the inverse correlation for promoter regions for example in the context of cancer (Ando et al., 2019), mechanistically achieved via interfering with transcription factor binding (Domcke et al., 2015). Up-regulated genes with hypomethylated promoter regions include *Phosphoenolpyruvate carboxykinase 1* (*Pck1*), which plays a role in insulin signalling and free fatty acid metabolism (Millward et al., 2010), and *Melanocortin 2 receptor accessory protein* (*Mrap*), associated with lipolysis function and important in adipocyte responses to dietary conditions (Berruien & Smith, 2020; X. Zhang et al., 2018). In contrast, *Acyl-coenzyme A thioesterase 9* (*Acot9*), associated with mitochondria function, fatty acid oxidation, and high-fat diet-induced weight gain (Steensels et al., 2020; Tillander et al., 2017), was down-regulated and its promoter was hypermethylated (Figure 2.2G, 2.2H).

In summary, hypermethylated and up-regulated genes were involved in lipid and energy metabolism including lipogenesis, and hypermethylated and down-regulated genes were associated with immune responses, potentially lower inflammatory tone.

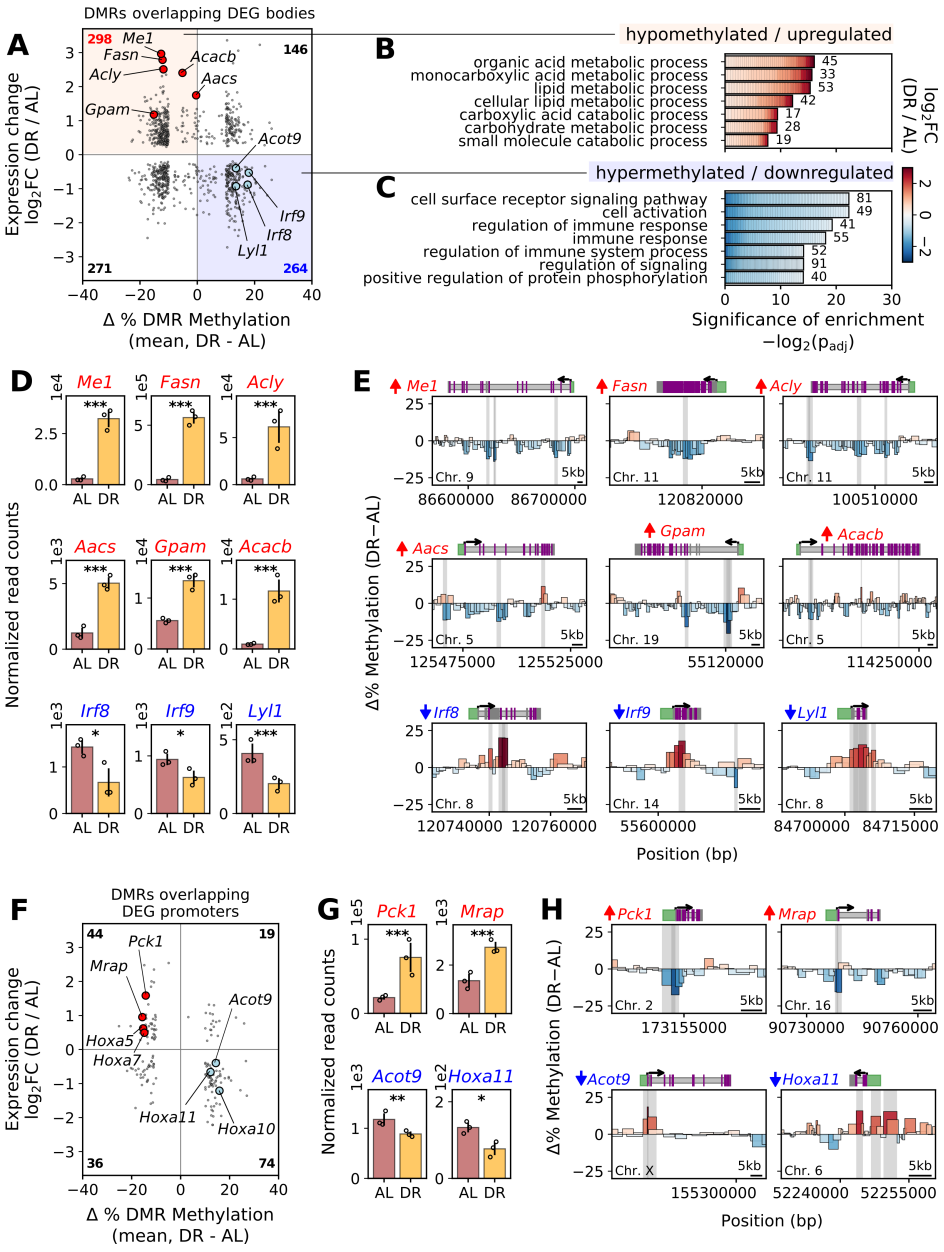


Figure 2.2 | **Gene level methylation and expression responses to long-term DR in aged white adipose tissue.** (A) Methylation level (as mean of all overlapping DMRs) and expression level changes of all 979 DEGs that overlap DMRs. (B, C) Top enriched GO terms by p_{adj} of gene sets in highlighted quadrants of (A). The number of genes of the enriched terms is annotated and expression level changes are displayed within each bar. (D) Transcription level changes (normalized read counts, *: $p_{adj} < 0.05$, **: $p_{adj} < 0.01$, ***: $p_{adj} < 0.001$) of chosen genes with an inverse trend of gene body methylation and transcription levels. (E) Methylation pattern changes over gene bodies of the same genes. DMRs are highlighted in gray within the tracks. Gene features are annotated on top of each track (green: promoter region (TSS -3000 bp, +100 bp); purple: coding sequence (CDS); light grey: intronic region, dark gray: untranslated region). (F) Mean DMR methylation level and expression level changes of all 173 DEGs with DMRs overlapping their promoters (TSS -3000 bp, +100 bp). (G) Transcription level changes of chosen genes with an inverse trend of promoter methylation and transcript levels. (H) Methylation pattern changes of the same genes. Top-left numbers in (E) and (G) reflect the exponential of 10 of y-axis values.

2.3.3 DR induces differential methylation of Hox clusters and is associated with HoxA gene regulation

Among DEGs with altered promoter methylation by long-term DR and inverse transcriptional regulation, we identified four of the twelve protein coding *Homeobox cluster A* (*Hoxa*) genes. *Hoxa5* and *Hoxa7* were up-regulated and their promoter regions were hypomethylated, and *Hoxa10* and *Hoxa11* were down-regulated and their promoter regions were hypermethylated (Figure 2.3A, 2.3B, 2.3H). Hox genes are widely active in adipose tissue (Cantile et al., 2003), and *Hoxa5*, *Hoxa10*, and *Hoxa11* are differentially expressed between adipose tissue deposits, defining their identity by steering cell differentiation (Gesta et al., 2006; Karastergiou et al., 2013; Procino & Cillo, 2013; Yamamoto et al., 2010). *Hoxa5* specifically was silenced following a high-fat diet in young adult mice, affecting adipose differentiation, and up-regulation of *Hoxa5* was suggested to potentially negatively regulate the formation of adipose tissue by inhibiting angiogenesis (W. Cao et al., 2018; Parrillo et al., 2016). *Hoxa5* also was more recently found to be a candidate gene modulating epigenetically induced adipocyte differentiation (Holzman et al., 2021; Kumar et al., 2021). Interestingly, three hypomethylated DMRs in the *Hoxa5* promoter overlapped a CCCTC-Binding Factor (CTCF) binding site (Figure S2.2A), and CTCF is associated with shaping the DNA methylation landscape (Damaschke et al., 2020). Collectively, these findings suggest a methylation-steered role of *Hoxa5* function in the WAT in a dietary context.

We next tested the genomic location of the HoxA cluster for DMR enrichment and identified a 16.6 fold enrichment ($p < 0.001$) of DMRs in the HoxA region compared to all bins as background (Figure 2.3A, 2.3B, 2.3F). Interestingly, we observed significant enrichment of DMRs also in HoxB, HoxC, and HoxD clusters (Figure 2.3C-F), but with statistically significant differential gene expression exclusive to the four HoxA cluster genes (Figure 2.3H). In contrast, DMR enrichment in the liver was only present in the HoxA cluster, and expression changes in the liver were comparatively mild (Figure 2.3G, S2.2B-E), indicating that a role for epigenetic changes in Hox gene regulation is exclusive to the WAT, and most pronounced in the HoxA cluster. To further define the putative functions of the DR-mediated Hox cluster changes, we investigated the cell-type-specific expression of Hox genes using the publicly available *Tabula muris* single-cell sequencing dataset (Schaum et al., 2018) and found them to be expressed mainly in adipose mesenchymal stem cells (MSCs) and endothelial cells (Figure 2.3I).

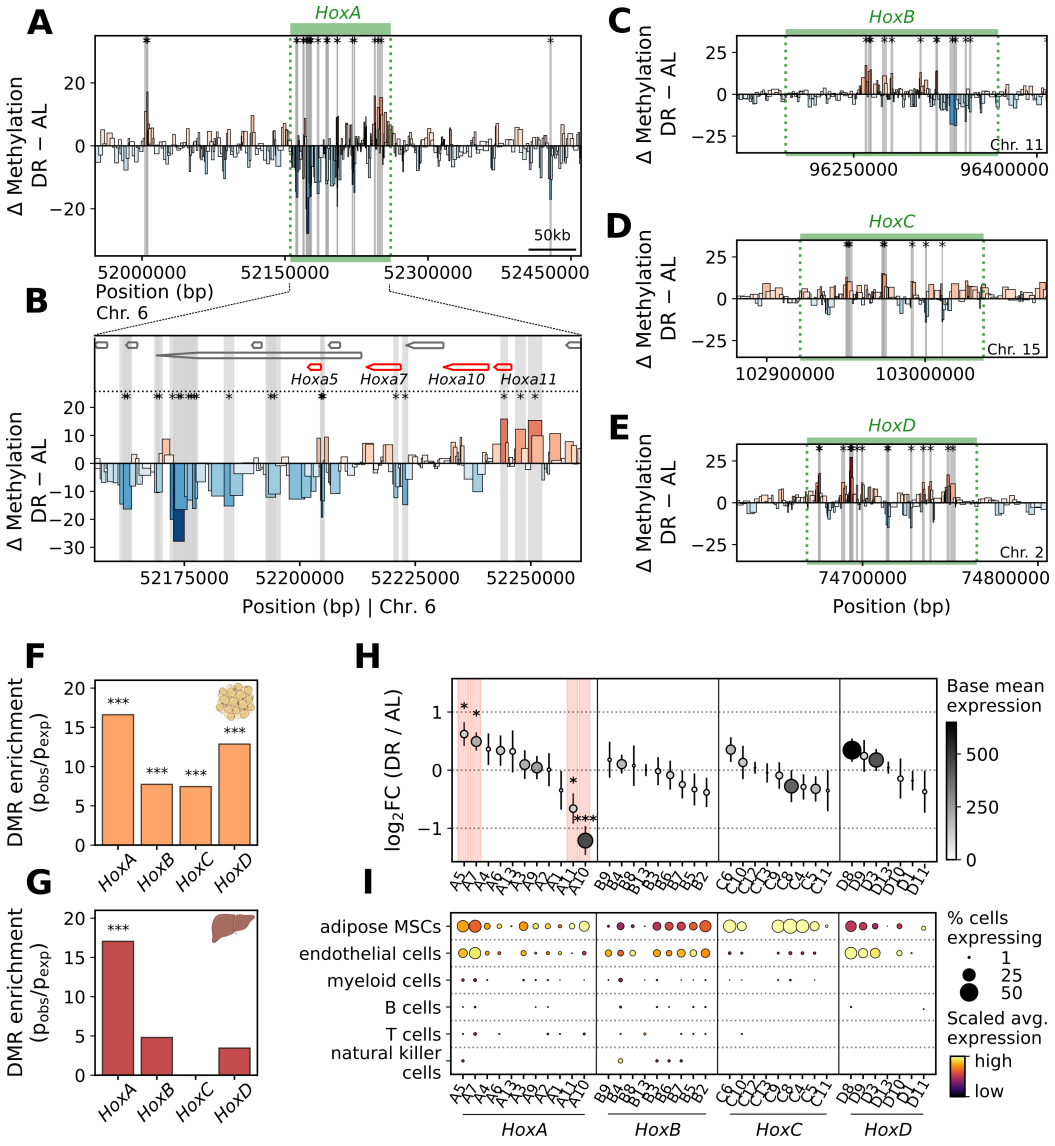


Figure 2.3 | **Hox cluster DNA methylation and Hox gene expression in aged mouse liver and white adipose tissue.** (A) WAT HoxA cluster methylation pattern changes between long-term AL and DR feeding with DMRs highlighted in grey. (B) Detail-view of the HoxA cluster region with highlighted HoxA gene positions. Differentially expressed HoxA genes are highlighted in red ($p_{adj} < 0.05$). (C-E) WAT methylation pattern changes between long-term AL and DR at the loci of the Hox clusters B, C, and D. (F, G) Fold enrichment of DMRs on Hox clusters in WAT and liver (against all available bins as control background, ***: two-sided Fisher's exact test $p < 0.001$). (H) Expression level changes of Hox genes in WAT of AL and DR fed mice (*: $p_{adj} < 0.05$, ***: $p_{adj} < 0.001$). (I) Single-cell expression profile of Hox genes of FACS sorted fat cells of the publicly available *Tabula Muris* dataset.

This is in line with the Hox gene function of defining fat deposit identity. In summary, we suggest that DR-mediated DNA methylation changes in the Hox cluster could ultimately steer Hox gene expression to control cell differentiation in response to diet also in aged WAT.

2.3.4 Persistent DNA methylation in late-life dietary switches does not explain the global transcriptional memory

There was a transcriptional and metabolic memory of diet in the WAT, but not the liver, after dietary switches at 24 months of age from AL to DR (ALDR), and a less pronounced one also in DRAL (Hahn et al., 2019). We investigated if this memory effect could be a consequence of persistent DNA methylation patterns. In the WAT, because there were 4 dietary conditions, fewer CpGs passed the coverage filtering, and we retained 23.5 million CpGs forming 939,928 bins with a median bin size of 4,647 bp. Notably, the decrease in global resolution also reduced the number of DMRs we identified in DR and AL from the 10,676 seen previously to only 4,606. In the liver, we integrated the diet switch WGBS data with life-long AL and DR data (Hahn et al., 2017) and generated 1,280,220 bins spanning 32 million covered CpGs with a median bin size of 3,289 bp.

We found 420 DMRs (9.1%) in the WAT and 638 DMRs (35.3%) in the liver that were present in the AL vs. DR contrast and conserved in the ALDR vs. DR contrast, thus exhibiting an AL feeding memory. In contrast, 844 DMRs (18.3%) in the WAT and 369 DMRs (20.3%) in the liver exhibited a DR feeding memory (Figure 2.4A). Thus, at least a part of the DNA methylome was not altered by late-in-life dietary switches, regardless of the feeding switch direction. Furthermore, most genes with switch-resistant expression had no association with persistent DNA methylation marks, as in the WAT only 38 of the 1978 genes with AL feeding memory, and 18 of the 600 genes with DR feeding memory, overlapped switch-resistant DMRs (Figure 2.4B, 2.4C). Hence, DNA methylation did not globally explain the previously observed transcriptional memory.

We next investigated individual genes with a "shared" DNA methylation and transcriptional memory, as they could promote functionality in response to late-in-life dietary switches. In the WAT, genes with a shared AL feeding memory included the four lipogenesis genes *Me1*, *Acly*, *Gpam*, and *Fasn* (Figure 2.4D, 2.4E, S2.3). All of them had a distinct DNA methylation pattern across their whole gene body in AL which was maintained following an ALDR switch. In contrast, in the liver we observed an uncoupling of DNA methylation and transcriptional memory, with key genes of lipogenesis exhibiting whole-gene-body, persistent hypermethylation (Figure S2.4A, S2.4B) but remaining transcriptionally responsive (Figure S2.4C). Aside from lipogenesis genes, another candidate for a shared memory was *Tissue inhibitor of metalloproteinase-4* (*Timp4*), an extracellular matrix (ECM) gene known for

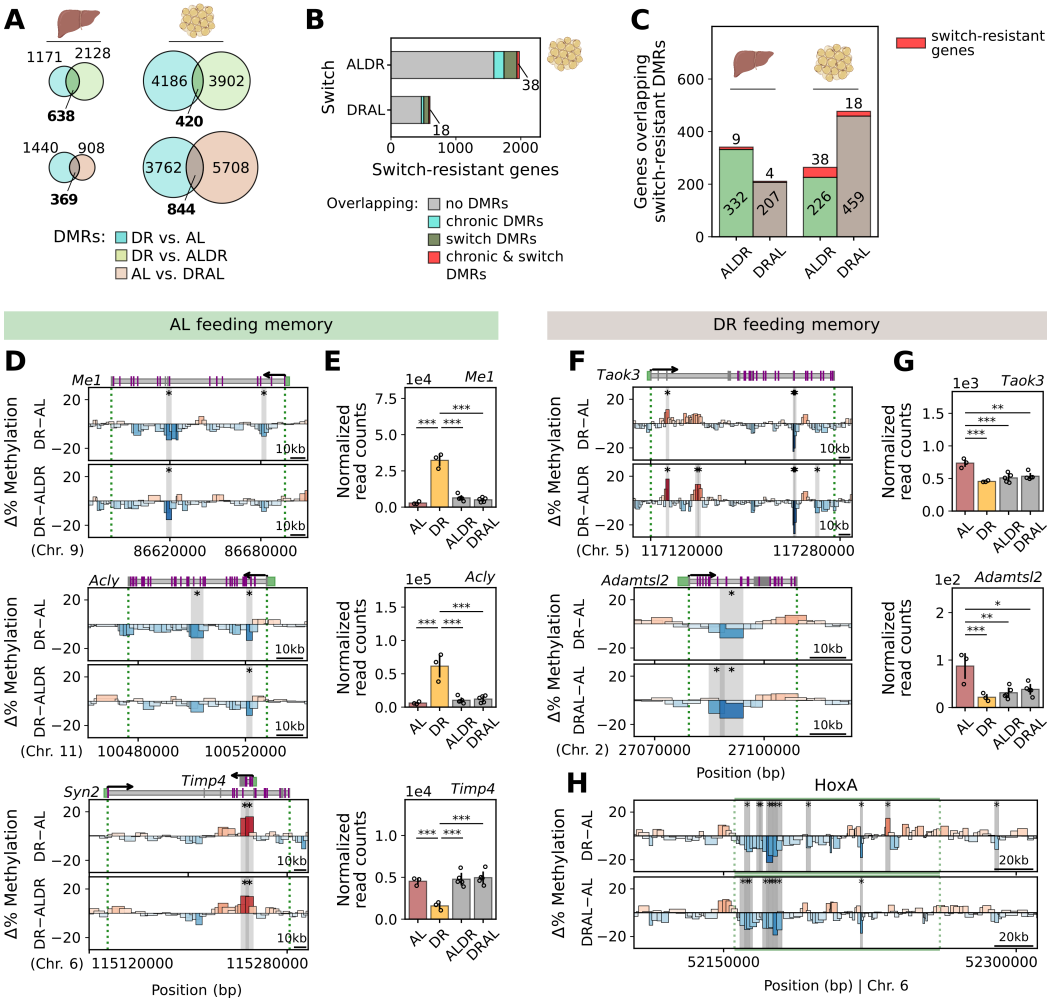


Figure 2.4 | **Dietary choices introduce persistent DNA methylation marks which rarely correlate with transcriptional memory.** (A) Shared DMR numbers between the long-term feeding contrast (AL vs. DR) and long-term to switches contrast (AL vs. DRAL; DR vs. ALDR). (B) Switch resistant genes (shared set of DEGs from AL vs. DR to AL vs. DRAL, or from AL vs. DR to DR vs. ALDR) and their overlap with DMRs in the switch condition, and with switch-resistant DMRs. (C) Number of genes that overlap switch-resistant DMRs in the liver and the WAT. Of these, the number of switch-resistant genes is annotated. (D) Chosen genomic regions with persistent methylation patterns across long-term feeding and the WAT ALDR switch. DMRs (χ^2-test, $p_{adj} < 0.05$; minimum region methylation difference > 10%) are highlighted in methylation tracks in grey and by asterisks. (E) Respective expression level changes. (*: $p_{adj} < 0.05$, **: $p_{adj} < 0.01$, ***: $p_{adj} < 0.001$). (F, G) Methylation pattern changes and expression level changes of genes with persistent methylation patterns in DRAL switches. (H) Methylation pattern changes of the HoxA cluster. Top-left numbers in (E) and (G) reflect the exponential of 10 of y-axis values.

its importance in the ECM turnover process, a major axis for WAT health (Mariman & Wang, 2010). *Timp4* deficiency was also reported to rescue high-fat diet-induced obesity effects in WAT due to defective lipid absorption (Sakamuri et al., 2017), possibly indicating a DR-mediated role for *Timp4* in the fat deposit reduction process.

Only 18 genes had a shared memory of DR feeding. These genes included

TAO kinase 3 (*Taok3*) and *A disintegrin and metalloproteinase with thrombospondin motifs like 2* (*Adamtsl2*) (Figure 2.4F, 2.4G). *Taok3* deficiency was previously observed to improve glucose clearance and affect insulin sensitivity in the WAT in high-fat diet-fed mice from nine to twelve weeks of age (De Coninck et al., 2018; Maes et al., 2018), and *Taok3* hypomethylation was associated with childhood obesity (R. C. Huang et al., 2015). *Adamtsl2* function in the adipose tissue is still under-explored, however, a related gene, *Adamts1*, was found to be essential for adipocyte differentiation (S. Z. Chen et al., 2016) which could at least suggest comparable roles for *Adamtsl2* which are mediated by DR. We also observed a DR methylation memory of the hypomethylated flank in the HoxA cluster (Figure 2.4H). While we could not link this to altered gene expression for HoxA genes, the refractory methylation landscape is in line with the finding of pronounced changes of the Hox cluster methylation with life-long feeding, and further supports a yet unknown diet-mediated role of Hox cluster methylation in the WAT.
In summary, we observed signs of refractory DNA methylation in both late-life switches, whereas they did not explain the global transcriptional feeding memory. However, the persistent transcriptional regulation of some genes with important WAT function was associated with persistent DNA methylation, indicating that DNA methylation could mediate part of a transcriptional feeding memory effect.

2.3.5 Late-life dietary switches induce DNA methylation changes that are mostly uncoupled from expression changes

DNA methylation was globally not associated with a transcriptional feeding memory, but could have a role in steering more short-term responses to late-life dietary switches. Hence, we investigated DNA methylation changes induced by late-life dietary switches and their functional associations. In the WAT we identified 6,728 DMRs in ALDR and 5,840 in DRAL, with a strong tendency towards hypomethylation over hypermethylation. In the liver, changes were less pronounced with 1,656 DMRs in ALDR and 959 DMRs in DRAL, however still with a higher proportion of hypomethylated DMRs (Figure 2.5A). This indicates that a late-life dietary switch likely increases the accessibility of the DNA via hypomethylation. However, in both tissues, this pattern was not explained by altered expression patterns of DNA methylation enzymes (Figure S2.5), which suggests either the presence of refractory hypomethylation patterns following earlier enzymatic expression changes or a passive loss of CpG methylation (Ginno et al., 2020; Kagiwada et al.,

2013). Matching this, de-methylation was found to be a frequent reaction in forcefully methylated CpGs (de Mendoza et al., 2022), suggesting that the de-methylation of DMRs, instead of methylation maintenance, could be a more efficient way to adapt to novel environment stress.

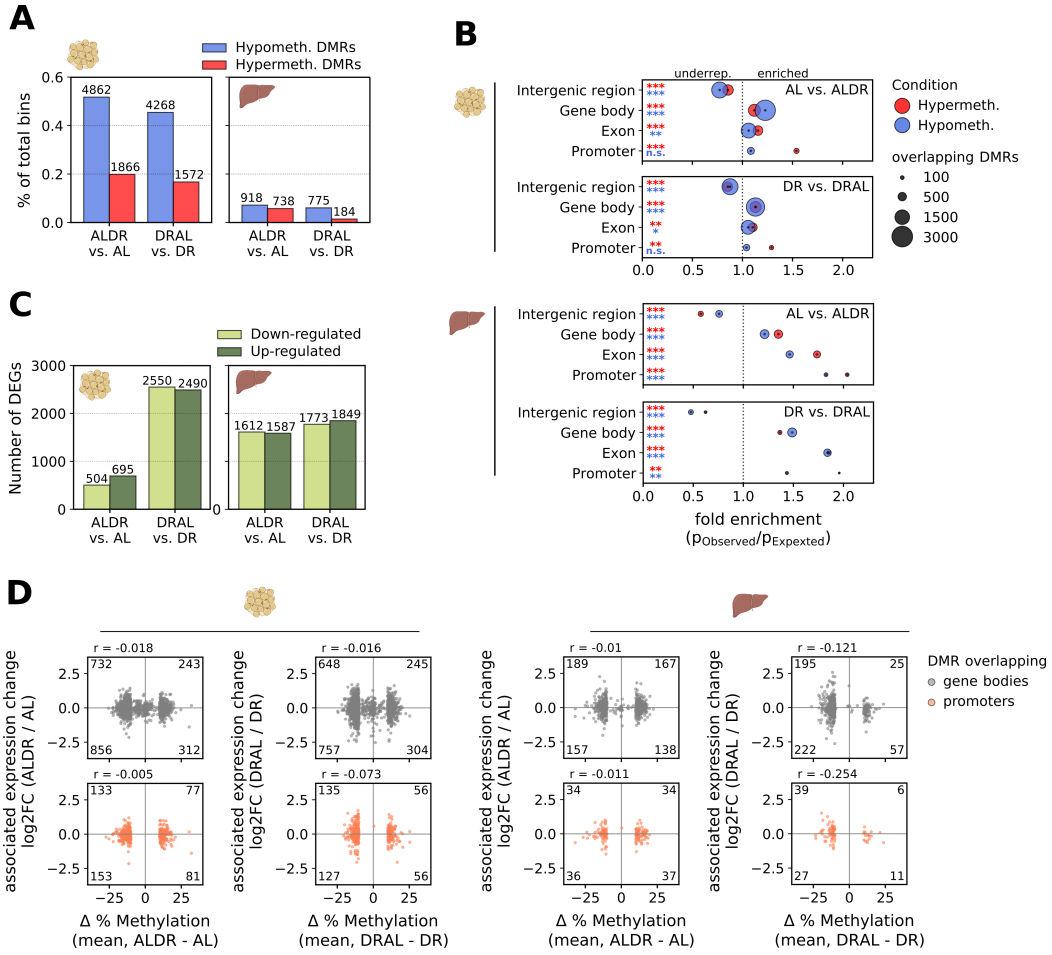


Figure 2.5 | **Late-life dietary switches induce DNA methylation changes that are uncoupled from direct transcriptional regulation.** (A) Number of DMRs in WAT and liver (numbers above bars) in late-life dietary switches (ALDR to AL; DRAL to DR), additionally normalized on total bin number. (B) Enrichment of hypermethylated and hypomethylated DMRs overlapping genomic features (overlapping base pairs >30% of bin or genomic feature length) against all bins as control background (two-sided Fisher's exact test; n.s.: not significant; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$). (C) Number of differentially expressed genes ($p_{adj} < 0.05$) in the WAT and liver in both late-life switches. (D) (A) DNA methylation level (as mean of all overlapping DMRs) and expression level changes of all genes that overlap DMRs on their gene bodies (gray) and on promoters (orange). Numbers of genes in each quadrant and Pearson's correlation coefficient (r) are annotated.

DMRs were significantly under-represented in intergenic regions but significantly enriched on gene bodies, exons, and promoters (except for hypomethylated promoters in WAT) (Figure 2.5B), suggesting a general regulatory potential. Thus, we next asked if changes in the DNA methylation landscape were associated with differential expression. However, there was no trend

of association between hypomethylation and global up-regulation or vice versa in response to dietary switches in either tissue, as both showed approximately equal numbers of up- and down-regulated genes (Figure 2.5C), nor did genes with their gene body or promoter overlapping DMRs show a directed correlation of methylation change and expression change in either tissue ($|r| < 0.3$ in all cases), with approximately equal numbers of up- and down-regulated genes in hyper- and hypomethylated genes (Figure 2.5D). Together with the absence of a global DNA methylation-promoted transcriptional memory, our findings collectively suggest an uncoupling of switch-induced DNA methylation and expression changes.
As we observed strong alterations in the WAT DNA methylation landscape unconnected to differential expression, we probed the potential origins. We focussed on genes that overlapped DMRs but were not differentially expressed. Hypomethylated DMRs overlapped with 1,412 genes in ALDR and 1,255 genes in DRAL (388 genes shared, $p_{adj} < 0.001$), and hypermethylated genes overlapped with 373 genes in ALDR and 405 genes in DRAL (56 shared, $p_{adj} < 0.001$) (Figure S2.6A). The resulting gene sets were involved, for examle, in "cell migration", "actin filament-based process", and "animal organ morphogenesis" (Figure S2.6B). This GO profile was shared across hyper- and hypomethylated genes, and all 4 dietary regimes, but was mostly absent in DEGs (Figure S2.6C). This indicates the presence of DNA methylation mediated processes that are not directly reflected on the gene expression level.

2.4 Discussion

The DNA methylome in WAT has important roles in defining fat depot identities (Bradford et al., 2019), in regulating various metabolic processes (Benton et al., 2015) and in metabolic disease including metabolic syndrome (Castellano-Castillo et al., 2019) and type 2 diabetes (Barajas-Olmos et al., 2018; Orozco et al., 2018), and in ageing (Rönn et al., 2015). We investigated the role of DNA methylation in life-long DR-mediated transcriptional changes, and whether those DNA methylation landscapes could explain the presence of a transcriptional memory previously found in WAT in response to late-in-life dietary switches.

In response to both life-long DR and late-life dietary switches, DMRs were enriched on functional genomic elements including exons, promoters,

gene bodies, and CGIs. This non-random distribution of DMRs suggests a regulatory role. However, the functional interpretation of the altered DNA methylation landscape is not trivial, as the interplay of DNA methylation and transcriptional regulation is complex (de Mendoza et al., 2022; X. Ma & Kang, 2019; Moore et al., 2013; Ziller et al., 2013). Importantly, DNA methylation can affect regulation of distal genomic elements (Aran et al., 2013), for example by mechanically altering DNA accessibility (Basu et al., 2022). The prediction of such indirect effects remains difficult, and we therefore limited our functional interpretations to altered DNA methylation spatially close to genes that were differentially expressed.

We first asked for the functional association of DNA methylation changes induced by life-long DR, and found that it induced an up-regulation of hypomethylated key genes of lipogenesis and energy homeostasis. Specifically *Acly*, *Fasn*, *Me1*, *Gpam*, *Aacs* and *Acacb* were interesting candidates of DNA methylation-mediated regulation as they all including their upstream master regulator *Srebf1* showed a similar regulative pattern. In the liver, the same lipogenesis genes were reported to be hypermethylated and down-regulated (Hahn et al., 2017; Kuhla et al., 2014), which further supports that DNA methylation is inversely associated with lipogenesis gene expression across both tissues. In the WAT, up-regulation of lipogenesis in the WAT as a DR-response was already reported (Chujo et al., 2013; Fujii et al., 2017; Higami et al., 2004; Okita et al., 2012), and we find strong evidence for a regulative role of DNA methylation here, which is in line with growing evidence of an extensive role of epigenetic regulation in energy homeostasis and metabolic processes (Park et al., 2021).

Reduced tissue inflammation is a consequence of DR (Kökten et al., 2021). Following life-long DR, we found promoter and gene body hypermethylation of down-regulated genes involved in immunity and inflammation responses, suggesting that DNA methylation regulated WAT inflammatory processes. In line with this, DNA hypomethylation is frequently associated with increased inflammation (Das et al., 2021).

Finally, long-term DR caused large-scale DNA methylation alterations of genomic Hox regions, including loci of *Hoxa5*, *Hoxa7*, *Hoxa10*, and *Hoxa11*. Developmental genes, including Hox genes, are regulated by DNA methylation for example in skeletal muscle (Tsumagari et al., 2013; Turner et al., 2020), and also in the WAT as a response to a high-fat diet (Parrillo

et al., 2016). Hox genes are also differentially methylated and expressed between human adipocytes of different fat depots (Bradford et al., 2019) to determine fat pad identity. Two of our candidates, *Hoxa5* and *Hoxa10*, regulate WAT in obesity, and Hoxa5 is involved in adipocyte differentiation (W. Cao et al., 2018), with this process possibly linked to the WAT metabolism master regulator PPARG (Kumar et al., 2021). Furthermore, both genes alongside other Hox genes promote fat accumulation in differentiated adipocytes (Singh et al., 2016). Our observations indicate both that Hox genes are differentially expressed, potentially to steer adipocyte differentiation, function, and tissue composition, and that this process is associated with DNA methylation. Interestingly, the hypomethylation in the upstream flank of the HoxA cluster was persistent to a DRAL late-life dietary switch, thereby being a candidate for the associated DR feeding memory. This maintenance of hypomethylation was uncoupled from differential expression, but could still have minor regulatory effects on Hox gene expression that were missed because of the generally low expression of Hox genes in the WAT.

We found that the previously reported transcriptional memory for diet could not be explained by persistent DNA methylation patterns on a global scale. We only observed rare candidates of a shared DNA methylation and transcriptional memory for AL feeding in the WAT, which included *Me1*, *Acly*, *Gpam* and *Fasn*. We hypothesise that life-long AL induces a WAT-exclusive regulatory buffer of increased lipogenesis mediated by persistent DNA methylation. Thereby, the WAT-exclusive DNA methylation memory could enable the WAT to react to sudden dietary changes more robustly, as parts of the lipogenesis pathway would be soft-locked in a state which represents prior dietary inputs. Contrasting observations in the WAT, in the liver, lipogenesis genes showed a persistent hypermethylation following AL, but remained transcriptionally responsive to a dietary switch. This could indicate that the transcriptional regulation of liver lipogenesis is less affected by DNA methylation. In line with this, the liver as metabolic hub of an organism has a significantly higher metabolic rate as the WAT (Z. M. Wang et al., 2010) and therefore could also require faster adaption to dietary changes. While DNA methylation could still have long-term suppressive effects on liver lipogenesis, they could be outweighed by additional regulative factors.

Finally, we observed large-scale DNA methylation changes in response

to dietary switches that were uncoupled from gene expression changes. We found this surprising, as similarly to changes in response to long-term DR, late-life switch induced DMRs were still mostly hypomethylated, and enriched on functional genomic elements. Interestingly, genes with DNA methylation changes in the WAT were functionally enriched for tissue remodelling in the WAT. As we only measured DNA methylation and transcriptional changes after the adipose tissue recomposition by weight gain or loss took place (Hahn et al., 2019), we hypothesized that these DNA methylation marks could have had a prior function to organize short-term transcriptional control of tissue reorganization. In consequence, the observed uncoupling of DNA methylation and transcriptional changes could indicate that transcriptional regulation adapts to dietary switches immediately, while resetting the DNA methylation patterns either requires a longer time or happens incompletely. Matching this assumption, more immediate transcriptional changes in structural genes were reported in datasets reflecting short-term dietary alterations, including in whole nematodes 4 days post a dietary switch (Rollins et al., 2019), pig fat following 3 days of fasting (Lkhagvadorj et al., 2009), or human subcutaneous adipose tissue after 28 days of caloric reduction (Clément et al., 2004; Viguerie et al., 2005). These processes could be sufficient to induce and complete regulatory processes, rendering persistent DNA methylation marks that are uncoupled from transcriptional changes as a remaining artefact of long-term regulation. Our study is limited to observations only three months after diet switches, and increasing the time-scale resolution and also the effective time after dietary switches could help to disentangle the observed dynamics of desynchronized regulation of DNA methylation and transcriptional changes in future.

2.5 Material and Methods

2.5.1 Mouse keeping, dietary protocols, and tissue collection

Mouse keeping and tissue collection were performed as described in (Hahn et al., 2019; Hahn et al., 2017). C3B6 F1 hybrid female wild-type mice were used, and DR was introduced at twelve weeks of age to avoid developmental effects. DR-treated mice received 60% of the food amount consumed by AL, applied stepwise with a reduction of the total amount of food by 10% per week for 4 consecutive weeks. Late-life diet switches from AL to ALDR and from DR to DRAL were introduced at 24 months of age. Mice were

sacrificed at the age of 27 months, corresponding to 24 months of AL or DR feeding, with subsequent three months of late-life switched feeding conditions if applicable, and organs of interest were immediately harvested and flash-frozen.

2.5.2 WGBS-sequencing and library preparation

DNA extraction and WGBS-sequencing, sequence trimming, alignment, deduplication, and methylation calling were performed as previously described in (Hahn et al., 2017) for the aged WAT of three animals each for AL, DR, ALDR, and DRAL conditions, and for the aged liver of three animals of DRAL feeding, and two animals of ALDR feeding. In addition, libraries of three aged AL and three aged DR liver samples from (Hahn et al., 2017) were acquired through GEO ID GSE92486.

2.5.3 Differential methylation analysis and annotation of switch-resistant DMRs

Differential methylation analysis was performed per tissue using a sliding binning approach as described in (Hahn et al., 2017) with the following changes and additions. Analogously to the previous study, CpGs were first filtered for a minimum of three observations per CpGs in all conditions. However, because the inclusion of each additional dietary condition group led to fewer CpGs passing this filtering, we performed differential methylation analysis for the long-term contrasts (WAT AL vs. DR / liver AL vs. DR) individually to achieve an increase in resolution before adding late-life diet switches in a second analysis. A newer release of SeqMonk (v1.45.1) was used as compared to the previous study. For the long-term comparison set of the WAT and liver, DMRs were identified for the contrast AL vs. DR. For the second comparison set including ALDR and DRAL conditions, DMRs were identified for all possible contrasts. In the second comparison set, switch-resistant DMRs were assumed if a DMR was shared between AL vs. DR and AL vs. DRAL (ALDR switch-resistant) or AL vs. DR and DR vs. ALDR (DRAL switch-resistant).

2.5.4 Enrichment of DMRs on functional genomic elements

To annotate genomic regions, we utilized annotation and positions as described in the following. Gene body positions including intron and exon positions were acquired from Ensembl *Mus musculus* mouse genome release 96. First introns were annotated by grouping all introns that are closest

to a gene body start. Unless stated otherwise, promoters were defined as 3000 bp upstream and 100 bp downstream of each gene start of the used Ensembl genome. CGI positions were acquired from (Illingworth et al., 2010) and classified into promoter CGIs, gene body CGIs, or first intron CGIs if they overlapped with the specific element and the overlap area was at least 50% of either the CGI or element length. All unannotated CGIs were finally annotated as intergenic CGIs, and every genomic region not covered by the above annotations was annotated as intergenic region. To test if overlapping DMRs are enriched on a genomic element, we first counted all DMRs that overlap with the genomic elements. However, because many genomic elements varied greatly in size and spread we went for a more conservative approach and counted an overlap only if a minimum of 30% of either the DMR or the overlapped feature are part of the overlapping area to reduce noise in our data. For DMR enrichment on Hox clusters, we count a DMR as overlapping on a genomic region if the overlap size is at least one bp. We went for this less conservative approach because of the relative shortness and less fragmentation of the respective regions as compared to all genomic regions. To evaluate a significant enrichment or under-representation of DMRs on a genomic region of specific genomic elements, we applied a two-sided Fisher's exact test on all DMRs overlapping with a feature or region, with all available bins as background.

2.5.5 Identification of genes with promoter or gene body methylation changes

We assumed differential methylation of a gene or promoter region if their genomic location overlapped with at least one DMR. Genes and promoters that overlapped with DMRs (AL vs. DR and AL vs. ALDR contrast, respectively) on their genomic location were annotated using the positional information of the Ensembl release. To focus only on regions with strong methylation changes, the methylation change per genomic element was calculated as the mean methylation change over all overlapping DMRs.

2.5.6 Differential expression analysis and annotation of switch-resistant genes

To ensure comparability between previously executed studies, count tables that were pre-processed and annotated as described in (Hahn et al., 2019; Hahn et al., 2017) were acquired for young and old AL and DR liver replicates (GEO IDs GSE92486) and for young and old AL and DR WAT

replicates and late-life switch ALDR and DRAL liver and WAT replicates (GEO ID GSE124772). Consequently, we obtained data of three replicates each for young and old WAT AL, WAT DR, liver AL, liver DR, liver ALDR, and liver DRAL. Furthermore, we obtained data of five replicates each for WAT ALDR and WAT DRAL. Differential expression analysis was executed per tissue by using custom R scripts, RStudio, and the following packages: Bioconductor (v3.11), DESeq2 (v1.28.2), org.Mm.eg.db (v3.11.4), and clusterProfiler (v3.16.1) (G. Yu et al., 2012). The p-values were adjusted by applying "Benjamini Hochberg" multiple testing correction implemented in DESeq2. Unless stated otherwise, DESeq2-normalized counts are used in figures of transcription levels. Unlike the described workflow in (Hahn et al., 2019), we did not apply a fragments per kilobase of transcript per million mapped reads filter to account for genes as "expressed", but counted them as "expressed" if they passed the independent filtering of DESeq2 (Love et al., 2014). This less restrictive approach increased the count of expressed genes but also the count of switch-resistant genes in our recalculation of switch-resistant genes in the WAT. Analogously to (Hahn et al., 2019), switch resistance was assumed if a DEG was shared between aged AL vs. DR and AL vs. DRAL (ALDR switch-resistant, AL feeding memory) or aged AL vs. DR and DR vs. ALDR (DRAL switch-resistant, DR feeding memory).

2.5.7 Single-cell dataset and investigation of cell-type specific expression

Pre-processed and annotated single-cell RNA-sequencing data of FACS sorted adipose tissue (Robject files accessed via https://doi.org/10.6084/m9.figshare.5821263.v3) was acquired from the publicly available *Tabula Muris* consortium dataset (Schaum et al., 2018). The dataset included scaled average expression values for expressed genes in sux different pre-annotated cell types. Using RStudio and the package Seurat (v3.2.2), values for scaled average expression and percent of expression per cell type were exported for all Hox genes present in our and the single-cell dataset. Data visualization was then performed using custom python scripts.

2.5.8 Bulk RNA-seq expression dataset and investigation of trends in ageing tissue

The pre-processed and annotated bulk RNA-sequencing raw count table of mouse tissues (accessed via https://doi.org/10.6084/m9.figshare.8286230.v1) was acquired from the publicly available *Tabula Muris* consortium

dataset (Schaum et al., 2020). Counts were normalized using RStudio and the packages Bioconductor (v3.11), and DESeq2 (v1.28.2) as described previously. Normalized counts of chosen genes were plotted as a function of origin tissue and age. To test rough trends of age-dependent incline or decline of expression in genes, a linear regression was performed using the python package scipy (v1.5.2) and the direction and significance of the regression slope were investigated.

2.5.9 GO term enrichment analysis and visualization

Significantly enriched GO terms were identified as previously described in (Srivastava et al., 2022), using the python package AGEpy (v0.8.2) (Metge et al., 2022) based on the DAVID api (D. W. Huang et al., 2009) and simplified using REVIGO (Supek et al., 2011). For the dotplot visualization, the top 22 unique GO terms p_{adj} value were identified by picking top GO terms from DR and ALDR groups alternatingly. For all performed enrichment analyses, all expressed genes in the respective tissue were used as background.

2.5.10 General data processing and visualization

Unless stated otherwise, data analysis and visualization was performed using python (v3.7.6), the packages matplotlib (v3.3.2) and seaborn (v0.11.1), pandas (v.1.2.0) and custom python scripts. Venn diagrams were generated using the package matplotlib-venn (v0.11.5). Density scatter plots were generated using the package datashader (v0.14.2). Icons of the WAT and liver tissues were taken from BioRender.

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2.7 Supplementary Figures

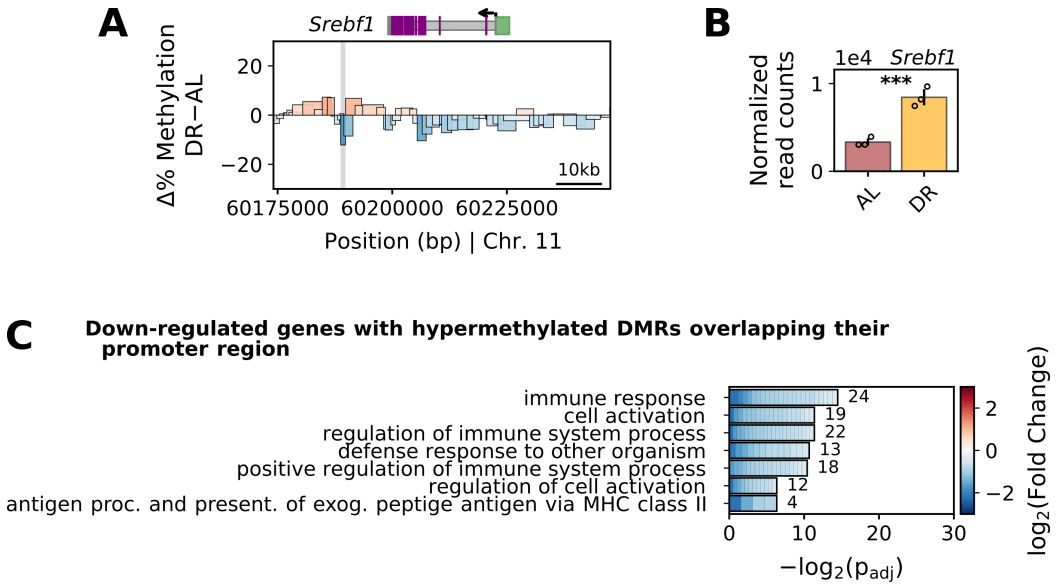


Figure S2.1 | **Additional data on DR-induced DNA methylation and expression changes in the WAT.** (A) Methylation pattern change of *Srebf1*. DMRs are highlighted in gray within the tracks. Gene features are annotated on top of each track (green: promoter region (TSS -3000 bp, +100 bp); purple: coding sequence (CDS); light grey: intronic region, dark gray: untranslated region). (B) Transcription level change (normalized read counts, ***: $p_{adj} < 0.001$) of *Srebf1*. (C) Top enriched GO terms of down-regulated genes with hypermethylated DMRs overlapping their promoter region. GO terms are chosen by p_{adj}. The number of genes of the enriched terms is annotated and expression level changes are displayed within each bar. Top-left numbers in (B) reflect the exponential of 10 of y-axis values.

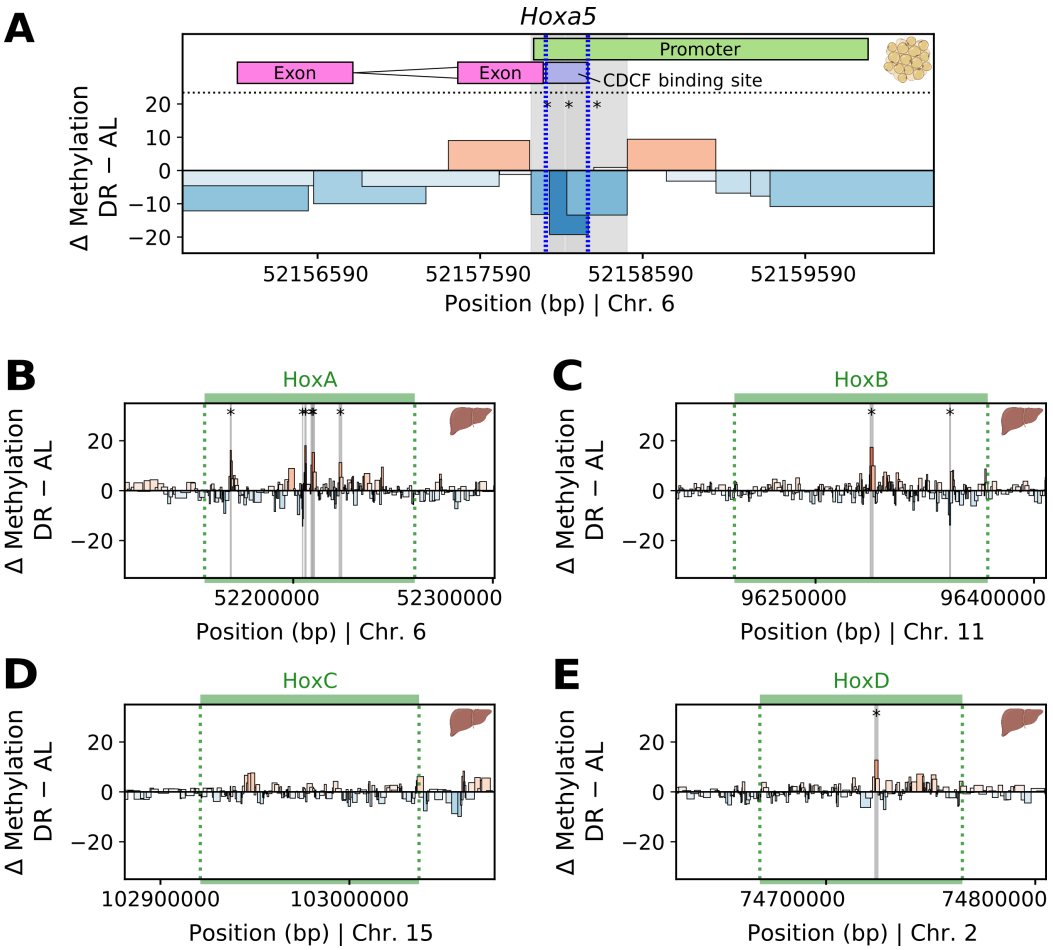


Figure S2.2 | **Additional data of DR-induced DNA methylation changes of Hox clusters in the WAT and liver.** (A) Detailed view of the methylation pattern changes of the *Hoxa5* locus in the WAT. DMRs are highlighted in grey and with asterisks. The location of a CTCF binding site (Ensembl regulatory build) (Zerbino et al., 2015), and the *Hoxa5* promoter (-100 bp to +3000 bp from TSS), exons, and introns are highlighted. (B-E) Methylation pattern changes in the liver at the genomic locations of Hox clusters A to D.

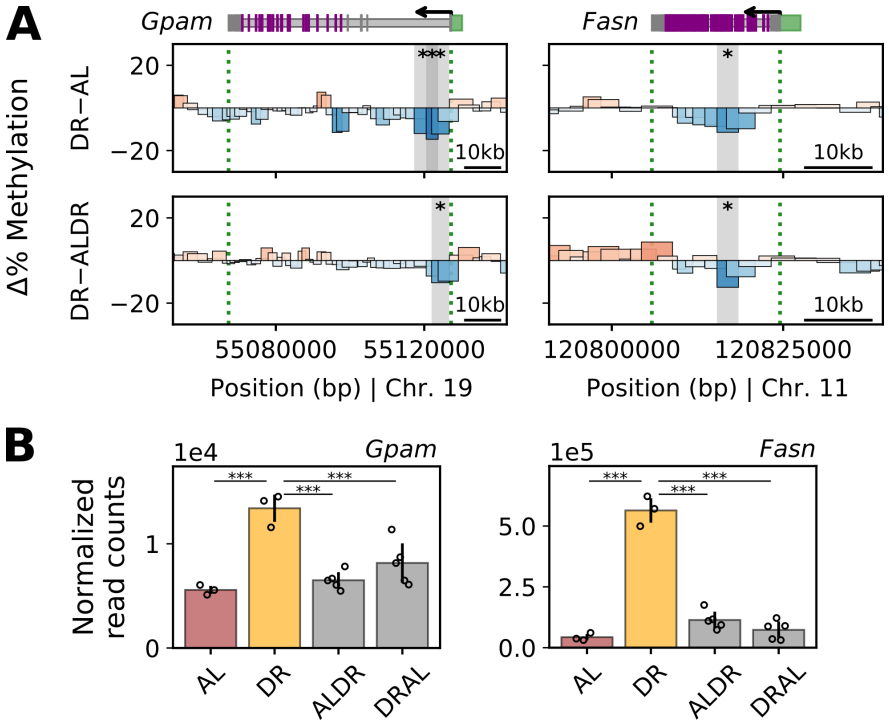


Figure S2.3 | **Additional lipogenesis genes with shared AL feeding memory in the WAT.** (A) Methylation pattern changes in DR vs AL and DR vs ALDR of *Gpam* and *Fasn*. DMRs (χ^2-test, $p_{adj} < 0.05$; minimum region methylation difference > 10%) are highlighted in methylation tracks in grey and by asterisks. (B) Respective expression level changes (***: $p_{adj} < 0.001$). Top-left numbers in (B) reflect the exponential of 10 of y-axis values.

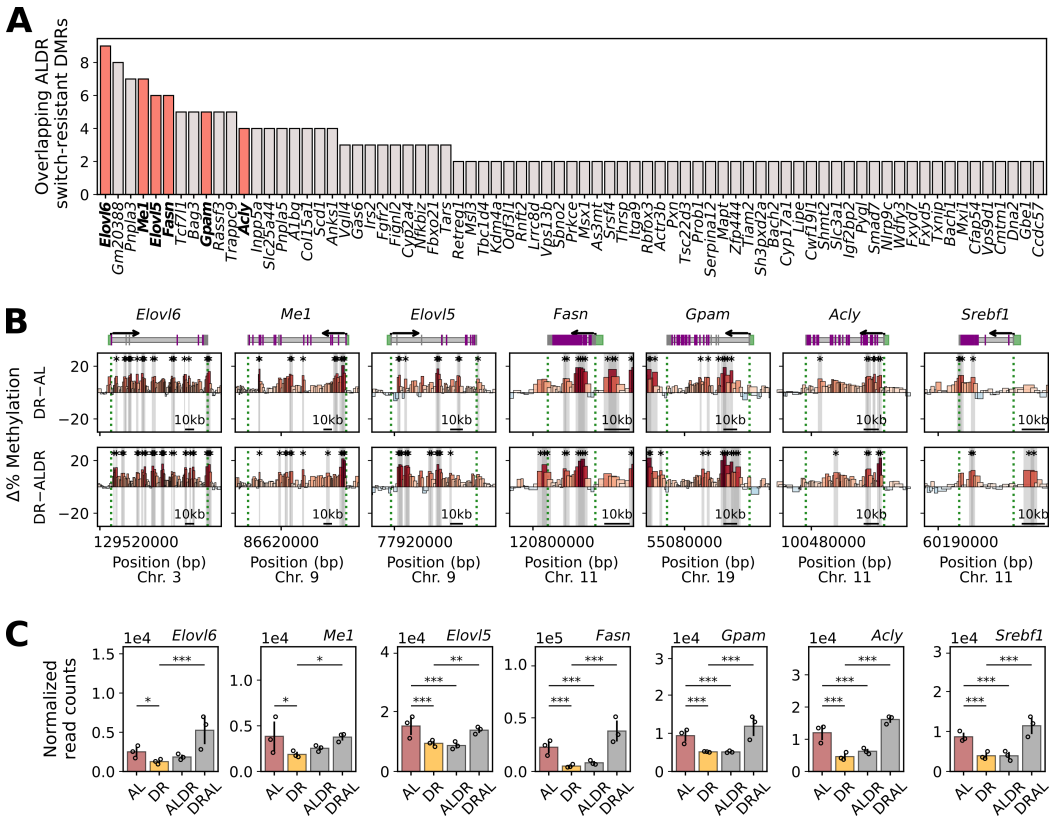


Figure S2.4 | **DR- and ALDR-induced DNA methylation and expression changes of lipogenesis genes in the liver.** (A) Number of the top 75 genes with overlapping ALDR switch-resistant DMRs. Key lipogenesis genes that were previously found to be hypermethylated and down-regulated in response to DR (Hahn et al., 2017) are highlighted. (B) DMRs of DR vs AL and DR vs. ALDR pairs. DMRs (χ^2-test, $p_{adj} < 0.05$; minimum region methylation difference > 10%) are highlighted in methylation tracks in grey and with asterisks. (C) Respective transcription levels and differential expression (against AL and DR only, *: $p_{adj} < 0.05$, **: $p_{adj} < 0.01$, ***: $p_{adj} < 0.001$). Top-left numbers in (C) reflect the exponential of 10 of y-axis values.

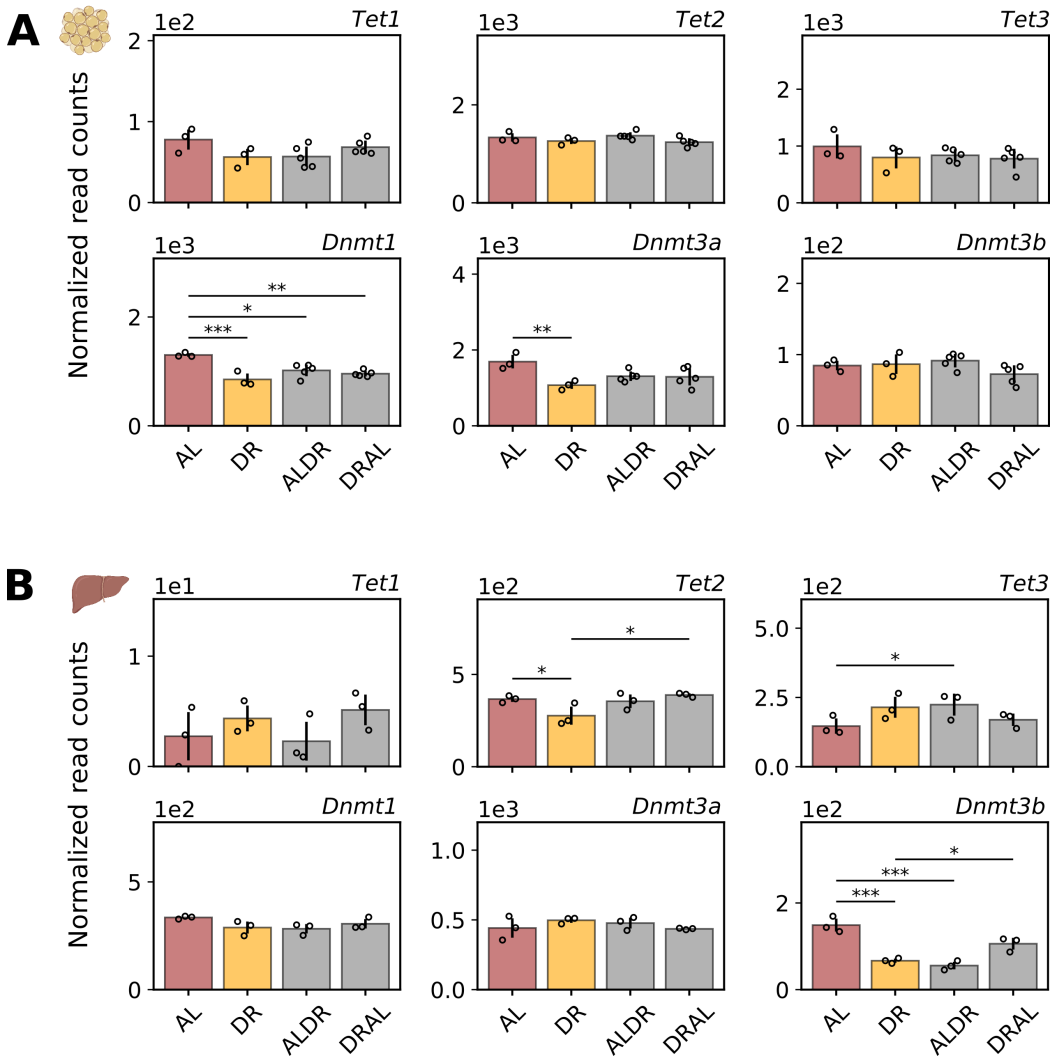


Figure S2.5 | **Expression levels of *Dnmt* and *Tet* DNA methylation enzymes in the WAT and liver.** (A) Expression levels in the WAT. (B) Expression levels in the liver. Transcript levels are annotated as normalized read counts. In addition, differential expression is annotated (*: $p_{adj} < 0.05$, **: $p_{adj} < 0.01$, ***: $p_{adj} < 0.001$). Top-left numbers reflect the exponential of 10 of y-axis values.

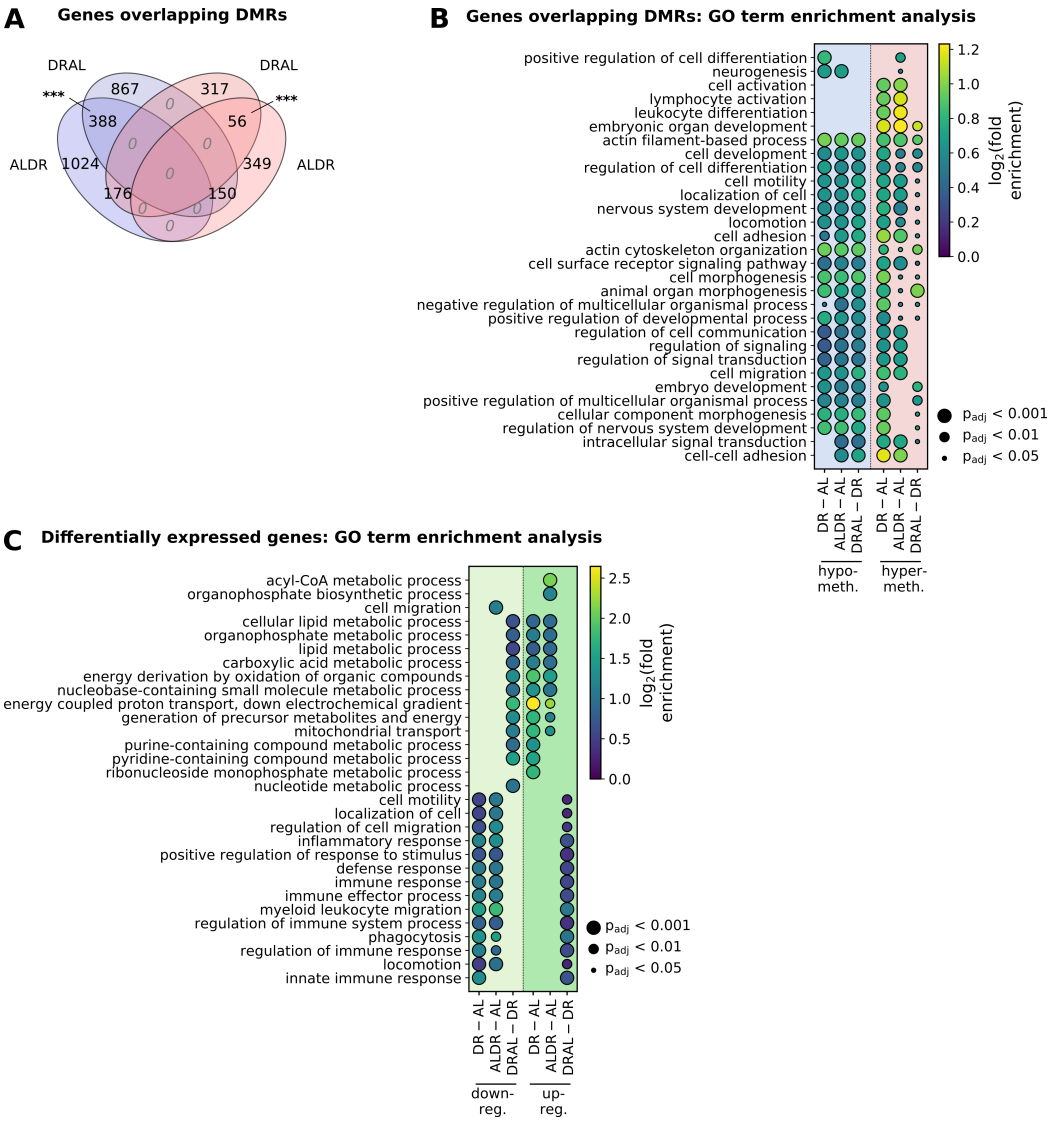


Figure S2.6 | **Additional data on DMR and DEG functional associations in the WAT.** (A) 4-way venn of the overlap of hypomethylated DMRs (blue) and hypermethylated DMRs (red) in ALDR vs AL and DRAL vs. DR in the WAT (***: Fisher's exact test, $p < 0.001$). (B, C) Dotplot representation of the top 30 significantly enriched GO terms of (B) genes that overlap DMRs in the WAT, and (C) DEGs in the WAT. The enrichment is calculated individually for hypo- and hypermethylated DMRs, and up- and down-regulated genes, and with all expressed genes in DR vs AL as background.

3 Project B:

Inter-tissue gene expression correlation declines with age and is improved by dietary restriction

(Unpublished manuscript)

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Author contributions: AB had the initial idea for the project. AB, DSG, LP, and SG conceived the project in more detail. DSG extracted RNA from mouse tissues for the subsequent RNA-seq experiments. DSG developed algorithms and performed dataset processing, analysis, and visualization of public and in-house data. AB, LP, and SG contributed to the dataset analysis by critical supervision. DSG wrote the manuscript. AB, LP, and SG critically reviewed the manuscript. Generated raw and processed data is stored on the server of the Max Planck Institute for Biology of Ageing, Cologne, Germany.

3.1 Abstract

Genes that are similarly expressed in two or more tissues may be indicators of regulatory homeostasis, for example promoted by the circadian rhythm. Ageing is known to deteriorate such regulatory processes which would in turn affect gene expression correlation between tissues. Here, we applied a correlation-based strategy to investigate gene-wise expression correlation between 17 mouse tissues of *Tabula muris senis*. We asked for the presence and functional origin of gene expression correlation, asked if gene expression correlation is declining with ageing, and asked how the longevity intervention dietary restriction (DR) alters these age-related effects in the kidney and liver. We observed gene expression correlation across most tissues, induced mostly by rhythmically expressed circadian genes, protein folding genes, and metabolic genes. Strikingly, this gene expression correlation profile was lost with ageing in mouse, and also the independent human GTEx dataset. In the kidney and liver, DR ameliorated gene expression correlation especially of nutrient sensing genes, and promoted the age-related maintenance of gene expression correlation of circadian genes. In summary, we herewith provide a correlation-based strategy to investigate gene expression correlation of tissues from the same mouse, show that ageing deteriorates this gene expression correlation, and find that DR limits the extend of this deterioration.

3.2 Introduction

The health and lifespan of multicellular organisms dependent on the homeostasis maintained by each of their tissues (Billman, 2020; Kotas & Medzhitov, 2015). Dysfunction in one tissue can impair the functionality of others (Oishi & Manabe, 2020; Tian et al., 2023), leading to tissue-derived systemic pathologies such as metabolic diseases (Diedrich et al., 2015; Houtkooper et al., 2010; Riera & Dillin, 2015), systemic inflammation (Franceschi et al., 2018), or the loss of hormonal homeostasis due to deterioration of the endocrine system (C. M. Jones & Boelaert, 2015; van den Beld et al., 2018). Ageing is a key driver of such functional deterioration in most (if not all) tissues of an organism, including the liver (Anantharaju et al., 2002; I. H. Kim et al., 2015), renal tissue (O'Sullivan et al., 2017), and adipose tissues (Mancuso & Bouchard, 2019), impairing organismal homeostasis (López-Otín et al., 2023). During ageing there is also deterioration in tissue-intrinsic processes such as the circadian rhythm (Acosta-Rodríguez et al., 2022; Tahara et al., 2017) with effects on hormonal (T. W. Kim et al., 2015; Lamia et al., 2011) and metabolic (Manella et al., 2021) processes that can impair an organisms health- and lifespan (Dubrovsky et al., 2010; Gale et al., 2011; Gan et al., 2015).

The deleterious effects of ageing can be delayed by interventions such as dietary restriction (DR). DR increases health- and lifespan in a range of organisms (Green et al., 2022), probably due to its combined effects on several hallmarks of ageing (V. D. Longo & Anderson, 2022; López-Otín et al., 2023), for example, on nutrient sensing (Piper et al., 2011), tissue inflammation (Morgan et al., 2007), and proteostasis. Exposure to DR rewires organismal metabolic processes systemically (R. Anderson & Weindruch, 2007; Bruss et al., 2010; Matai et al., 2019), but especially in tissues involved in nutrient-sensing (Swindell, 2009), inducing the remodelling of the transcriptome in adipose tissues (Hahn et al., 2019), the liver (Hahn et al., 2019; Hahn et al., 2017; Kuhla et al., 2014), and the kidney (Afsar et al., 2021; Xu et al., 2015). Importantly, all of these organs are both metabolic hubs, and have endocrine functionality. Furthermore, the liver and kidney to control detoxification processes (Bechmann et al., 2012; Kellum et al., 2021; van den Berghe, 1991; F. Wang et al., 2021), potentially imperative in the organism-wide responses to DR. Importantly, the timing of feeding during DR can entrain circadian rhythm, and a timed feeding in addition to DR further enhanced effects on healthspan and lifespan (Acosta-Rodríguez et al., 2022).

Transcriptional and other omics datasets can be used to monitor the effects of ageing or DR on different tissues and to test for sets of genes that are differentially expressed across individual tissues (Srivastava et al., 2020). A more quantitative approach is to assess inter-tissue expression correlation, by comparing the expression levels of the same gene (or set of genes) in two (or more) tissues originating from the same individual (hereafter referred to as 'paired tissues'). This strategy has already been applied to several RNA-seq datasets, for example to compare cancerous tissues and healthy tissues originating from the same individual (Petti et al., 2021), to reveal insulin-related co-expression networks between tissues from obese and non-obese patients (Kogelman et al., 2016), and to show age-related convergence in expression levels of tissue-specific genes reflecting a loss of cell type identity (Izgi et al., 2022). Functionally, genes with inter-tissue expression correlation could reflect periodically oscillating genes of the circadian clock (Dobrin et al., 2009; R. Zhang et al., 2014), but could also reflect a coordinated upstream regulation of specific sets of genes.
In this study, we analysed the inter-tissue gene expression correlation between murine tissues, and its changes with age and in response to DR. We also determined the functional associations of correlated genes across tissues. To do this, we examine the *Tabula muris senis* bulk RNA-seq dataset (Schaum et al., 2020), which contains longitudinal data of 17 paired tissues (or 136 tissue pairs) from 1 to 27 month old mice. We reveal a global trend of coordinated expression of genes across most tissues that is lost with ageing. Thereby, the number of correlated genes in most tissue pairs gradually decreases, and some genes lose correlation across multiple tissues. The trend of age-related loss of expression correlation was also present in the independent human GTEx dataset (Lonsdale et al., 2013) and an independently generated RNA-seq dataset of kidney and liver from young (5 months) and old (20 to 28 months) C3B6 F1 hybrid female wild-type mice fed ad libitum (AL) and DR. Genes differentially regulated by DR were more strongly correlated across tissues and between liver and kidney, DR ameliorated the loss of correlation with age of genes involved in circadian rhythm.

3.3 Results

3.3.1 RNA expression profiles of mouse tissues are positively correlated

We initiated our analysis by testing whether gene expression is correlated between tissues of individual mice. Therefore, we used RNA-seq data from tissues extracted from the same individual mice of the *Tabula muris senis* bulk RNA-seq dataset, which contains gene expression data from 17 tissues, corresponding to 136 unique tissue pairs, from male and female C57BL/6JN mice from the age of 1 to 27 months (Schaum et al., 2020) . To investigate similarities in expression profiles independent of age and sex, we performed a tissue-pair-wise partial correlation analysis, accounting for the covariates "age" and "sex" (Figure 3.1A). Partial correlation and significance were then calculated for each expressed gene per unique tissue pair (136 tissue pairs with a median of n = 49.5 tissues per pair, Figure S3.1). As sample collection could potentially bias expression correlation, we also tested for the presence of corresponding batch effects caused by sacrificing groups of mice but did not observe any obvious pattern globally, or across individual tissue pairs (Figure S3.2).
Significantly correlated genes ($p_{adj} < 0.05$) were found in all tissue pairs, with a median of 51 positively and 2 negatively correlated genes per tissue pair, indicating a strong bias towards positive correlation of gene expression (Figure S3.3). The highest number of significantly correlated genes was found between the three white adipose tissue depots, directly followed by the kidney-liver pair (Figure S3.3A, S3.3B) with 1 negatively and 436 positively correlated genes (Figure 3.1B). To address whether the observed correlations in gene expression between tissues were not simply caused by chance, we repeated the analysis but randomly shuffled the mouse IDs for each tissue pair (n = 1000 iterations) before calculating gene-wise Pearson correlation. The significance of the median correlation shift per tissue-pair was then calculated by comparing it to the random-pairing control (Figure 3.1C, 3.1D). The median correlation of 35 out of 136 tissue pairs (25.7%) was significantly shifted towards a positive correlation (Figure 3.1E), revealing an organism-wide bias towards a positive inter-tissue expression correlation.

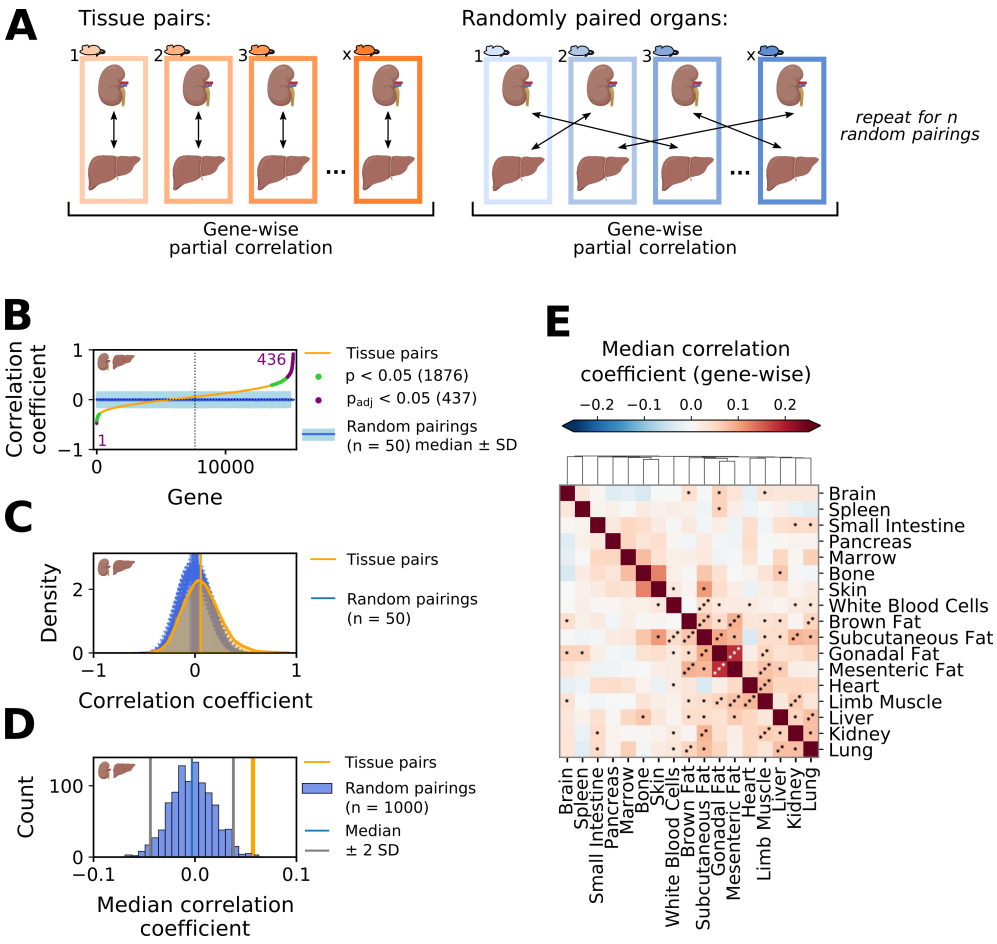


Figure 3.1 | **Expression profiles of tissue pairs are correlated.** (A) Analysis workflow to identify gene expression correlation between tissue pairs. Results for kidney vs. liver are highlighted in B-D. (B) Correlation coefficient distribution of tissue pairs compared to random pairings background (n= 50 iterations). Medians are highlighted with vertical bars. (C) Median correlation coefficient of tissue pairs compared to random pairings background (n = 1000 iterations). (D) Gene-wise correlation coefficient compared to random pairings background (n = 50 iterations). Genes are sorted by their correlation coefficient. (E) Heatmap representation of median correlation coefficients for all tissue pairs in the dataset. Significance of the median correlation shift compared to the random pairing background is indicated (CDF, n = 1000 iterations with subsequent BH-correction, *: $p_{adj} < 0.05$, **: $p_{adj} < 0.01$, *** $p_{adj} < 0.001$).

3.3.2 Correlated genes are organized in clusters that reflect tissue functionality and identity

In order to identify genes that were correlated across tissue pairs, we calculated an 'interaction score' as the sum of positive significant correlation ($p_{adj} < 0.05$) events per gene and tissue. Ward clustering of this score revealed 3 tissue-specific and 4 gene-specific clusters (Figure S3.4). The three major gene clusters with the highest median interaction scores (referred to as clusters 1 to 3) strongly shaped the tissue clusters (Figure 3.2A). To learn more about the functional aspects of these clusters, we performed a

gene-cluster-wise GO term enrichment analysis (Figure 3.2B).

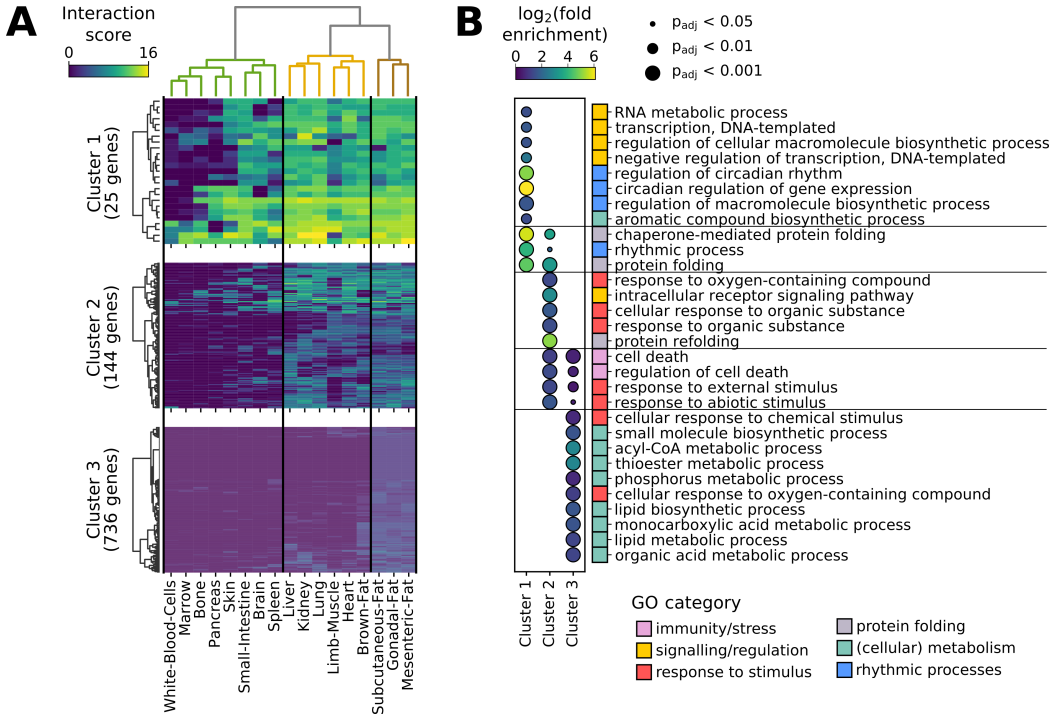


Figure 3.2 | **Genes correlated between tissues are organized in functional clusters.** (A) The interaction score was used to cluster genes that were correlated between multiple tissue pairs. Three functional clusters were identified, whereas cluster 1 includes correlated genes across all tissues (n = 25), cluster 2 includes correlated genes across metabolically more active tissues (n = 144), and cluster 3 includes correlated genes between white adipose tissues (n = 736). (B) Dotplot representation of the top GO terms that were significantly enriched across all clusters. Colored boxes represent manually annotated broader GO categories for all terms.

Cluster 1 contained 25 genes that were positively correlated across most analysed tissues. Genes in cluster 1 showed a strong enrichment of terms associated with chaperone-mediated protein-folding, gene expression regulation and rhythmic processes. Seven genes associated with regulation of circadian rhythm were previously described to periodically oscillate across multiple tissues (R. Zhang et al., 2014), potentially indicating synchronisation of the circadian clock among tissues of the same animal (Figure S3.5A). Consistent with this hypothesis, periodically expressed genes were among the top correlated genes across all tissues especially in more metabolically active tissues (Figure S3.5B), and comprised several key drivers of the two major feedback loops of circadian gene regulation (Rijo-Ferreira & Takahashi, 2019), including *D-Box Binding PAR BZIP Transcription Factor* (*Dbp*), *Period circadian clock 1* (*Per1*), *Per2*, *Per3*, *Nuclear receptor subfamily 1 group D member 1* (*Nr1d1*), *Nr1d2*, *Nuclear factor interleukin 3* (*Nfil3*),

Cryptochrome 1 (*Cry1*), *Cry2*, and the core genes *circadian locomotor output cycles kaput* (*Clock*) and *Aryl hydrocarbon receptor nuclear translocator like* (*Arntl*).

Cluster 2 contained 144 genes that were mainly correlated across more metabolically active tissues including liver, kidney, brown fat, and muscle, and was enriched for genes broadly related to protein folding. Together with the protein folding genes in cluster 1, we found a total of 19 well correlated protein folding genes (Figure S3.5C), which were also tightly linked at the protein level (Figure S3.5D). These genes included several targets of the heatshock chaperone families *Hsp90*, *Hsp70* and the co-chaperone *Dnaj* and *Ahsa* families, which are abundantly expressed in eukaryotic cells and are essential for protein folding and degradation processes, orchestrating the maintenance of proteostasis (B. Chen et al., 2005; Dean & Johnson, 2021; Hoter et al., 2018; Shemesh et al., 2021). Their synchronised expression specifically in metabolically active tissues could reflect their response to dietary intakes, for example increased gene expression levels following nutrient availability or to cope with higher metabolic demands. Consistent with this assumption, we also observed an enrichment of correlated genes related to responses to organic substances and oxygen compounds in the same clusters which include many of the above-mentioned proteostasis and circadian genes, but also further targets. These included *DNA methyltransferase 3a* which encodes DNMT3A which regulates metabolic processes on the epigenetic axis, for example in adipocytes (You et al., 2017), genes encoding metabolic proteins, including *pyruvate dehydrogenase kinase 4* (S. Zhang et al., 2014), and the transcription factor genes of the metabolic mediators *Krüppel-like 9* (*Klf9*) (Gans et al., 2021; Sweet et al., 2019) and *Klf11* (Lomberk et al., 2013; Mathison et al., 2015).

Finally, cluster 3 contained 736 genes which were mainly correlated across white adipose tissues. These genes were specifically enriched for functions associated with white adipose tissue such as lipid- or organic acid metabolism. This signal could be caused by the abundantly present cells of different depots which express genes of similar pathways in response to whole-body upstream signals such as nutrient sensing or circulating factors. Thus, the correlation may be driven mainly by tissue identity, i.e. the expression of similar genes in similar cell types in response to the same upstream signals. Consistent with this assumption, correlated genes were enriched for a variety of pathways (Figure S3.5E), including pyruvate metabolism, which regulates energy metabolism downstream of hormonal signals including insulin (Coore et al., 1971; Jungas, 1971), the AMP activated protein

kinase (AMPK) signalling pathway that mediates fatty acid oxidation and can be regulated, for example, by circulating leptin (Martínez-Sánchez, 2020; Minokoshi et al., 2002), and the steroid biosynthesis pathway, which is dependent on cholesterol (Chung & Parks, 2016; J. Li et al., 2014).
Because of the strong circadian gene signal we observed in well correlated genes of cluster 1, we suspected that at least a part of the signal of all correlated genes could originate from genes that are rhythmically expressed either aside from, or downstream of the circadian rhythm. Thus, by combining rhythmic process genes of the Gene Ontology database (Carbon & Mungall, 2018) with genes annotated by the public datasets RhythmicDB (Castellana et al., 2022) and CGDB (S. Li et al., 2016), we defined a list of n = 2,444 genes with putative rhythmic expression in at least two mouse tissues simultaneously, representing approximately 11% of all expressed genes across tissues. In line with the circadian gene enrichment, 22 of the 25 (76%) genes in cluster 1 overlapped with this rhythmic expression gene set. In cluster 2, 73 of the 144 (50%) genes overlapped with this set, and included aforementioned heat shock proteins and chaperones of the *Hsp90*, *Hsp70*, *Dnaj*, and *Ahsa* families, and both *Klf9* and *Klf11*. Finally, in cluster 3, 205 of 736 genes (27%) overlapped with the set of oscillating genes. Thus, the large overlap of rhythmically expressed genes with gene expression correlation indicates that at least part of the functional signal originating from correlated gene expression could be caused by their oscillating gene expression patterns. However, rhythmic gene expression can also not explain the full signal we observe.
In summary, we found inter-tissue correlations for gene expression that included periodically oscillating genes across the whole body, which includes genes involved in proteostasis and in metabolism across metabolically active tissues, and genes regulating lipid metabolism and energy homeostasis and that respond to common upstream regulators across different adipose depots.

3.3.3 Correlation of gene expression between tissues declines with age in mice

We next determined whether inter-tissue expression correlation changed with age. We grouped samples from significantly correlated tissue pairs (Figure 3.1E, n = 35) by age, thereby creating 7 overlapping ageing bins, each consisting of samples from 4 age groups (Figure 3.3A). This way, we balanced the analysis between the statistical power required for the

correlation analysis (with a median of 21 samples per tissue pair) and a wide distribution across age groups. The number of significantly correlated genes ($p_{adj} < 0.05$) per ageing bin was then calculated via partial correlation while accounting for within-ageing-bin covariates 'sex' and 'age'. As our correlation signal is vulnerable to outlier samples, which could introduce multiple-sampling bias due to our overlapping binning approach, we also applied a conservative filtering step. We removed significantly correlated genes if the signal only came from individual samples and if their removal led to a loss of significant correlation (Figure S3.6A, S3.6B), thereby removing approximately 20% of all previously significant genes across all age groups (Figure S3.6C). The remaining significantly correlated genes varied greatly in number between the tissue pairs (Figure S3.7A), but overall followed a striking trend of loss of expression correlation with age in most tissue pairs (Figure 3.3B, 3.3C). The median coefficient of determination (R^2) over all genes per age group that were significantly correlated in at least one age group also declined with ageing (Figure S3.7B).

The decline in inter-tissue gene expression correlation with age could be explained by technical or biological artefacts. We tested whether a decrease in statistical power due to a lower number of samples in the last two ageing bins (including months 15 to 27, Figure S3.8A) might introduce a bias. By repeating the analysis with down-sampled data to achieve equal number of samples in all ageing bins, we confirmed the decrease in age-related inter-tissue gene expression correlation (Figure S3.8B), indicating that the lower number of samples in the two oldest ageing bins was not responsible for the observed trend. We also assessed the possible role of age-related gene expression changes. Most age-related expression changes (Pearson correlation: age, normalised read counts, $p_{adj} < 0.05$) were rarely present in both tissues of a pair at the same time, but were more likely to occur in only one of the two tissues individually (Figure S3.9A). However, removing genes with age-associated expression changes did not show a different ageing trajectory, nor did their removal strongly affect the overall trend (Figure S3.9B). In summary, we found a global trend of age-related correlation loss that cannot be explained by technical biases or age-related expression level changes.

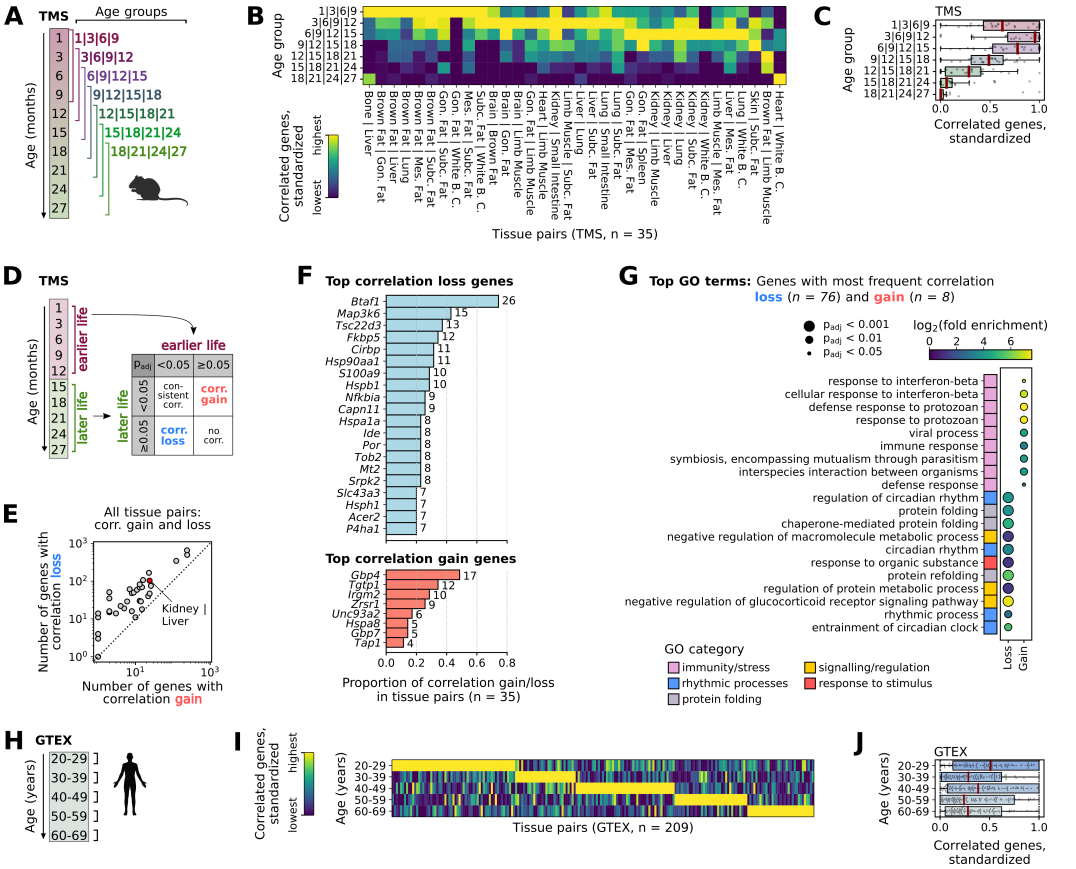


Figure 3.3 | **Global expression correlation declines with ageing.** (A) Age-dependent correlation changes are evaluated by binning mice across their age by summarizing all mice of four subsequent time points. For all resulting bins, partial correlation is calculated. (B) Heatmap representation of the standardized number (column-wise min-max normalization) of correlated genes per time bin for all tissue pairs with a significant median correlation shift ($p_{adj} < 0.05$, see Figure 3.1E). (C) Boxplot representation of the min-max standardized median signal of all pairs per time point in B. (D) Schematic representation of classifying a gene as a gene with correlation gain, or correlation loss in the *Tabula muris senis* dataset. Genes with age-dependent correlation change are assessed by calculating the partial correlation for mice in their earlier (months 1 – 12) and later (months 15-27). (E) Tissue-pair-wise count of genes with age-dependent correlation gain and loss. (F) Genes with most frequent age-related correlation loss (top) and loss (bottom) across all tissues. (G) Dotplot representation of the top GO terms (BP FAT ontology, after revigo redundancy reduction) that are significantly enriched in genes with age-related correlation loss (n = 76) and gain (n = 8) in at least 4 tissue pairs. Colored boxes represent manually annotated broader GO categories for all terms. (H-J) The same as A-C, but repeated for a subset of the GTEX dataset. Abbreviations for tissues in (A): Gon. Fat: Gonadal Fat, Mes. Fat: Mesenteric Fat, Subc. Fat: Subcutaneous Fat, White B. C.: White Blood Cells.

3.3.4 Genes with age-related loss of expression correlation have roles in regulation of gene expression, proteostasis, circadian rhythm and inflammatory processes

We next asked which molecular processes might be affected by the age-related decrease in inter-tissue gene expression. To avoid potential bias from overlapping age bins, we divided the dataset into two non-overlapping

bins, 'early life' (months 1 to 12) and 'late life' (months 15 to 27). Age-related correlation loss was defined as genes that that were significantly correlated in early life, but not in late life, while age-related correlation gain was defined as significant correlation in late but not early life (Figure 3.3D). Consistent with the global age-related correlation loss observed before, all analysed tissue pairs had more genes with correlation loss (Figure 3.3E). Interestingly, when re-investigating genes with age-related correlation change by using the resolution of overlapping ageing bins, we observed that (as shown for example in the kidney-liver pair) genes showed different dynamics of age-related correlation change, with some changing their correlation continuously with ageing and others more abruptly (Figure S3.10), implying different underlying causes.

To identify groups of genes whose inter-tissue coordination was most affected by ageing, we asked which genes most often lost or gained correlation between tissues. To avoid a potential bias from age-associated gene expression changes, only genes that did not show significant age-associated expression changes within each tissue pair were included in the analysis. Again, we observed a global bias towards age-related correlation loss as compared to gain (Figure 3.3F), with 76 genes frequently losing correlation and 8 genes frequently gaining correlation (in at least 4 of the 35 tissue pairs) spread mostly across adipose and metabolically active tissues (Figure S3.11). Of note, while our filtering removed significant effects of age-related expression change, we still observed at least mild age-related expression change in some of our top candidates. In the 8 genes with age-related correlation gain, this age-related expression change was positively biased (Figure S3.12A) which could contribute to age-related correlation gain. In the top correlation loss genes, the signal was less clear, but candidates including *B-TFIID TATA-box binding protein associated factor 1* (*Btaf1*) or *Cold-inducible RNA-binding protein* (*Cirbp*) still showed consistent mild age-related expression trends across tissues that we could not remove (Figure S3.12B). The age-related correlation loss in these top candidates was thus sufficiently strong to counteract the potential correlation-amending effects of age-related expression changes.

GO term enrichment (Figure 3.3G) of the top loss of correlation genes revealed a functional profile of general signalling and gene expression processes derived from, for example, *Btaf1*, *Cirbp*, *Mitogen-activated protein kinase kinase kinase 6* (*Map3k6*), and *Insulin degrading enzyme* (*Ide*) which all regulate further downstream processes. Specifically *Map3k6* and *Ide* are implicated in ageing, as MAP3K6 is a potential regulator of mammalian

Target Of Rapamyin (mTOR) (Guo et al., 2015) which is central to the mediation of ageing specific pathways (Papadopoli et al., 2019), and IDE can degrade insulin (Pivovarova et al., 2016) which is a core messenger central to the nutrient-sensing hallmark of ageing (López-Otín et al., 2013). We also found a functional enrichment in genes associated with circadian rhythm and protein folding, the two functional categories of consistent correlation across tissues previously identified. Circadian genes included central oscillators such as *Nr1d2*, *Cry2*, and *Per2* but also *Cirbp*, which contributes to regulated clock-gene expression in response to sleep-wake cycles (Hoekstra et al., 2019), and protein folding genes including various heatshock proteins, suggesting an age-related loss of proteostasis and disruption of the circadian rhythm. Furthermore, all mentioned gene targets aside from the top candidate *Btaf1* were also part of the set of 2,444 rhythmically expressed genes in mice, which indicates that ageing generally deteriorates rhythmic expression of genes aside from the core clock itself, which is represented also on expression correlation level.
In contrast, the top genes with global correlation gain were enriched exclusively for stress- and immunity related functions and included mainly innate immune genes such as *T-cell specific GTPase 1* (*Tgtp1*) and *Immunity-regulated GTPase family M member 2* (*Irgm2*), two genes important in viral and bacterial defence responses. Also, the top gene with age-related correlation gain in 17 of 35 (> 48.5%) correlated tissue pairs, *Guanylate binding protein 4* (*Gbp4*), is associated with pathogen defence and interferon response (Hu et al., 2011). White adipose tissues, liver, kidney, and lung were the main tissues in which these genes showed a correlation gain (Figure S3.11B), all of which share immune functionality including the presence of immune cells and the age-related increase in immune cell infiltration (Almanzar et al., 2020). The increase in correlation with age among immune related genes might therefore be explained, by an increase in systemic inflammation in aged individuals or by an infiltration of immune cells across several tissues. Of note, our filtering of age-related genes, the top candidates of age-related correlation gain also show strong biases towards age-related increase in expression levels (Figure S3.12A).

3.3.5 Correlation of inter-tissue gene expression declines with age in humans

Given our findings in the mouse, we next asked whether gene expression is also correlated between tissues in humans. We used the GTEx dataset

(Lonsdale et al., 2013), which contains bulk RNA-seq data of tissues obtained from humans aged between 20 and 79 years, binned every 10 years. To confirm the general age-independent correlation signal we observed in *Tabula muris senis*, we initially focussed on tissue pairs that approximate those of *Tabula muris senis* and used Pearson correlation to confirm the presence of a strong positive correlation bias, which is even stronger than previously observed in *Tabula muris senis* (Figure S3.13A), possibly due to the high statistical power provided by the very large sample size of the GTEx dataset. Analogous to the previous analysis in mouse (Figure 3.2A), we initially performed a clustering analysis to find frequently correlated genes across tissue pairs. To avoid technical artefacts, we removed tissues with low sample counts (< 50 paired tissues) and additionally down-sampled to n = 66 samples (the minimum sample count available in the remaining tissue pairs) to balance the dataset. We found 409 genes that were frequently correlated across tissue pairs (Figure S3.13B). These were mostly enriched for immunity-related functions (Figure S3.13C). However, we found that genes that were frequently correlated in mouse (including circadian and protein folding genes) also showed frequent correlation in human data (Figure S3.13D).

We next asked if the age-related loss of correlation also was conserved in the human data (Figure 3.3H). We had to account for the strong imbalance in sample numbers per age group in GTEx, and down-sampled the data to balance the sample numbers per tissue pair. To ensure sufficient statistical power, we restricted our analysis to tissue pairs with ≥ 15 samples in each remaining ageing bin and removed the oldest age group (70-79 years old) as it rarely included a large number of paired samples. This resulted in 209 tissue pairs of 25 individual tissues in which we could also confirm the presence of a decrease in inter-tissue correlation with age, albeit milder than in *Tabula muris senis* (Figure 3.3I, 3.3J). Due to the lack of sufficient paired tissues across the ageing axis (especially in the oldest and youngest age group) and the rare overlap of remaining tissues in GTEx with *Tabula muris senis*, we could unfortunately not repeat the functional analysis of genes with age-related correlation change in human.

3.3.6 Correlation dynamics in kidney and liver expression profiles are robust across datasets

We next addressed whether DR, an intervention that promotes late-life health and extends lifespan in diverse animals including mice, affected

inter-tissue gene expression correlation and the age-related loss in correlation. As there was no published DR data set available with sufficient numbers of paired tissues, we generated a new RNA Seq data set focusing on gene expression correlation between the kidney and liver. This pair was chosen because it (1) showed a significant shift in expression correlation that decreased with age in *Tabula muris senis*, (2) was among the top pairs in terms of total number of correlated genes across ageing bins, (3) both tissues play an essential role in organismal homeostasis maintenance by orchestrating metabolic processes and detoxification (Bechmann et al., 2012; Kellum et al., 2021; van den Berghe, 1991; F. Wang et al., 2021).

RNA-seq was done on kidney and liver samples obtained from female C3B6 F1 hybrid wild-type mice with 18-20 samples per group of young mice (5 months of age) and old mice (20, 24, or 28 months of age) fed AL or DR (Figure 3.4A). The three older time-points were pooled to reach the required sample number for the correlation. PCA of the normalised read counts revealed separation of samples by tissue, age and diet (Figure S3.14A), verifying the quality of the data set. As the correlation analysis is susceptible to outliers, we next tested for the presence of outlier samples and found 4 samples per tissue in old AL samples and 5 samples in the DR group with aberrant expression patterns (Figure S3.15). To maximize statistical power, and because all outlier samples showed a comparable bias in expression levels, we decided to add the outlier samples as further covariates rather than removing them from the analysis.

First, we asked if the newly generated dataset confirmed previous findings in *Tabula muris senis* (Figure S3.14B). We calculated the partial correlation of all young and old AL-fed mice combined with "age" and the "outlier" samples as covariates. In line with previous findings, we found a general trend of positive expression correlation, and the gene-wise partial correlation coefficients of *Tabula muris senis* and our new AL dataset were weakly, but significantly correlated ($r > 0.16$, $p < 0.001$, Figure S3.14C, S3.14D). Furthermore, we observed a significant overlap of 91 genes (Fisher's exact test, $p < 0.001$) with significant positive correlation in both datasets (Figure S3.14E), which remained consistent also after removing genes with age-associated expression changes from both datasets (Figure S3.14F). Similar to *Tabula muris senis*, significant negative correlation remained rare ($n = 11$). Furthermore, gene ontology analysis identified shared functional terms in both data sets including circadian rhythm, protein folding and response to organic substances and interferon beta (Figure S3.14G). Thus, despite differences such as mouse genetic background, diet, sample number

and the inclusion of only female mice, we were able to verify the positive gene expression correlation between the liver and kidney pair affecting similar cellular processes in our new data set, indicating that these effects are robust and reproducible.

3.3.7 DR induces a transcriptional profile in kidney and liver that is robust to age-related correlation change

To determine how DR affected the correlation profile of mice during ageing, we performed the analysis individually for the age groups (AL young, AL old, DR young, DR old), using within-group "age" and "outlier" samples as covariates. In young AL animals, correlation across all genes was significantly positively shifted compared to the random pairing control ($p = 0.011$, median $r = 0.056$) (Figure 3.4B). An even stronger shift was observed in young DR animals ($p < 0.001$, median $r = 0.136$), suggesting that the DR treatment increases gene expression correlation between the liver and kidney compared to AL control animals already at young age. While there was no significant shift in correlation observed in old AL animals ($p = 0.06$), DR animals also showed a significantly positively shifted gene expression correlation at old age ($p < 0.001$, median $r = 0.132$), indicating that the DR treatment maintained gene expression correlation between the liver and kidney with age. We hypothesised that this trend was driven by genes specifically regulated by DR and found that DR-regulated genes (Bonferroni-adjusted $p_{adj} < 0.01$ in either tissue) were generally significantly more highly correlated than non-DR-regulated genes (two-tailed t-test, $p < 0.001$) (Figure 3.4C). Importantly, this trend was also present in the AL groups albeit not as strong. This suggests that diet-responsive genes are in general more highly correlated between kidney and liver, which might reflect coordinated transcriptional programs that are induced in kidney and liver simultaneously via neural cues or circulating factors in response to dietary changes. We also found that previously identified genes with frequent age-related expression correlation loss (Figure 3.3F) showed a similar signal of correlation loss in AL in the new dataset, whereas most of them maintained their correlation at older age with DR (Figure S3.16A, top).

As we previously observed a strong association of oscillating gene expression aside from circadian genes themselves and expression correlation, and DR specifically is known to be a Zeitgeber for circadian patterns (Acosta-Rodríguez et al., 2022), we next asked if gene expression correlation is

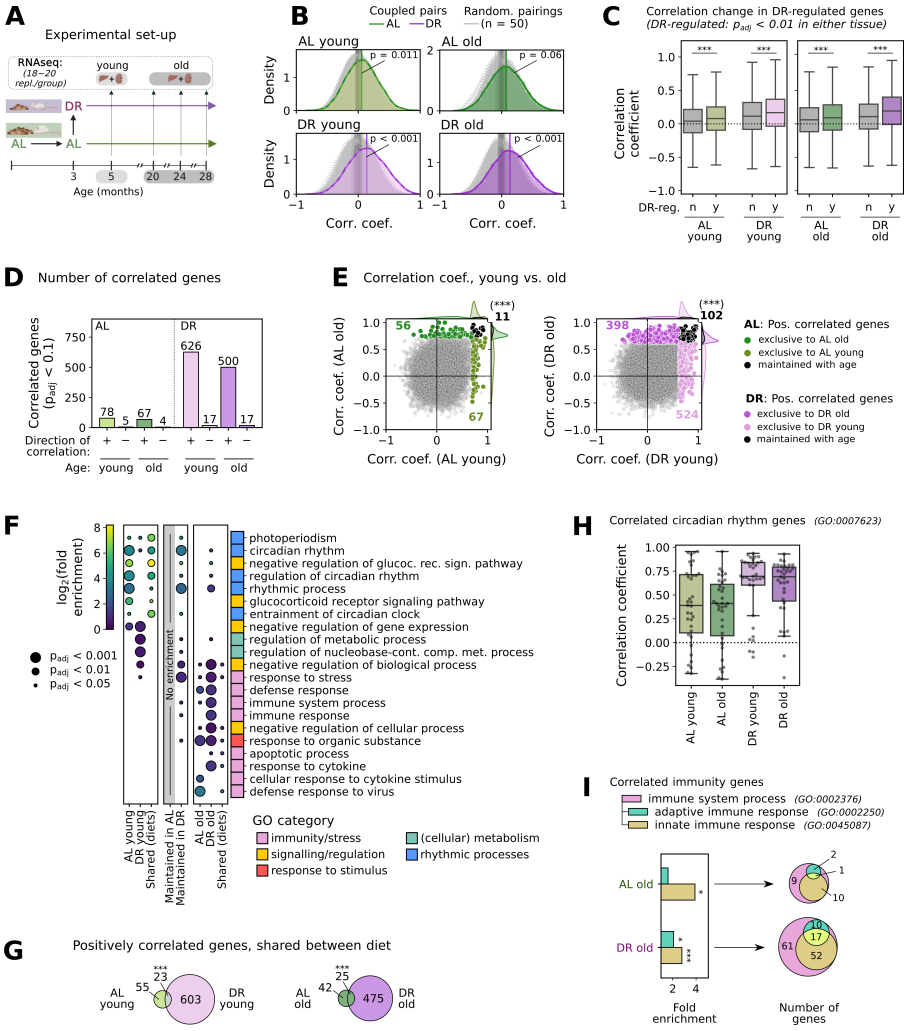


Figure 3.4 | **Gene expression correlation is improved in response to DR.** (A) Mouse cohort set-up for liver and kidney sample collection. (B) Median correlation shift in AL young, AL old, DR young, and DR old. Significance was determined by comparing the distribution to a random pairing background with n = 1000 iterations. (C) Distribution of correlation coefficients in genes that are DR-regulated (Bonferroni-adjusted p_{adj} < 0.01 in either kidney or liver, n = 5,319 genes in AL young vs DR young (left comparison); n = 6,230 genes in AL old vs DR old (right comparison)). Significance between non-DR-regulated (n) and DR-regulated (y) gene sets is indicated (two-sided t-test; ***: p < 0.001). (D) Number of positively (+) and negatively (-) correlated genes (p_{adj} < 0.1) in AL young, AL old, DR young, and DR old after removing genes with age-related expression change (Bonferroni-adjusted p-value < 0.05 in either kidney or liver, n = 1,508 genes in AL with 12/41 overlapping with correlated genes in young/old; n = 942 genes in DR with 63/118 overlapping with correlated genes in young/old). (E) Correlation coefficient in young vs. old groups in both feeding groups. Significance of overlaps is indicated (one-sided (alternative: "greater") Fisher's exact test; ***: p-value < 0.001). (F) Dotplot representation of the top GO terms (BP_ALL ontology after revigo redundancy reduction) that are significantly enriched in positively correlate genes of gene sets displayed in Figure 3.4E. Colored boxes represent manually annotated broader GO categories for all terms. (G) Overlap of significantly correlated genes between diets within the same age group. Significance is indicated (one-sided (alternative: "greater") Fisher's exact test: ***: p-value < 0.001). (H) Correlation coefficients of all correlated circadian rhythm genes in either age or feeding group. (I) Fold enrichment and number of genes of correlated in old AL and DR associated with GO terms "immune system process", "adaptive immune response", or "innate immune response".

increased specifically in rhythmically expressed genes. We overlapped the 2,444 previously defined oscillating genes with sets of DR-regulated genes and found that 1,197 of 6,230 DR-regulated genes (19.2%) in young, and 1,047 of 5,319 DR-regulated genes (19.6%) in old were also part of the oscillating gene set. In DR, oscillating genes thereby had a significantly higher correlation as compared to non-oscillating genes (two-tailed t-test, $p < 0.001$ in all comparisons), with median correlation shifts of 0.114 (diet-independent), 0.158 (non-oscillating), and 0.215 (oscillating) in young, and 0.112 (diet-independent), 0.17 (non-oscillating), and 0.24 (oscillating) in old. In summary, the DR treatment increased gene expression correlation between the liver and kidney already at young age and maintained correlation at old age and this effect seems to be mainly driven by diet-responsive genes. While oscillating genes are more strongly contributing to the signal of increased gene expression, they are not explaining the DR-induced effects alone.

3.3.8 DR ameliorates circadian gene expression correlation and induces a unique metabolic profile of expression correlation

To obtain functional information on genes with correlated expression in response to DR, we performed a GO term analysis of genes that were correlated between the liver and kidney of young and old AL and DR animals. Genes with age-associated expression changes (Bonferroni-adjusted $p_{adj} < 0.05$ in either tissue) were removed from the dataset and a less conservative cut-off ($p_{adj} < 0.1$) was used in order to include a larger number of genes in the analysis, which roughly doubled the amount of significantly correlated genes across all groups as compared to numbers with a cut-off of $p_{adj} < 0.05$, facilitating downstream functional analysis. Among positively correlated genes, we found slightly fewer significantly correlated genes in old animals in both AL (78 vs. 67) shared) and DR (626 vs. 500) (Figure 3.4D). Thereby, aside from the 11 genes in AL and 102 genes in DR that were shared across age, correlated genes in either young or old were distributed across the total gene population (Figure 3.4E), indicating that their significant correlation was at least in part age-group-specific.

Strikingly, a more than 5-fold higher number of genes was significantly correlated in DR as compared to AL. These genes are candidates for a DR-specific expression profile which is regulated in coordination in the liver and kidney. However, surprisingly, despite the general increase in number of

correlated genes, we did not find a significant functional enrichment when exclusively investigating all genes significantly correlated only in young or old DR, but not AL. This could suggest that the general increase in the number of correlated genes in response to DR might be a consequence of multiple upstream mechanisms that induce a more coordinated expression between tissues in general. To still shed light on potential functional influences on DR despite the absence of a unique functional signal, we thus next asked for functions of genes that are correlated in response to AL, and how they compare to DR (Figure 3.4F).

In young AL, we observed a significant enrichment of genes associated with circadian genes, including *Arntl*, *Dbp*, *Per1*, and *Per3* (Figure S3.16A, bottom). In old AL, the circadian signal is lost, and the correlation profile is mainly shaped by genes related to immunity and stress pathways. Both the circadian gene correlation in young AL, and the immune gene correlation in old AL are in line with our findings in *Tabula muris senis*. Next, we asked how DR affects this functional profile. Compared to young AL, there was no strong difference in the functional signal in young DR, which also consisted mainly of a circadian gene signal. In line with this, we observed a significant overlap of 23 genes correlated both in young AL and DR (Figure 3.4G), which included the previous circadian genes alongside further previously non-correlated candidates such as *Cry1*, *Nr1d1*, or *Nfil3* (Figure S3.16A, bottom). In old DR, we observe a strong profile of immunity and stress related genes similar to old AL. Contrasting old AL, however, the circadian profile was maintained with age, suggesting that DR maintains the gene expression correlation of circadian genes in general, and its maintenance with ageing. In line with this, the median expression correlation of circadian genes in general was increased in DR as compared to AL (Figure 3.4H). Thus, we observe a strong correlation-amending effect of DR on the level of circadian gene expression, which is in line with recent findings of a study investigated the beneficial effects of DR and timed feeding on lifespan, mouse behaviour, and oscillating gene expression patterns (Acosta-Rodríguez et al., 2022) Furthermore, aside from circadian genes, we found that approximately 26% of the genes with expression correlation DR young (163 of 626) and old (133 of 500) respectively were also part of the 2,444 genes with rhythmic expression. Thus, also on a more downstream level, rhythmic expression levels remained relatively stable.

We next investigated the finding that old DR tissue, despite the known inflammation inhibiting potential DR has(Morgan et al., 2007), showed an increased number of correlated inflammation and immunity related genes

(140 in DR, 22 in AL) mainly shaped by innate immunity genes (Figure 3.4I). Albeit the higher number of correlated genes in DR in total, the proportion of the immunity and stress related signal was comparable (28% of all correlated genes in DR, 32.8% in AL), which indicates that the "stronger" functional signal in DR might just be due to the generally higher number of correlated genes in general. Another factor contributing to the "stronger" signal in DR could be an increased expression variance in AL, which we observed especially in immune genes of the kidney and the liver (Figure S3.16C). In contrast, in DR a lower general variance across both tissues indicated a tighter regulation of genes in both tissues and less drop outs of gene correlation as compared to AL.
Finally, we asked which genes retain their correlation with ageing in DR, as these genes are potential candidates for a DR-specific programme of stably coordinated expression across tissues. Potentially, such genes could contribute to the DR-mediated pro-longevity phenotype. In DR, 102 genes were maintained in DR, 83 which were not correlated at all in the AL diet. These genes were mainly enriched both for the circadian and immunity signals as discussed already. However, DR-maintained genes also included key mediators of longevity pathways such as *Forkhead-box-protein O1* (*Foxo1*) and *Foxo3* (Figure S3.17) and further mediators of several nutrient sensing pathways including *Sterol regulatory element binding transcription factor 1* (*Srebf1*), *Klf13*, and *Klf15* (Rui, 2014; Takeuchi et al., 2021). In summary, we found that DR not only ameliorated circadian gene expression correlation, but also induces a correlation profile of genes linked to *FoxO*-mediated insulin signalling regulation (or nutrient signalling in a broader sense) which was coordinated across the liver and kidney and was robust to ageing.

3.4 Discussion

The inter-tissue expression correlation signal that we detected in our analyses could have several potential sources. In practice, a gene would be classified as correlated if it is consistently up- (or down-) regulated in two tissues in response to any upstream cue. Conversely, a loss of expression correlation could imply a loss of coordinated expression in one or even both tissues of the same tissue pair. As our analysis is limited to the transcriptional level, we do not specifically track the upstream cues. However, we have found that we can infer functional associations by examining individual profiles of correlated genes within a single tissue pair, or even

across several tissue pairs.

Throughout our analysis, we considered several factors that could potentially introduce systemic bias into our data. In general, to ensure sufficient statistical power throughout our analysis we prioritised maximising confidence over resolution. To further avoid bias introduced by within-group composition, we used partial correlation and included sex and within-group age as confounding variables. As correlation could be introduced by individual outlier samples, we systematically excluded significantly correlated genes between tissues if they did not persist after removing individual time points from a bin. To ensure that our results were not confounded by other effects, we then repeated our analysis while including other putative biasing factors. Addressing the imbalance of statistical power based on less available samples in aged *Tabula muris senis* samples via downsampling, and addressing effects of age-related expression changes via filtering did not significantly alter our results. Importantly, the trend of age-related correlation change was also present in the independent GTEx dataset, and in our newly generated kidney-liver dataset. In summary, due to the stability of the signal despite addressing these biases, and the presence of a comparable signal across datasets, we are confident that the observed signal of age-related correlation change is robust and biologically meaningful.

A strong correlation of expression between tissues may be a consequence of large-scale, well-coordinated organismal processes. An example of this is the circadian rhythm, which is known to be generally well synchronised across tissues of an organism (R. Zhang et al., 2014). Circadian gene expression is essential for controlling a wide range of organismal responses, including metabolic coordination and sleep (Rijo-Ferreira & Takahashi, 2019). In mammalian systems, this regulation is synchronised, for example, in response to day/night cycles and feeding times (Acosta-Rodríguez et al., 2022), and is centrally controlled in the suprachiasmatic nucleus, but also oscillates at the cellular level (Mohawk et al., 2012; Nakamura et al., 2015; Pickel & Sung, 2020). Consistent with the notion that circadian genes are inherently similarly oscillating across tissues, and likely reinforced by the fact that all mice were kept under controlled laboratory conditions, we found that key oscillating genes were highly correlated across almost all tissues. Correlated genes included for example *Arntl*, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Nfil3*, *Nr1d1*, *Nr1d2*, and *Clock*. All of these genes are making

up a large proportion of the main circadian clock framework (Takahashi, 2017) and the strong expression correlation that we observe across all tissues strongly reflects its tight global regulation. Maintenance of this framework is thereby essential for an organisms homeostasis, as it controls hormonal (T. W. Kim et al., 2015; Lamia et al., 2011) and metabolic (Pickel & Sung, 2020) processes on a large scale, and is estimated to indirectly influence up to half of the transcriptome (Patel et al., 2015).

Strikingly, we found that this correlation signal deteriorated with age, as we consistently observed a decrease in circadian gene correlation, including globally and in kidney and liver. Consistently, *Cirbp* in particular, which was previously shown to play a role in synchronising clock-gene expression globally in response to sleep-wake cycles (Hoekstra et al., 2019), was among the top genes with global age-related expression loss. The signal we report is independent of a previous analysis of the *Tabula muris senis* dataset, which revealed age-related differential expression of some targets of the circadian circuit (Schaum et al., 2020) and indicates an age-related perturbation of the formerly tight circadian regulation. Consistent with our findings, ageing has been shown to alter the periodicity of the mammalian circadian rhythm on the transcriptional level, but also on the level of processes it controls such as temperature, behaviour, and hormonal control (Hood & Amir, 2017). Furthermore, peripheral molecular clocks, which are regulated cell-autonomously by the core genes *Per1* and *Per2*, are known to be affected by age in a tissue-specific manner (Sellix et al., 2012; Tahara et al., 2017; Yamazaki et al., 2002), which could directly cause a decrease in correlation between tissues. Functionally, impairment of circadian expression is associated with shortened lifespan and metabolic disease (Froy, 2011; Kondratov et al., 2006; Penev et al., 1998; Q. Wang et al., 2010), and lack thereof significantly reduced mouse lifespan as demonstrated in a CLOCK deficiency model (Dubrovsky et al., 2010). Interestingly, and consistent with previous notions that DR may partially mediate its healthspan benefits via circadian clock maintenance (Acosta-Rodríguez et al., 2021), we found that DR counteracted age-related correlation loss in circadian genes in the liver and kidney, including in aforementioned core circadian genes *Clock*, *Cry1*, *Per1*, *Per3*, *Nfil3*, and *Arntl*. Unfortunately, we cannot answer how much of this signal could be induced by the DR feeding regime which could strongly contribute to circadian alignment with beneficial effects even for lifespan due to timed feeding, as demonstrated recently (Acosta-Rodríguez et al., 2022). However, overlapping results of

our analysis with a list of potentially rhythmically expressed genes based on public dataset annotations (Carbon & Mungall, 2018; Castellana et al., 2022; S. Li et al., 2016) strongly suggests that aside from the circadian clock itself, we find that part of the signal of correlation and its loss is originating from these oscillating genes. In summary, our results strongly suggest that circadian gene expression is tightly coordinated between tissues on the transcriptional level, their expression correlation is perturbed with age, but this deterioration can be counteracted with DR, which could contribute to its age-ameliorating effects.

In addition to the circadian genes, genes from the core heat shock protein families *Hsp70* (*Hspa5*, *Hspa8*) and *Hsp90* (*Hsp90aa1*, *Hsp90ab1*, *Hsp90b1*), as well as associated co-chaperones such as *Ahsa1* and genes from the *Hsp40* family (*Dnaja1*, *Dnajb1*), were well correlated across most of the tissues in *Tabula muris senis*. The role of heat shock proteins include, for example, stress-related systemic transcription factor regulation (Tower, 2009), and general maintenance of proteostasis (Díaz-Villanueva et al., 2015). We observe the protein folding signal predominantly in metabolically active tissues, which could indicate that these tissues express heat shock proteins in response to a joint metabolic signal correlation signal to facilitate metabolic downstream processes. For example, an increase in heat shock protein expression across tissues in response to feeding would facilitate the correct folding of newly generated proteins with increased nutrient availability. Alternatively, increased heat shock protein expression could buffer the stress caused by increased metabolic activity. Both hypotheses are consistent with the notion that HSPs are known to be regulated in response to metabolic stress and modulated by general dietary composition (Gomez-Pastor et al., 2018; Moura et al., 2018).

Interestingly, we observe a loss of expression correlation in several heat shock proteins, with *Hsp90aa1*, *Hspb1*, *Hspa1a* and others even among the top global candidates with loss of correlation across tissues. This suggests an age-related deterioration in the coordinated expression of protein folding genes in general. It is well known that loss of efficient protein folding is generally associated with ageing (Basaiawmoit & Rattan, 2010), whereas long-lived rodents such as the naked mole rat (Pérez et al., 2009) and bat species (Salmon et al., 2009) have previously been associated with increased organismal protein stability. Furthermore, impaired proteostasis can lead to systemic diseases (Herczenik & Gebbink, 2008; Moreno-Gonzalez &

Soto, 2011), including neurodegenerative diseases (Sweeney et al., 2017), amyloidosis in various tissues (Tasaki et al., 2021), cardiovascular diseases (Gouveia et al., 2017), and metabolic diseases such as diabetes mellitus (Westermark et al., 2011). *Hsp90aa1* in particular is known to be repressed in association with brain ageing and the onset of neurodegenerative diseases (Brehme et al., 2014). In addition, *Hsp90aa1* and *Hspa1a* have previously been linked to cellular survival in tumour cells (Rodina et al., 2016). In line with this, inhibition of *Hsp90* expression has recently been shown to attenuate cellular senescence with cell type specific effects, and in vivo treatment with an HSP90 inhibitor prolonged the lifespan of mice (Fuhrmann-Stroissnigg et al., 2017). These studies highlight the complex role of chaperone complexes, particularly those associated with *Hsp90*, in ageing. In summary, a well-coordinated expression of protein folding genes is essential in order to establish and maintain proteostasis, and a lack of this axis could negatively impact health and lifespan. Our results indicate an age-related loss of coordinated *Hsp* expression with respective implications for organismal homeostasis.

Genes with the most common age-related correlation loss also included *Btaf1*, *Map3k6*, and *Ide*. The top target with global age-related correlation loss is *Btaf1*, which encodes BTAF1. BTAF1 forms a complex with the transcription mediator *TATA-Box binding protein* (TBP) to control TBP-mediated transcriptional processes at the organismal level (Akhtar & Veenstra, 2011; Klejman et al., 2004). *Btaf1* knockdown studies have been limited to cell lines in which BTAF1-dependent modifying effects on TBP-chromatin binding affinity have been classified (De Graaf et al., 2010), and both the role of *Btaf1* and TBP in ageing remain to be elucidated. However, based on the potential broad regulatory impact of *Btaf1*, a lack of coordinated expression across tissues could have diverse effects on organismal ageing, and we therefore propose *Btaf1* as a potential target of future ageing studies. MAP3K6 is a serine/threonine kinase involved in the regulation of Vascular endothelial growth factor (VEGF) with roles in angiogenesis and tumourigenesis (Berra et al., 2000; Eto et al., 2009). *Map3k6* was previously identified in an *in vitro* screen as a candidate kinase for ameliorating cellular senescence (Ferrand et al., 2015). Furthermore, in a knockdown study, MAP3K6 was shown to suppress autophagy, likely by activating the mammalian Target Of Rapamyin (mTOR) (Guo et al., 2015), which is itself a key regulator of ageing-related processes such as insulin signalling, metabolism, cellular growth, and proliferation (Papadopoli et al., 2019; Yoon, 2017). Finally,

IDE has an insulin-degrading function, and insulin signalling is central to promoting an age-amending role in nutrient-sensitive tissues (López-Otín et al., 2013; Pivovarova et al., 2016). A role for *Ide* has previously been implicated in age-related pathologies such as Alzheimer's disease and type 2 diabetes (Kochkina et al., 2015; Qiu & Folstein, 2006). Consequently, targeting IDE has been discussed as a putative therapeutic measure to address these diseases (Leissring et al., 2021; Pivovarova et al., 2016; Tang, 2016). Finally, albeit trends were mild, we found that DR also enhanced expression correlation of *Btaf1*, *Ide*, and *Map3k6* in kidney and the liver. In summary, all three listed targets consistently lose their coordinated expression across tissues with ageing and all have broad regulatory roles themselves, highlighting them as potential novel nodes of systemic loss of organismal homeostasis that we identified based on our expression correlation approach.

Apart from the loss of correlation, we observed a gain of correlation in genes related to immunity and inflammatory signalling throughout our analysis. This observation is consistent with signals from previous studies of the *Tabula muris senis* single cell (Almanzar et al., 2020) and bulk RNA-seq dataset (Schaum et al., 2020). This signal could thus be explained by inflammageing-related responses (Franceschi et al., 2018) that are common to multiple tissues, and sufficiently strong to not be removed completely by our filtering. Opposing this assumption, we observed that in our newly generated datasets in the kidney and liver of mice fed AL and DR diets, genes with age-related expression changes were more strongly enriched for adaptive immune responses, and correlated genes without age-related expression changed had a stronger innate immunity signal. This suggests that the genes that we remove by filtering for age-related expression change are mainly representing the adaptive immune system. Consistently, of the top genes with correlation genes across tissues, most were those responsible for innate immune responses, including *Gbp4* and *Gbp7* which both have orchestrating roles in cell-autonomous immune response (Tretina et al., 2019). In humans, other members of the *Gbp* family, for example GBP1 (C. J. Yu et al., 2011; Zhao et al., 2022), GBP2 (S. Yu et al., 2020), GBP3 and GBP4 (Liu et al., 2021) were previously associated with the mediation of immune cell infiltration processes in tumours. Furthermore, genes with top age-related correlation gain, including both *Gbp*s, *Transporter 1, ATP Binding Cassette Subfamily B Member* (*Tap1*), or *Irgm2* are expressed both in more common cell types such as epithelial cells, tissue-specific

cells such as hepatocytes in the liver, but also commonly expressed in immune cells (e.g. tissue-specific macrophages, B cells, T cells, natural killer cells) of several tissues (Almanzar et al., 2020). In summary, genes with age-related correlation change could represent an increased innate immunity response in aged tissues, potentially representing age-related immune cell infiltration processes. Of note, while it is difficult to derive functional consequences from the present findings, the innate immune system is known to be heterogenously dysregulated with ageing in a tissue-specific way, with potential consequences for the general immune response, inflammageing, and disease formation (Shaw et al., 2013).

Liver and kidney tissues were among tissue pairs with the strongest overall inter-tissue correlation of all pairs analysed, which was lost with age. Both tissues are well known to respond to insulin (Hale & Coward, 2013; Takeuchi et al., 2021), which is central to DR-mediated healthspan extension, and liver and kidney tissue health is known to be ameliorated by DR (Andrianova et al., 2021; Hahn et al., 2017). We found that DR-fed mice generally showed a stronger positive correlation signal at the whole-genome level that was robust to ageing. In addition to ameliorating correlation maintenance, DR induced a novel correlation profile in both young and old animals that was completely absent in DR. This profile included nutrient-sensing mediators such as *Foxo1*, *Foxo3*, *Klf13*, *Klf15*, and *Srebf1*, all of which are important in the signalling downstream of insulin, linking nutrient availability to metabolic reprogramming (Takeuchi et al., 2021). It was recently demonstrated that DR likely promotes its effects jointly via a combination of altered nutrient intake and daily timed feeding (Acosta-Rodríguez et al., 2022), which ameliorates circadian alignment. The DR-specific genes with maintained correlation could be a consequence of the DR-ameliorated circadian rhythm, as it is known that nutrient-sensing, metabolic control, and the circadian clock are strongly inter-connected (Acosta-Rodríguez et al., 2022; Gooley, 2016; Pickel & Sung, 2020).

In summary, we have revealed a striking loss of age-related gene expression correlation. This loss of correlation is an indicator for an age-related loss of homeostasis, reflected by a reduced coordination of transcription across tissues. DR treatment ameliorated this loss, likely via the amelioration of circadian regulation and linked nutrient-sensing pathways to bring about its age-delaying effects. This way, DR could contribute to a tighter

tissue-spanning transcriptional regulation which could bring about DR-specific age-delaying effects. In future, extending our analysis to further tissues or longevity interventions could provide valuable information about how age-related maintenance of homeostasis could be a consequence of transcriptional coordination across tissues.

3.5 Material and Methods

3.5.1 Mouse and human data acquisition and preprocessing

Mouse RNA-seq data from the *Tabula muris senis* bulk RNA-seq dataset (Schaum et al., 2020) were kindly provided by Dr. Oliver Hahn in the form of read count tables for all 17 mouse tissues (bone, brain, brown fat, gonadal fat, heart, kidney, limb muscle, liver, lung, marrow, mesenteric fat, pancreas, spleen, skin, small intestine, subcutaneous fat, and white blood cells, accessed via GSE132040) including the information from which mouse the tissues originated. Tissues that originate from the same mouse are referred to as "paired tissues". In total, gene expression correlation was analysed in 136 unique tissue pairs. Therefore, raw count matrices of all paired tissues were normalized using the standard workflow of the Bioconductor (v3.11) package DESeq2 (v1.28.2) (Love et al., 2014). These normalized read counts were then used for all subsequent pairwise analyses of the mouse data set.
Human RNA-seq data from GTEx (Lonsdale et al., 2013) were acquired from the official GTEx portal (dataset version GTEx V8) in the format of raw read counts (normalized using the standard workflow of DESeq2 (v1.28.2) (Love et al., 2014). Samples with a SMRIN score < 6 and samples with "cells" in their annotation were removed. Furthermore, genes with an average expression of < 100 were removed. Log2-transformed counts were batch-corrected using a linear model using the following design matrix: $\sim$ *SMTSISCH* + *SMGEBTCH* + *DTHHRDY* + *SMTSD* + *SEX*. The residuals of this model, which reflect batch-corrected values, were then used for the correlation analysis.

3.5.2 Tissue-pair-wise correlation analysis across age and sex

The tissue correlation analysis in mice was performed in a pair-wise approach for all 136 unique tissue pairs in the dataset. Genes with more than 20% of zero counts in any of the two tissues per pair were removed

to prevent signal artefacts from zero-count values. Partial regression was calculated per gene for normalized read counts, using the pairwise_corr() function of the pingouin (v0.5.1) python package (Vallat, 2018), and including mouse "age" and "sex" as covariates. Gene-wise partial correlation coefficients were assessed in all unique tissue pairs, defining genes with a $p_{adj} < 0.05$ as significantly correlated. As control, a random-pairing control was included per tissue-pair and the analysis was repeated 1000 times. For each iteration, mouse IDs were randomly shuffled within a tissue pair. Because information on sex and age was also randomized controlling for them became needless, and a Pearson correlation analysis on the normalized read counts per gene was performed using the function stats.personr() of the scipy package (v1.5.2). The significance of the median correlation coefficient roriginal was assessed by comparing its location relative to the normal distribution background, defined by the metrics of the median correlation coefficients of the random-pairing distribution, by using the cumulative distribution function (scipy functions norm(loc = Mean, scale = Standard Deviation); norm().cdf()).

3.5.3 Analysis of gene expression correlation with age

To analyse whether gene expression correlation changed with age, mice were grouped into the following ageing bins: 1 to 9 months, 3 to 12 months, 6 to 15 months, 9 to 18 months, 12 to 21 months, 15 to 24 months, and 18 to 27 months. Tissue-pair-wise correlation analysis on the binned data set was then performed as described above. To account for the over-representation of single age groups, which might bias the resulting values for example due to very high or low normalized count values, a "leave-one-out filtering" was performed. Therefore, the analysis was repeated for each bin, but in this analysis each age group was individually dropped. For example, for the ageing bin 1|3|6|9 months, the bins 3|6|9, 1|3|6, 1|3|9, and 1|6|9 were analysed individually. All genes that did not pass the significance cut-off of $p_{adj} < 0.05$ in all these bins were removed.

3.5.4 Clustering of genes by correlation across multiple tissues

To identify genes that were correlated across multiple mouse tissues, a custom gene-wise "interaction score" per tissue was used. Therefore, the sum of the number of positive significant correlation events ($p_{adj} < 0.05$, $r > 0$) of a gene across all tissues was calculated. The interaction score ranges from 0 (no significant correlation in any tissue pair) to 16 (significant correlation

with all other tissue pairs). Clustering of interaction score matrices was performed using "ward" clustering as implemented in the scipy.cluster package function hierarchy.linkage(optimal_ordering = True), and subclusters were identified and displayed by using a relative distance cut-off and the function hierarchy.fcluster(criterion = "distance"), and hierarchy.dendrogram(), and the seaborn function seaborn.clustermap().

3.5.5 GO term enrichment analysis and visualization

GO term analysis, including the generation of GO term dotplots, was performed as previously described in (Srivastava et al., 2022). In brief, significantly enriched GO terms were identified using the python package AGEpy (v0.8.2) (Metge et al., 2022) based on the DAVID api (D. W. Huang et al., 2009) and their redundancy was reduced using REVIGO (Supek et al., 2011). Significant functional terms were identified using the AGEpy function david.DAVIDenrich, specifying the "GOTERM_BP_FAT" ontology. Significant GO terms were filtered for redundancy by applying REVIGO (parameters "cutoff": 0.7, "valueType": "pvalue", "measure": "SIMREL").

3.5.6 Definition of rhythmically expressed genes

To identify genes that could be rhythmically expressed in mice (in addition to genes of the circadian clock), we downloaded annotations of rhythmically expressed genes from a total of three sources, (1) RhythmicDB (Castellana et al., 2022) (v1.1, accessed on 05th June 2023), (2) CGDB (S. Li et al., 2016) (v1.0, accessed on 05th June 2023), and (3) the GO ontology (Carbon & Mungall, 2018) (v2023-05-10).
For RhythmicDB, preprocessed data for mouse was acquired using the "BioCycle" algorithm across all oscillation periods, and genes were annotated as rhythmically expressed in one tissue when they passed a p-value threshold $p \leq 0.01$. Next, the tissue of each rhythmically expressed genes was annotated based on the origin dataset (E-GEOD-11516, E-GEOD-33726-POL, E-GEOD-33726-TOT, E-GEOD-3751-LI, E-GEOD-52333-HFD, E-GEOD-52333-NC: "Liver"; E-GEOD-3751-SM: "Skeletal muscle"; E-GEOD-414: "Aorta"; E-GEOD-70384: "SCN"; E-MEXP-3780: "Cartilage"). To reduce tissue biases specifically of the liver, we dropped all rhythmically expressed genes that were not present in at least two tissues, and that way acquired n = 957 rhythmically expressed genes. For CGDB, we acquired all mouse genes that were pre-annotated in the database as rhythmically expressed across datasets. We then collected all annotated genes of the following

tissues: "Liver" (including hepatocytes), "White adipose tissue" (including epididymal adipose tissue, epigonadal adipose tissue, visceral adipose tissue, inquinal adipose tissue), skeletal muscle, heart (including aorta), "Lung", "Kidney", "BAT", "Marrow" (including bone marrow), "Bone" (including femur, femur vertebra), "Brain" (including frontal cortex, midbrain, cerebellum, cortex, hippocampus, hypothalamus, prefontal cortex, amygdala), "Spleen", "Intestinal tissues" (including intestine, gut, jejunum, small intestine, ileum), and "SCN". Again, to reduce tissue-specific biases, we dropped all rhythmically expressed genes that were not present in at least two tissues, and that way acquired n = 1,461 rhythmically expressed genes, with an overlap of 211 genes between both datasets.
We combined the gene sets from both datasets with the n = 314 genes of GO:0048511 (rhythmic process) to achieve a final list of n = 2,444 rhythmically expressed genes that could potentially be oscillating in multiple tissues of the body.

3.5.7 Identification of genes with age-related correlation gain and loss

To identify genes with age-related correlation change, we split the dataset into two non-overlapping bins, representing earlier life (1 to 12 months) and later life (15 to 27 months). For both bins, we performed a tissue-pair-wise correlation analysis as described above, but without additional filtering. Genes that were significantly correlated ($p_{adj} < 0.05$) in earlier life, but not in later life were defined as "genes with age-related correlation loss", and genes that were significantly correlated in later life, but not in earlier life were defined as "genes with age-related correlation gain".

3.5.8 Clustering of genes with age-related correlation gain or loss

To identify sub-clusters in genes with age-related correlation gain and loss, we clustered them using their partial correlation coefficient of the bin-wise tissue-pair-wise correlation analysis. Clustering is then performed as described previously by using "ward" clustering with optimal_ordering.

3.5.9 Calculation of age-related expression changes in *Tabula muris senis*

Age-related expression changes per gene were calculated using Pearson correlation of age and normalized counts. Gene expression was scored as age-related when $p_{adj} < 0.05$ in either tissue of a tissue pair.

3.5.10 Mouse husbandry and DR protocol

The DR study was performed in accordance with the recommendations and guidelines of the Federation of the European Laboratory Animal Science Association (FELASA), with all protocols approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, Germany (84-02.04.2015.A437). Female F1 hybrid mice (C3B6F1) were generated in-house by crossing C3H/HeOuJ females with C57BL/6NCrl males (strain codes 626 and 027, respectively, Charles River Laboratories). Lifespans of AL and DR mice were previously published (Kaeser et al., 2021). Animals were weaned at 3-4 weeks of age and were then randomly assigned in groups of 5 females to individually ventilated cages under specific-pathogen-free conditions with constant temperature (21 °C), 50-60% humidity and a 12-hour light-dark cycle. For environmental enrichment, mice had constant access to nesting material and chew sticks. All mice received commercially available rodent chow (ssniff R/M-Low phytoestrogen, ssniff Spezialdiäten, Germany) and were provided with filtrated water ad libitum. DR animals received 60% of the food amount consumed by AL animals. DR treatment was started at 12 weeks of age by directly transferring mice from AL to 40% DR. DR animals were fed once per day in the morning, and all animals were checked daily for their well-being and any deaths. Organs were collected from 5 months (AL young, DR young), or 20, 24, or 28 months (AL old, DR old) old female mice. All mice were killed in the morning within a period of 3 h prior to the regular feeding time of the DR mice. Mice were killed by cervical dislocation, and tissues were rapidly collected and snap-frozen in liquid nitrogen.

3.5.11 RNA isolation from liver and kidney tissue

RNA was extracted from 30 mg of liver tissue, and 50 mg of kidney tissue using TRIzolTM (ThermoFisher Scientific, Germany). Briefly, tissue samples were homogenized in 750 µL Trizol and 100 µL of RNAse-free water using a handgun. Samples were then incubated for 5 minutes at RT, 200 µL of chloroform was added and again incubated for 10 minutes at RT. The RNA-yielding aqueous phase was retrieved using Phase Lock GelTM Heavy Tubes (Quantabio). RNA was precipitated by adding 550 µL of isopropanol and 55 µL of 3M NaOAc and incubation overnight at -20°C. The RNA was pelleted by centrifugation at 12,000 rpm for 15 minutes at 4°C, and washed three times with ice-cold 70% ethanol with subsequent centrifugation at 10,000 rpm for 10 minutes at 4°C. The pellet was then air-dried for 5 minutes, and

resuspended in 40 µL RNAse free water. RNA was treated with DNAse using the DNA-freeTM Kit (Thermo Fisher Scientific) to remove genomic DNA contamination. Isolated RNA sample quality was assessed by High Sensitivity RNA Tapestation (Agilent Technologies Inc., California, USA) and quantified by Qubit 2.0 RNA HS assay (ThermoFisher, Massachusetts, USA).

3.5.12 RNA-seq library preparation and differential expression analysis

RNA-seq library preparation and sequencing (50 million reads, 25 million in each direction, 150bp paired end) of kidney and liver RNA was performed by Admera Health (New Jersey, USA). poly(A)+ mRNA was isolated using the NEBNext® Poly(A) mRNA Magnetic Isolation kit (New England BioLabs Inc., Massachusetts, USA). Prior to first strand synthesis, samples were randomly primed (5’ d(N6) 3’ [N=A,C,G,T]) and fragmented. First strand synthesis was done using the Protoscript II Reverse Transcriptase for approximately 40 minutes at 42°C. All remaining steps for library construction were done according to the NEBNext® Ultra™ II Non- Directional RNA Library Prep Kit for Illumina® (New England BioLabs Inc., Massachusetts, USA). Final libraries quantity was assessed by Qubit 2.0 (ThermoFisher, Massachusetts, USA) and quality was assessed by TapeStation D1000 ScreenTape (Agilent Technologies Inc., California, USA). The final library size was about 430bp with an insert size of about 300bp. Illumina® 8-nt dual-indices were used. Equimolar pooling of libraries was performed based on QC values and sequenced on an Illumina® Novaseq S4 (Illumina, California, USA) with a read length configuration of 150 bp for 50 M paired-end reads per sample, i.e. 25M reads for each direction). Libraries read processing was automated using Flaski (v3.11.34) (Iqbal et al., 2021), developed by the Bioinformatics Core Facility at the MPI for Biology of Ageing (Cologne, Germany). Raw reads were mapped to the mm39 ENSEMBL build 105 using kallisto (v0.46.1) (Bray et al., 2016). Gene counts were quantified in the same step. Differential gene expression was analysed using DESeq2 (v1.24.0) (Love et al., 2014) in R (v3.6.3). Lowly expressed genes were excluded from the analysis if more than 80% of all samples per group (AL young and old joined; or AL young, AL old, DR young, DR old, respectively) had a normalized counts value < 10 for this gene. Inter-tissue gene correlation analysis of kidney and liver data was then performed as described previously. A gene was

defined as age-related when the DESeq2 Bonferroni-adjusted p_{adj} passed the threshold of 0.05 in either of the two tissues.

3.5.13 Multiple testing correction

Unless otherwise stated, p-values were adjusted using Benjamini-Hochberg multiple testing correction. For all adjustments, the package statsmodels (v0.13.2) and its function stats.multitest.multipletests() were used.

3.5.14 General data processing and visualization

General data processing and visualization were performed using custom python scripts and the following python packages: scipy (v1.5.2), numpy (v1.21.0), pandas (v1.2.0), seaborn (v0.11.1), matplotlib (v3.3.2), and its associated package matplotlib-venn (v0.11.5). Tissue icons (kidney, liver) and mouse and human icons were taken from BioRender.

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3.7 Supplementary Figures

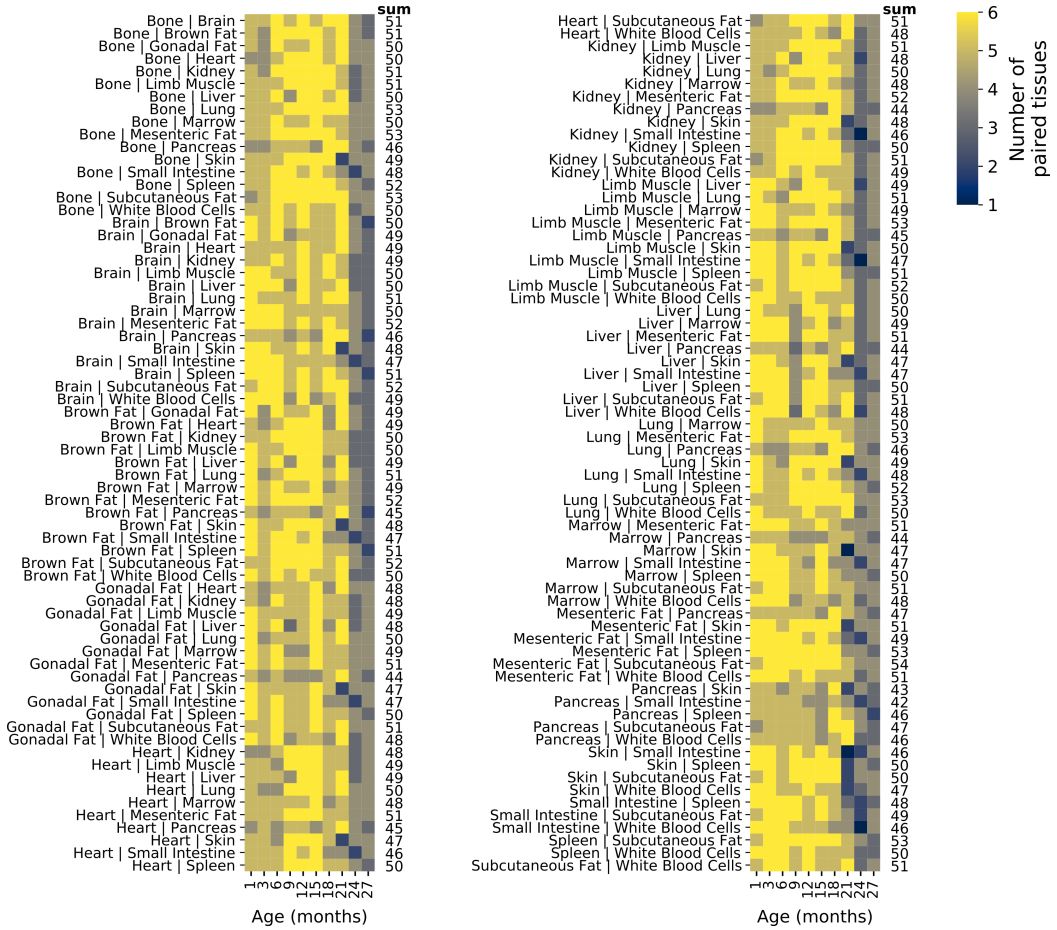


Figure S3.1 | **Number of paired tissues per unique tissue pair in the dataset.** Number of replicates are presented per age time point. The sum over all time points is annotated next to each row.

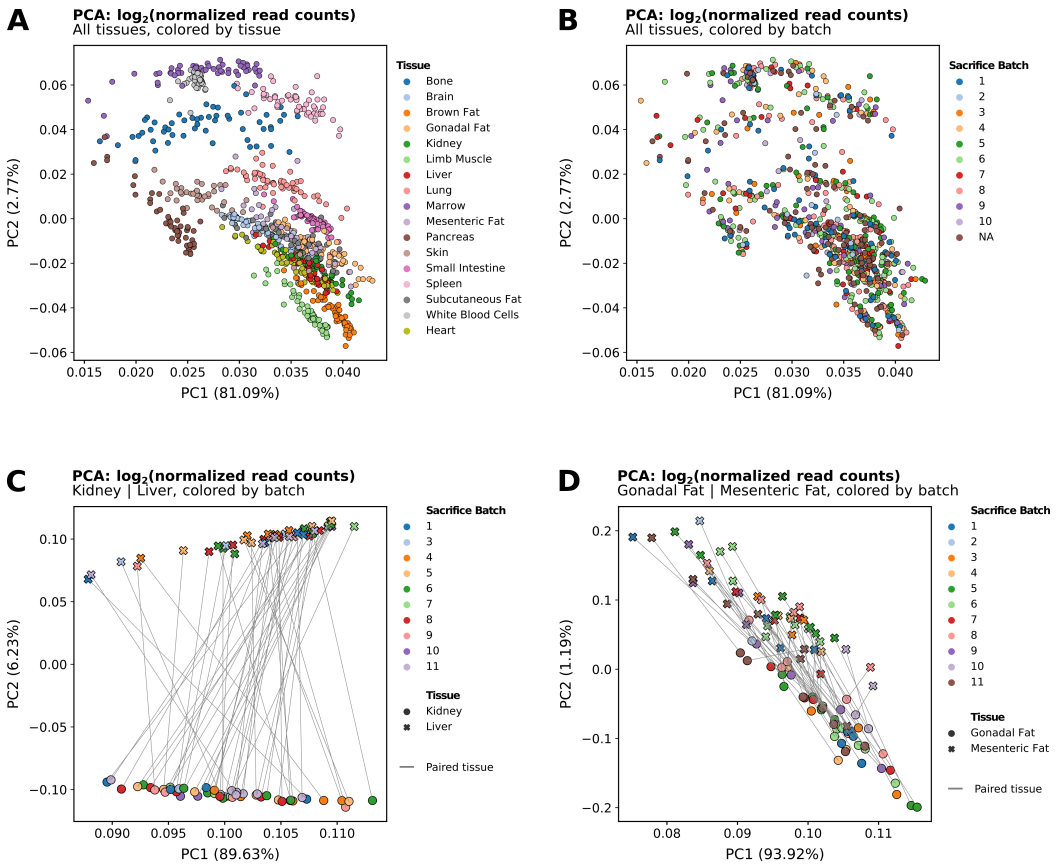


Figure S3.2 | **Effects of sacrifice batches on *Tabula muris senis* bulk RNA-seq samples.** All PCAs are based on log2 DESeq2 normalized read counts. Sacrifice batch data (one number indicates the same day of mouse sacrifice) was kindly shared by Dr. Nicholas Schaum. NA indicates samples for which no sacrifice batch data was available upon the time of request. (A) PCA of all available samples colored by origin tissue. (B) The same PCA, but colored by sacrifice batch. (C) Example of tissue-pair specific PCA in kidney | liver. Tissues that derive from the same mouse are connected with a line. (D) Example of tissue-pair specific PCA in gonadal fat | mesentaric fat.

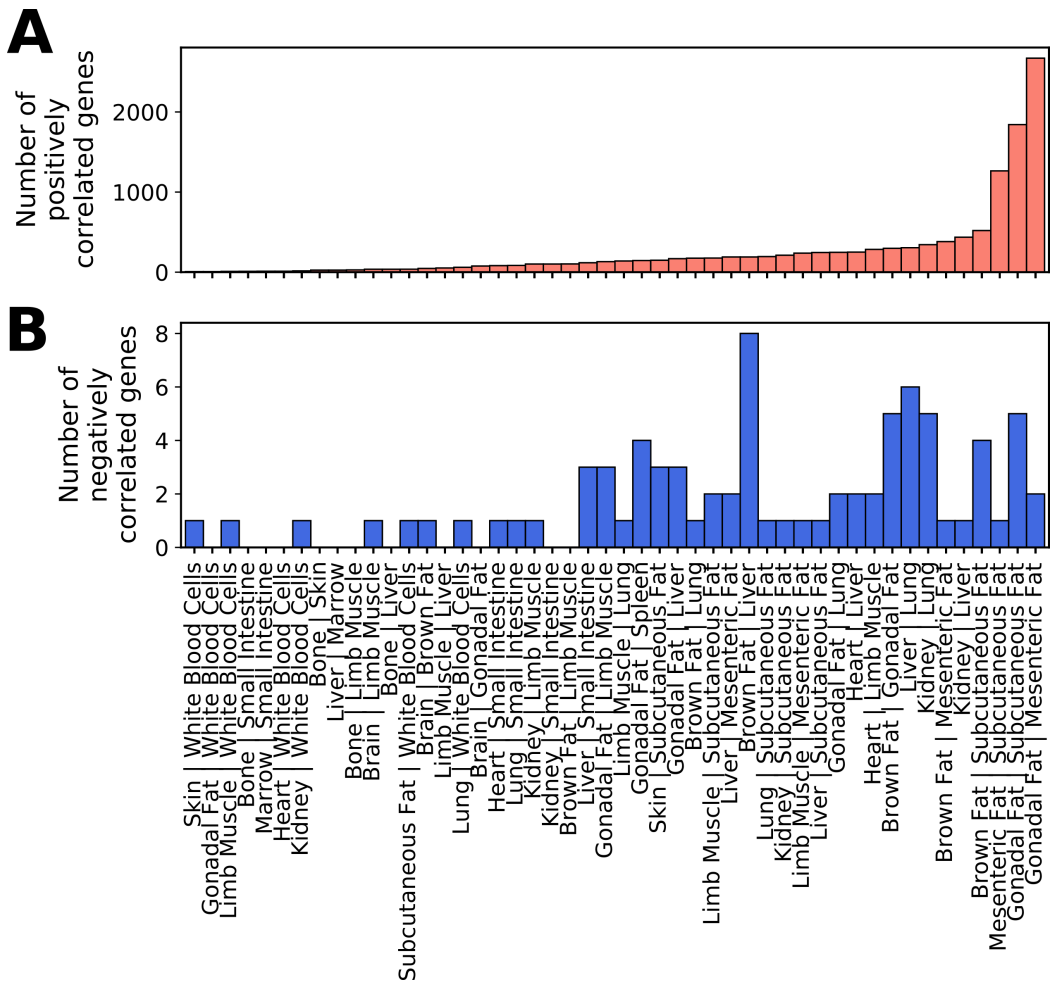


Figure S3.3 | **Number of genes with positive and negative correlation.** Bar plot representation of the number of (A) positively and (B) negatively correlated genes genes.

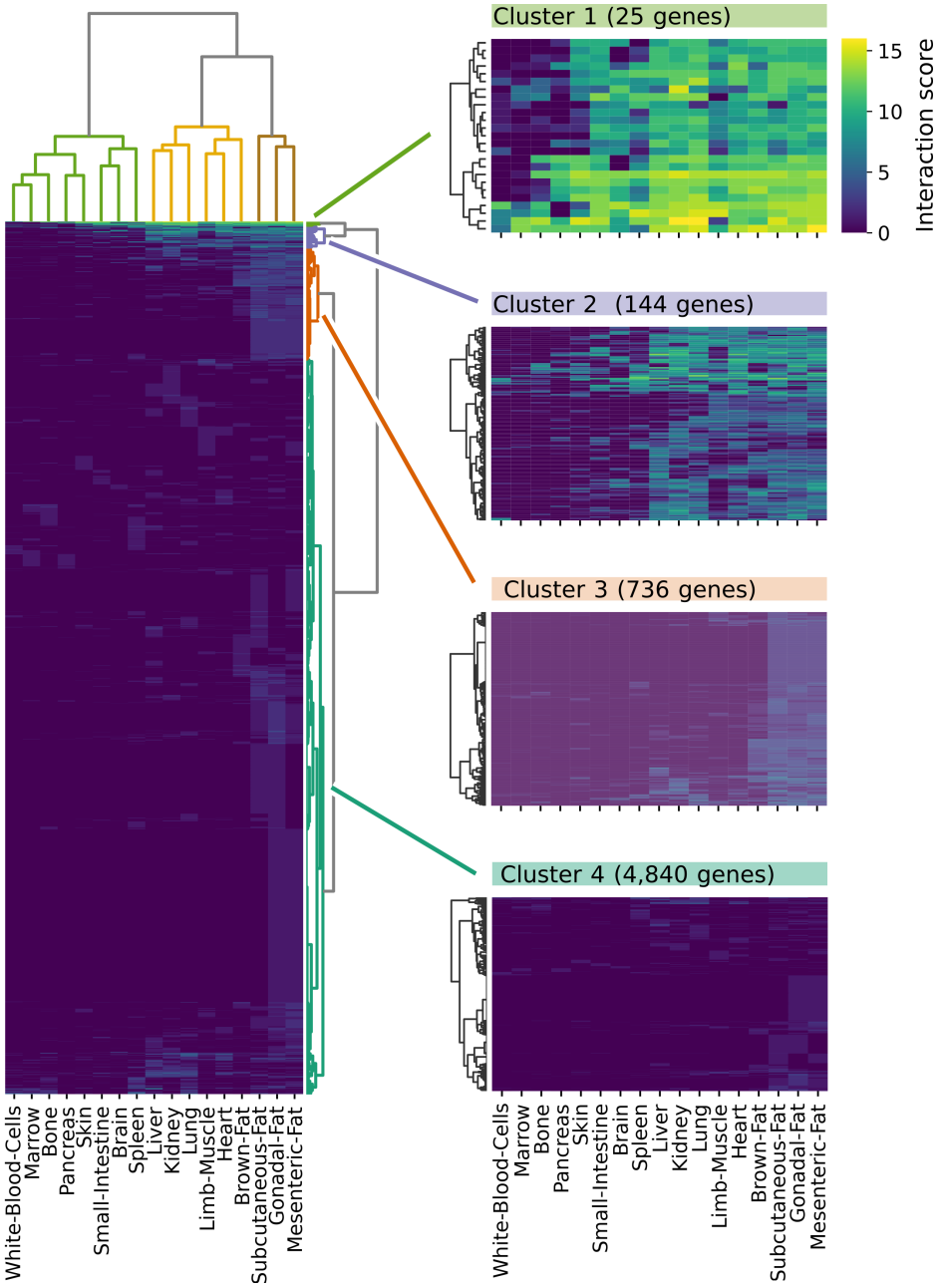


Figure S3.4 | **Gene correlation frequency across multiple tissues.** Correlated genes between multiple tissue pairs are identified by counting their significant correlation events ($p_{adj} < 0.05$) with all other tissues in the dataset ("interaction score"). Clusters of correlated genes are defined by ward clustering, defining a relative distance cut-off of 0.35 of the maximum for genes, and of 0.65 for tissues. Left: Clustermap representation of the interaction score of all expressed genes in the dataset with at least 1 occurrence of correlation between tissues (n = 4,840 genes). Right: Four clusters of correlated genes. Cluster 1: correlated genes across all tissues (n = 25). Cluster 2: correlated genes across metabolically more active tissues (n = 144). Cluster 3: correlated genes in white adipose tissues (n = 736). Cluster 4: remaining genes with only spurious correlation.

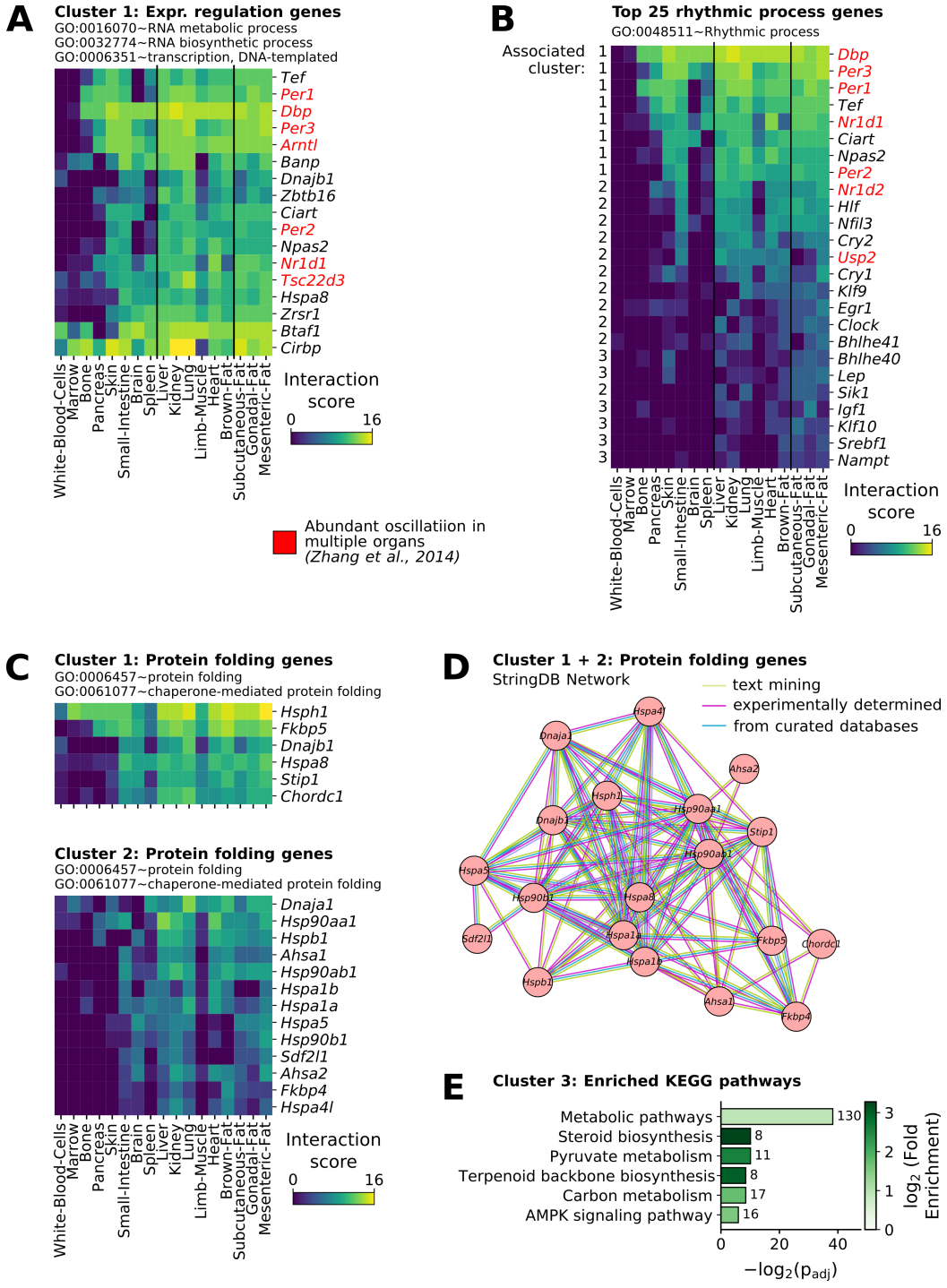


Figure S3.5 | **Functional association of genes in clusters 1 to 3.** (A) Expression regulation associated genes in cluster 1. Red represents genes that are found to be oscillating in multiple organs (S. Zhang et al., 2014). (B) Top 25 rhythmic process associated genes across all clusters, sorted by sum of their interaction score. The origin cluster of each gene is represented on the left. (C) Protein folding genes in clusters 1 (top) and 2 (bottom). (D) STRING (Szklarczyk et al., 2021) network of protein-protein interactions of the proteins of all protein folding genes in cluster 1 + 2. Nodes are displayed if they pass a minimum STRING score of 0.7.

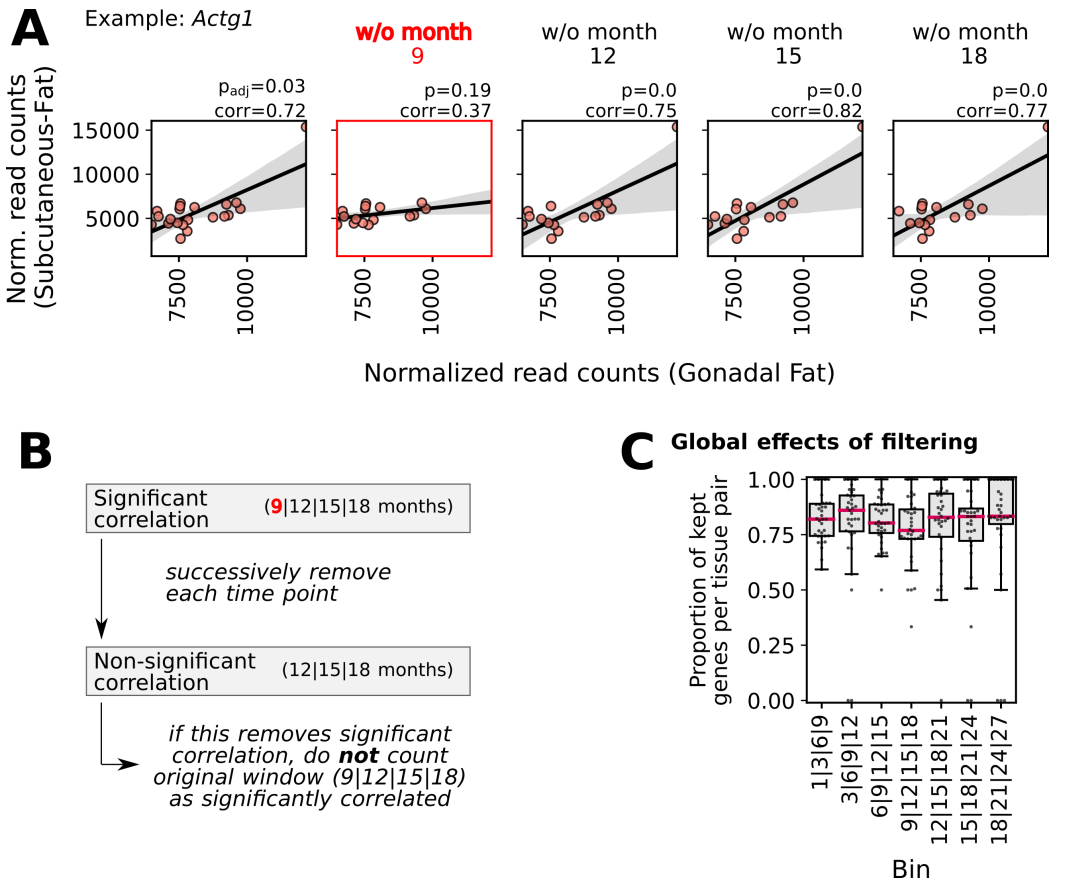


Figure S3.6 | **"Leave-one-out" filtering effect on count of correlated genes.** (A) Example correlation profile of Actg1 in the ageing bin 9|12|15|18 of gonadal and subcutaneous fat. Month 9 (red) is likely responsible for the significant signal as removing it also removes the significant signal ($p_{adj} > 0.05$). (B) Schematic representation of the filtering procedure. (A) is taken as example here, with 9 months as outlier group. (C) Proportion of kept genes after filtering for all tissue pairs with significant median correlation shift (Figure 3.1E, n = 35).

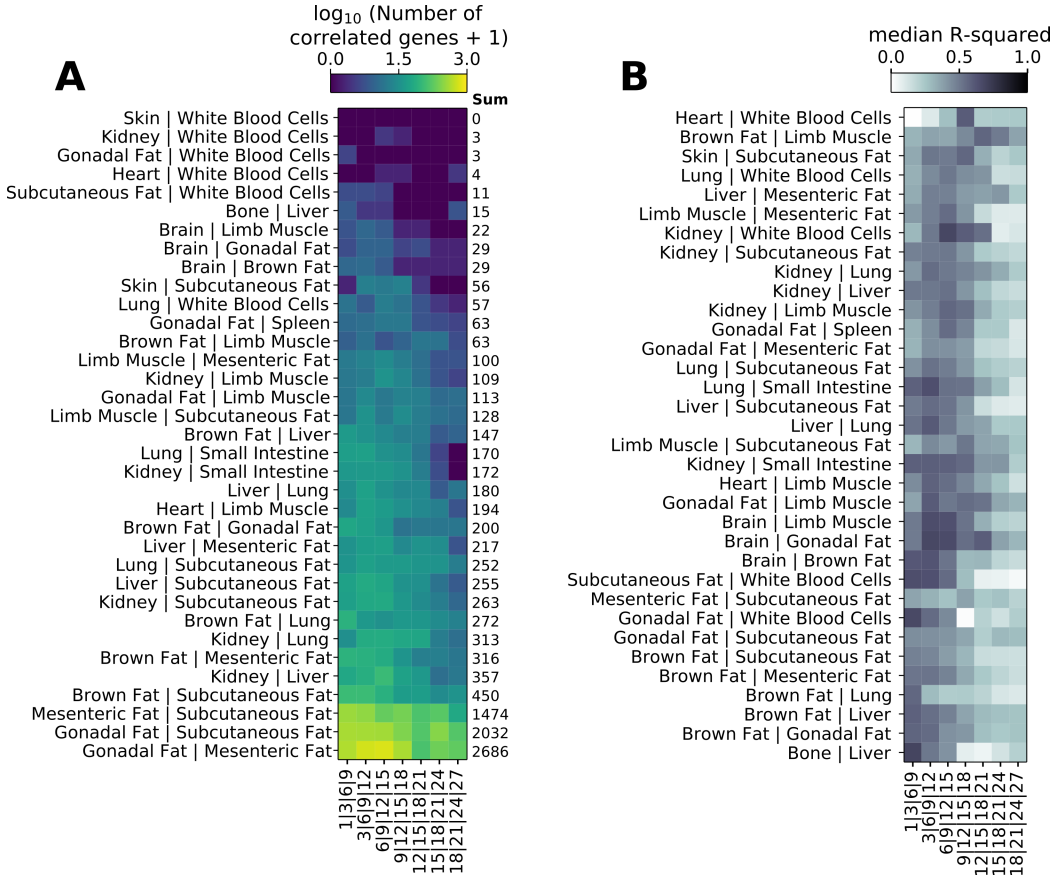


Figure S3.7 | **Additional information on age-related correlation change.** Represented are all tissue pairs with significant median correlation shift (Figure 3.1E, n = 35) after applying "leave-one-out"-filtering (Figure S3.6). (A) The total number of correlated genes ($p_{adj} < 0.05$) in all age groups. Numbers are given on a log10 scale per age group. Heatmap rows are sorted by the sum of the number of correlated genes per tissue pair, which are listed on the right side. (B) Median coefficient of determination (R^2) over all genes per age group that were significantly correlated ($p_{adj} < 0.05$) in at least one age group. Values are sorted similarly as in Figure 3.3B.

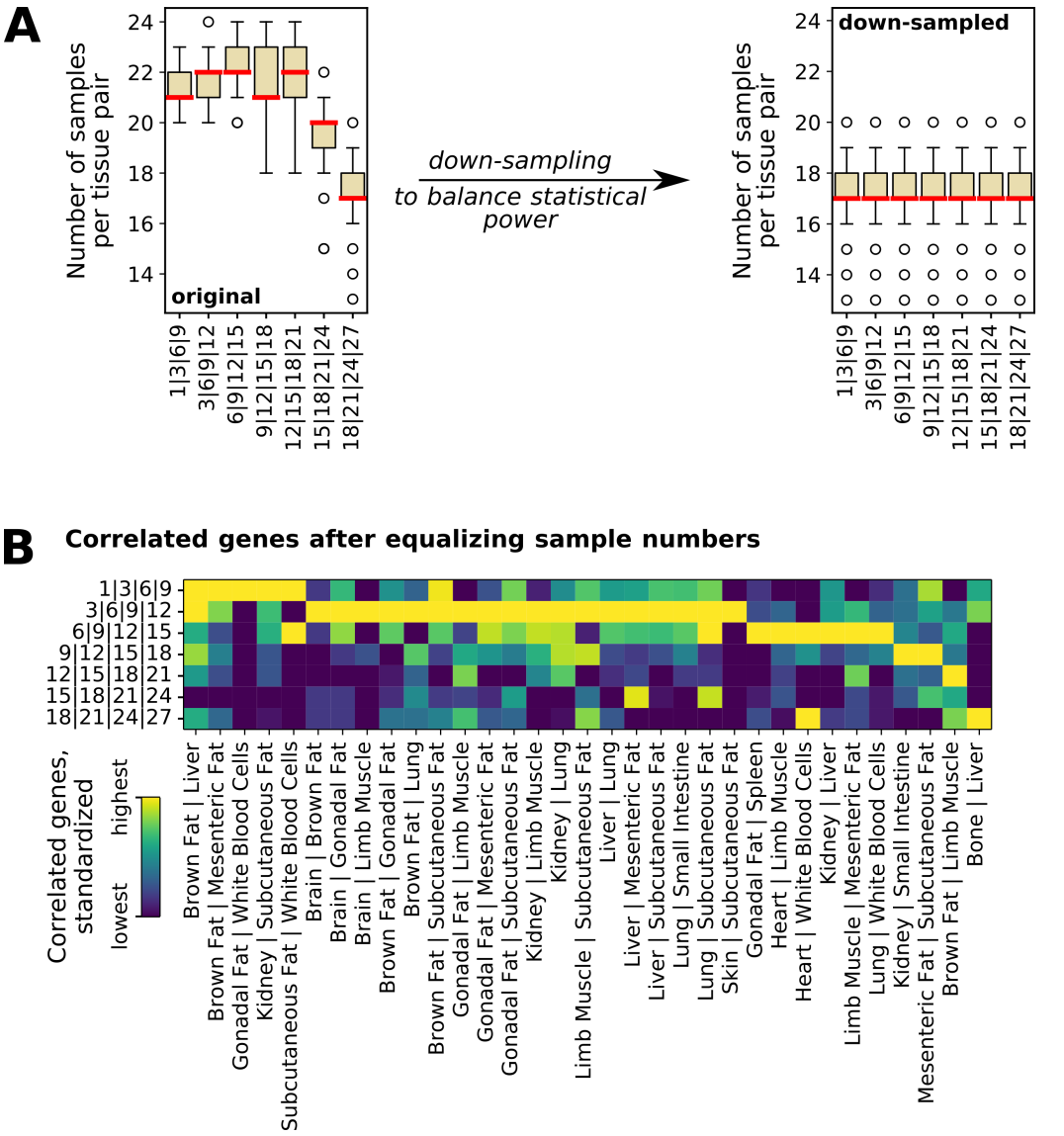


Figure S3.8 | **Effect of down-sampling on correlated genes.** (A) To test the effect of sample numbers and resulting variation in statistical power, sample numbers within ageing bins were removed in a random pattern to be equal to the number of samples in the ageing bin with the lowest amount of samples. (B) Standardized (min-max normalization) number of correlated genes across all tissue pairs with significant median correlation shift (Figure 3.1E, n = 35).

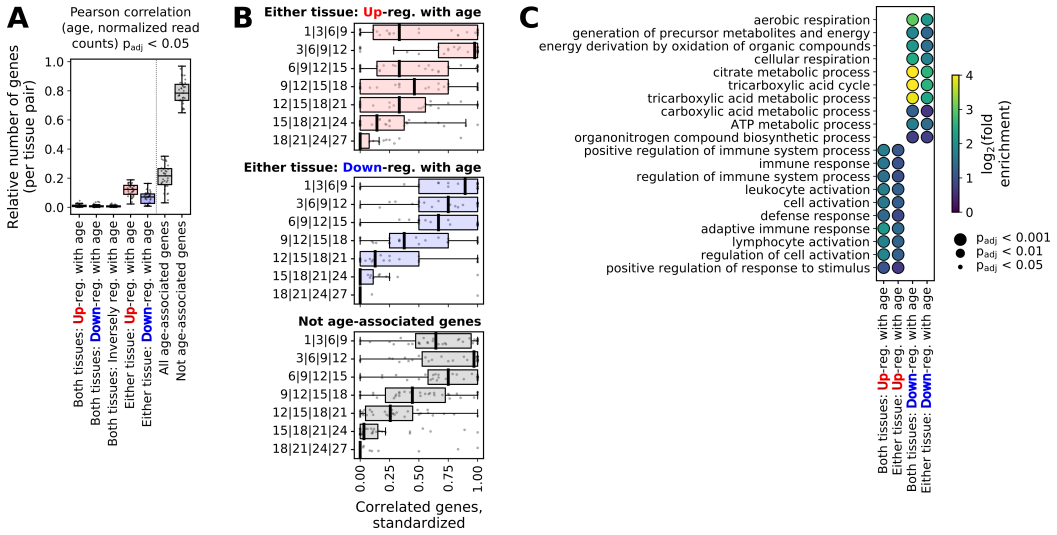


Figure S3.9 | **Functional role of age-associated genes in the dataset.** (A) Boxplot representation of the proportion of age-associated genes (significance of Pearson correlation of age and normalized read counts with subsequent BH p-value-adjustment; $p_{adj} < 0.05$). Genes are grouped by whether they are up- or down-regulated in both tissues (left three boxes), or up- or down-regulated in either tissue of a pair (following-up two boxes), and their sum is represented in "All age-associated genes". (B) Boxplot representation of the min-max standardized median correlation of all tissue pairs of genes that are (top, red) up-regulated with age in either of two tissues, (middle, blue) down-regulated with age in either of two tissues, and (bottom, gray) not associated with age in either of the two tissues. (C) Dotplot representation of the top GO terms (BP FAT ontology, after revigo redundancy reduction) that are significantly enriched. To reduce noise, only genes were used that were in the top 25% most frequent genes of all tissue pairs with age-related correlation gain in both tissues (n = 782) or in either tissue (n = 2,784), and genes with significant correlation loss in both tissues (n = 726) or in either tissue (n = 2,116).

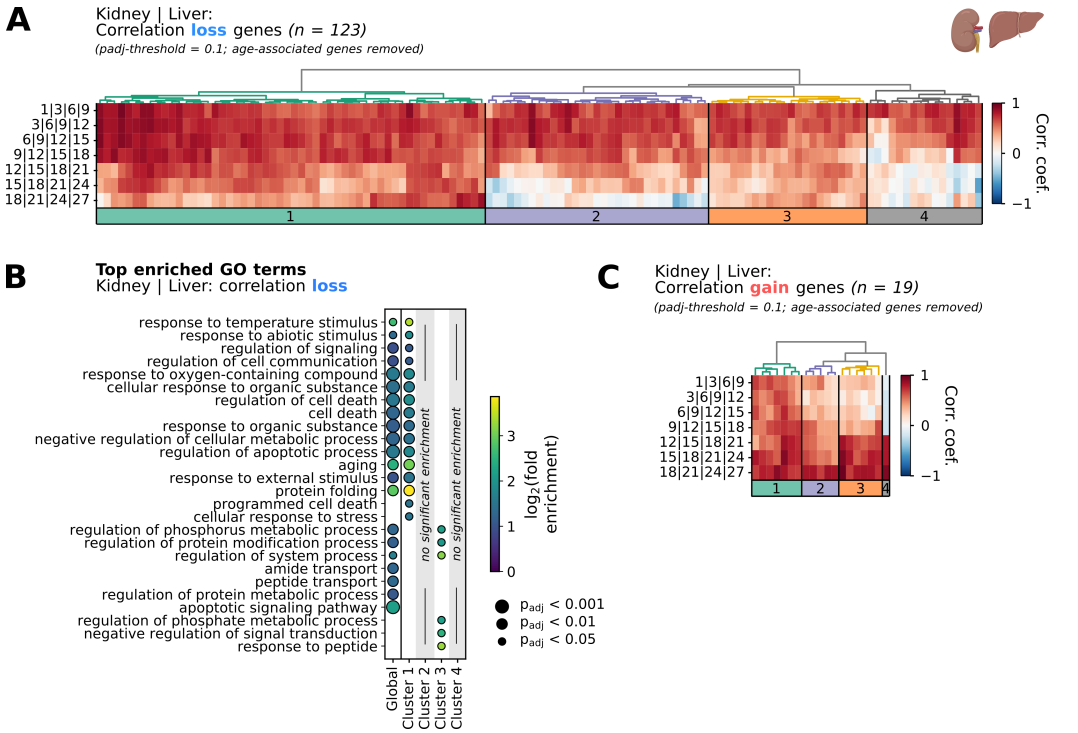


Figure S3.10 | **Genes with correlation loss in liver and kidney.** (A) Clustermap representation of genes with age-related correlation loss (p_{adj} < 0.1, age-associated genes removed). Gene clusters are highlighted, defined by a relative distance cut-off of 0.4 of the maximum distance. (B) Dotplot representation of enriched GO terms (BP Fat ontology, after revigo redundancy reduction) of all genes with correlation loss ("Global"), and cluster-specific genes ("Cluster 1" to "Cluster 4"). A local GO term enrichment was only present in clusters 1 and 3. (C) Clustermap representation of genes with age-related correlation gain.

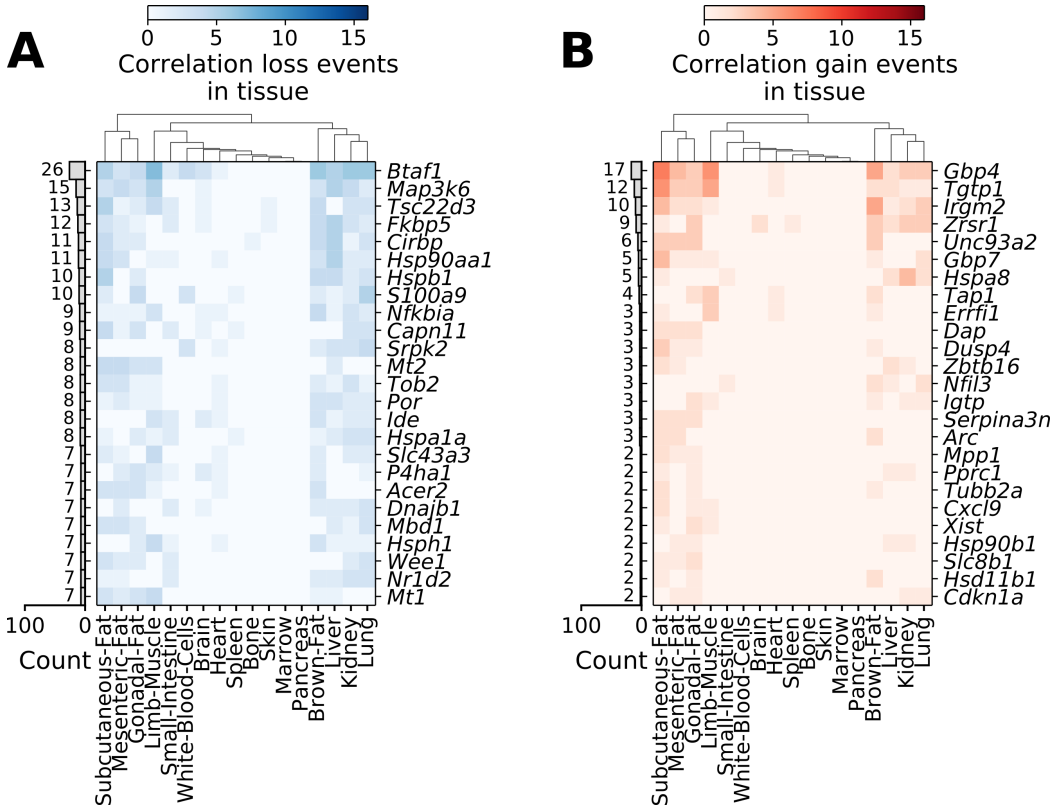


Figure S3.11 | **Tissue-specific signal of genes with age-related correlation change.** For the top 25 genes with (A) age-related correlation loss and (B) correlation gain across tissue pairs, their origin is represented on the level of individual tissues. The tissue clustering is based all genes with at least 5 significant correlation events across all tissue pairs.

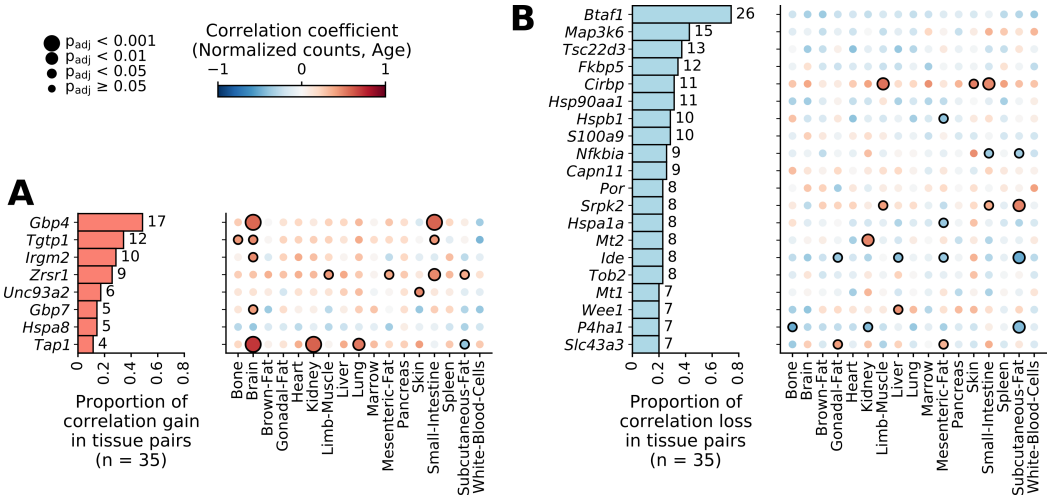


Figure S3.12 | **Age-related expression change trends of top genes with age-related correlation change.** Dotplot representation of the Pearson correlation statistics of normalized counts vs. age for the top genes with (A) age-related correlation gain and (B) correlation loss across tissues. Significant correlation ($p_{adj} < 0.05$) is indicated through black circles around individual dots. These tissues are filtered out and do not contribute to the frequency of age-related expression change annotated in the bar plots.

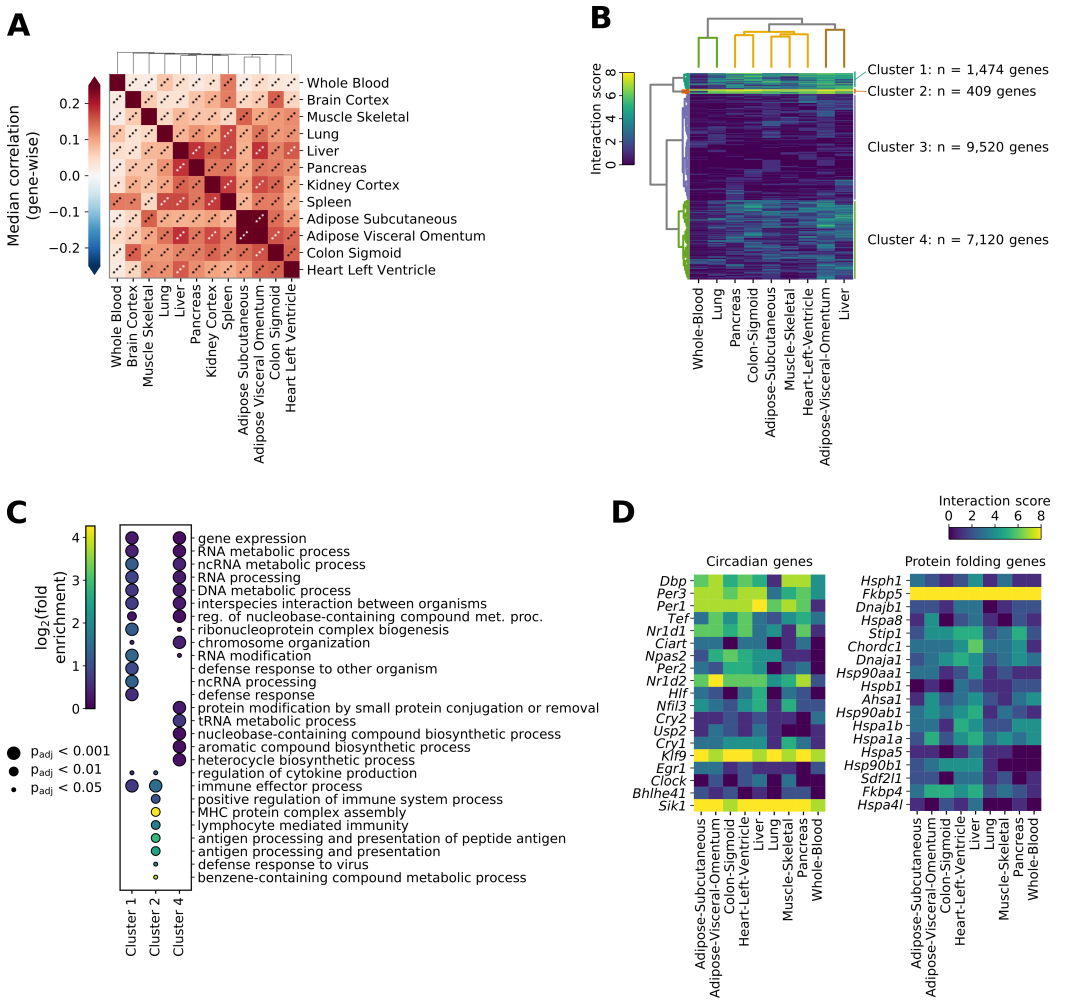


Figure S3.13 | **Inter-tissue expression correlation of the GTEx dataset.** (A) Heatmap representation of median correlation coefficients of 12 tissue pairs of the dataset. Significance of the median correlation shift compared to the random pairing background is indicated (CDF, n = 1000 iterations with subsequent BH-correction, ***: $p_{adj} < 0.001$). (B) Clustering of correlated genes between multiple tissues. Tissues with less than at least 50 paired tissue samples were removed from the analysis. Values were down-sampled to the minimum available paired samples across all tissue pairs (n = 66) to prevent a bias induced by statistical power. "Interaction score" is defined as the number of significant correlation events ($p_{adj} < 0.05$) per gene in one tissue with all other tissues in the dataset. (C) Dotplot representation of the top GO terms (BP FAT ontology, after revigo redundancy reduction) that are significantly enriched across clusters 1, 2, and 4. (D) Interaction score of the top correlated genes in the mouse dataset, including circadian genes (left, from Figure S3.5B) and protein folding genes (right, from Figure S3.5B).

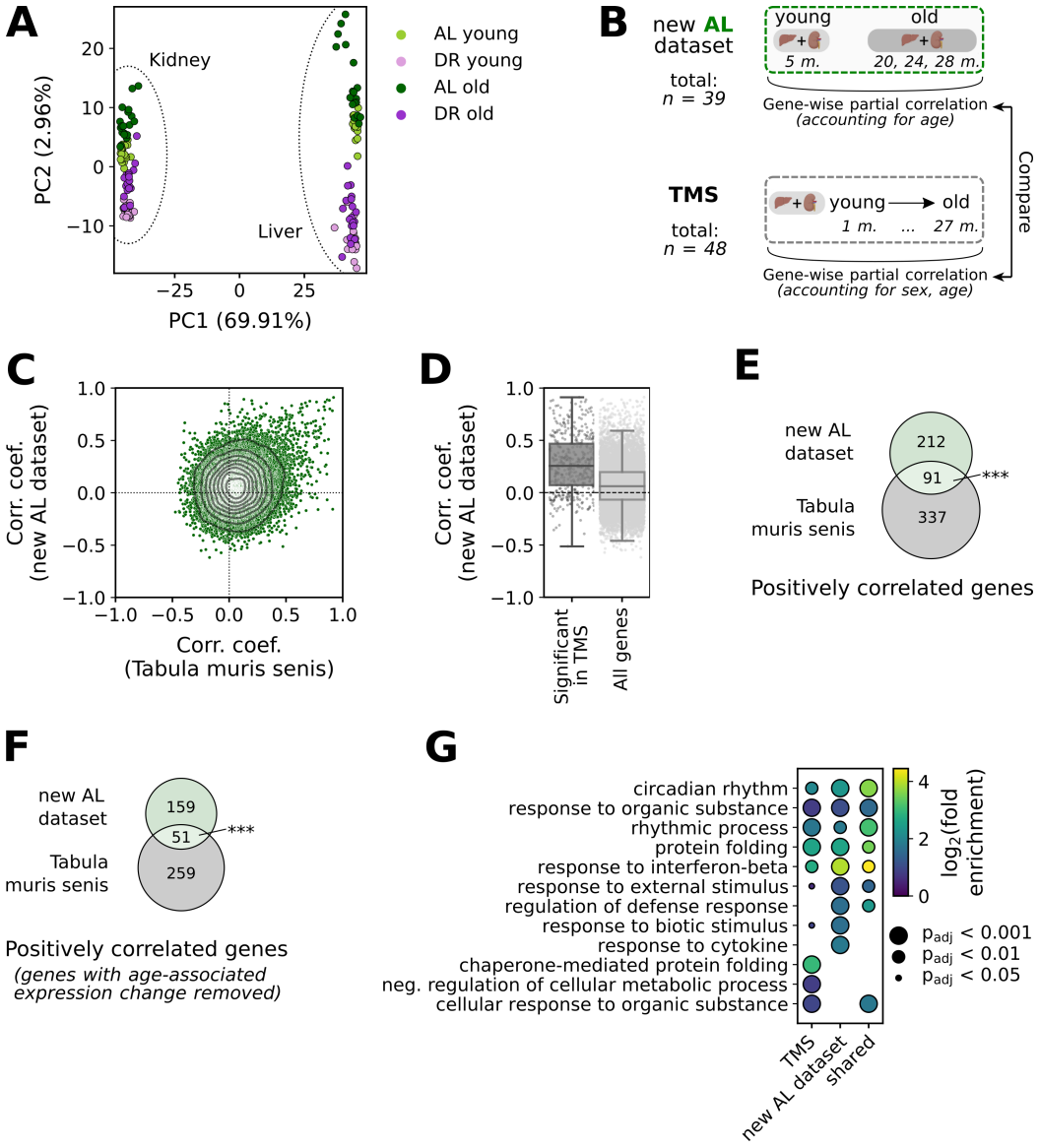


Figure S3.14 | **A positive correlation shift in kidney-liver is conserved across datasets.** (A) PCA of all samples in the dataset based on the DESeq2 normalized counts. (B) Schematic representation of metadata differences and applied partial correlation parameters of the new AL dataset and the *Tabula muris senis* (TMS) dataset. (C) Gene-wise correlation coefficients in liver and kidney of mice of *Tabula muris senis* versus the new AL dataset. (D) Correlation coefficient in the new AL dataset of genes that are significantly correlated in *Tabula muris senis* (TMS). (E) Overlap of significantly positively correlated genes ($p_{adj} < 0.05$) in *Tabula muris senis* and AL mice of the new dataset. (F) The same representation as in E, but with genes with age-associated expression change removed (TMS: Pearson correlation of age and normalized read counts, $p_{adj} < 0.05$ in either tissue; new AL dataset: DESeq2 with subsequent Bonferroni-correction, $p_{adj} < 0.05$ in either tissue). (G) Dotplot representation of the top GO terms (BP FAT ontology, after revigo redundancy reduction) of the gene sets in (F).

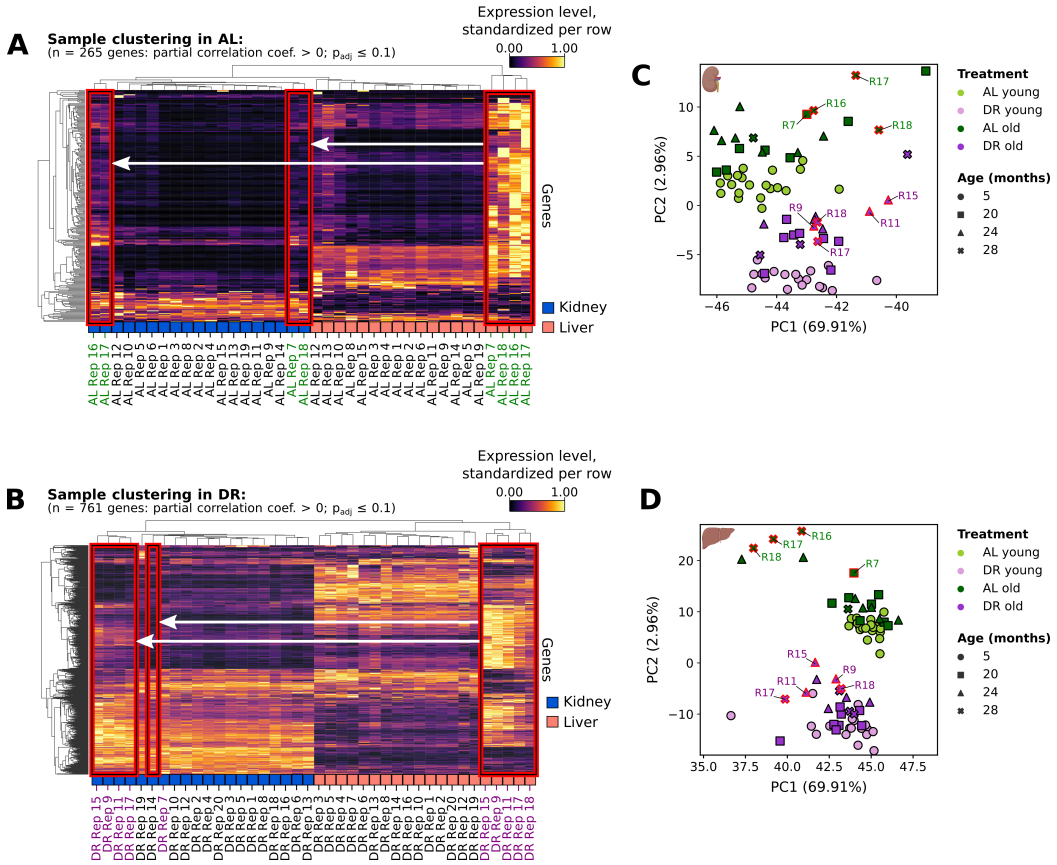


Figure S3.15 | **Detection of outlier samples in old AL and DR groups.** (A, B) Clustermap representation of the relative expression levels (row-wise min-max standardization) of the 256 positively correlated in old AL (A, $p_{adj} \leq 0.1$) and the 761 correlated genes in old DR (B, $p_{adj} \leq 0.1$). Red boxes highlight defined outlier samples that were defined based on clustering differences (dendogram on top) in liver samples (salmon) that also showed sub-clustering in kidney samples (blue). (C, D) PCA of all samples, zoomed on kidney (C) and liver (D) samples respectively. Previously defined outlier samples are highlighted.

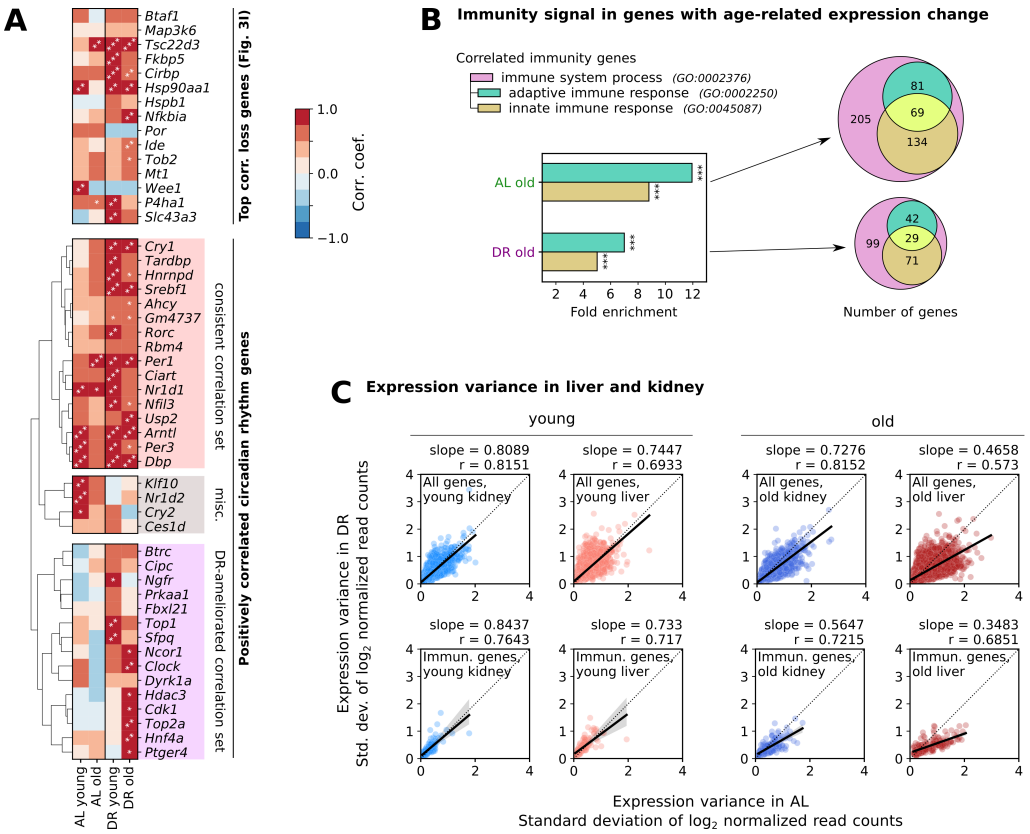


Figure S3.16 | **Additional information on circadian and immune signal in correlated genes of kidney and liver.** (A) Clustermap of the correlation coefficient of chosen genes. Top: top genes with frequent correlation loss (from Figure 3.3F) that passed all filterings. Bottom: circadian genes with significant correlation ($p_{adj} < 0.1$) in either group (*: $p_{adj} < 0.05$, **: $p_{adj} < 0.01$, ***: $p_{adj} < 0.001$). In the bottom group, major clusters of genes are formed which are manually annotated as "consistent correlation set" (genes with generally strong correlation in AL and DR), "misc." (genes with stronger correlation in AL, or generally noisy coefficients across groups), and "DR-ameliorated correlation set" (genes with stronger correlation in DR groups). (B) Fold enrichment and number of genes with age-related expression change (Bonferroni-adjusted p-value < 0.05 in either kidney or liver, n = 1,508 genes in AL, n = 942 genes in DR) in old AL and DR associated with GO terms "immune system process", "adaptive immune response", or "innate immune response". (C) Expression variance (standard deviation of log2 read counts in AL and DR) of all expressed genes (top), or all immunity-related genes with significant correlation in either AL old or DR old (bottom). Parameters of the linear regression are annotated on top.

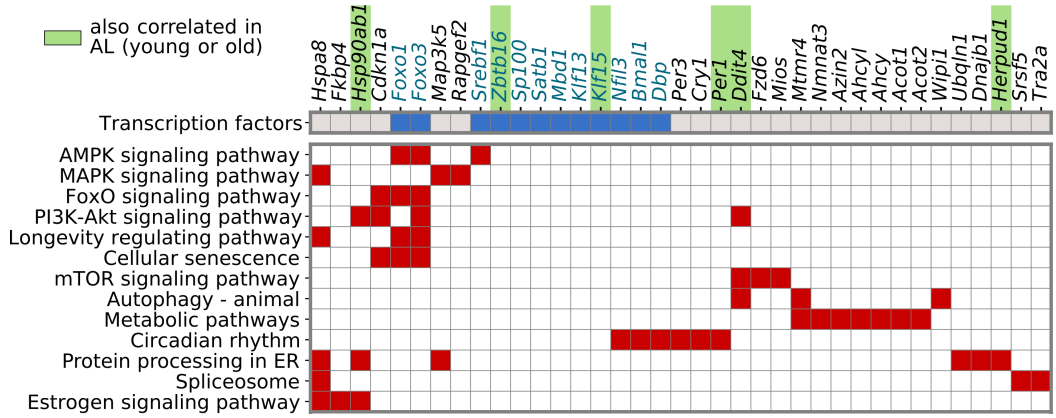


Figure S3.17 | **Pathway association (KEGG) of genes with maintained expression correlation in DR.** The association of all genes with maintained correlation in DR (n = 102) was mapped using the KEGG BRINE (for transcription factor annotation) and KEGG PATHWAY database. All genes that could be mapped to pathways with at least 3 total mapped entries are shown.

4 General Discussion

Ageing affects healthspan and lifespan in almost all species by contributing to a progressive functional decline that disrupts the homeostasis of an organism. Even minor perturbations can trigger a cascade leading to the development of age-related diseases (López-Otín et al., 2023). DR and other ageing interventions can delay, attenuate, or counteract age-related functional decline (Fontana et al., 2010). Due to the systemic effects that DR has on ageing, it is inherently difficult to unravel how it specifically induces the plethora of its age-improving effects. The analysis of biological datasets using computational approaches is a great tool to address this problem, allowing researchers to study and compare the affected biological processes as a whole, while simultaneously examining individual processes in detail (Wieser et al., 2011). In my PhD thesis, I applied *in silico* strategies to address open questions about how ageing alters the transcriptional and epigenetic profiles of different mouse tissues, and how DR affects these changes. Doing so, I have further elucidated the beneficial effects of DR on two axes of the ageing mouse: the interplay of epigenetic and transcriptional regulation in diet-responsive tissues, and the contribution of DR to maintaining a tissue-spanning homeostasis as reflected at the level of transcriptional regulation. As the project-specific results have already been discussed in detail in the respective project manuscripts, in the following sections I will summarise the key findings with regard to DR in each chapter, and then highlight open questions and limitations of both projects.

4.1 DR and its influence on the DNA methylation landscape

The WAT is associated with age-related disease formation (Johnson & Stolzing, 2019; Oishi & Manabe, 2020; Palmer & Kirkland, 2016) and, next to the liver (S. X. Cao et al., 2001; Hunt et al., 2019; Rhoads et al., 2018), has emerged as a key tissue in mediating responses of dietary feeding patterns such as DR (Bruss et al., 2010; Okita et al., 2015; Valle et al., 2010). In the liver, DR-induced changes of the transcriptional profile are associated with changes in its DNA methylation landscape (Hahn et al., 2017), highlighting the methylome as an important axis of mediating DR responses in the liver.

The WAT is also known to be regulated by DNA methylation changes (X. Ma & Kang, 2019), but how DR in particular affects the WAT methylome was not yet known.

In the first project (project A), I found that chronic DR remodels the DNA methylation landscape of the WAT, with differential methylation enriched on gene bodies, promoters and CGIs. Hypomethylated genes were associated with up-regulation of lipogenesis and energy metabolism genes, and hypermethylated genes with down-regulation of inflammation-related genes. WAT-specific changes in lipogenesis and energy metabolism are thereby opposing the trends of DR-induced down-regulation and hypermethylation of the same genes in the liver (Hahn et al., 2017), indicating that DNA methylation is involved in the DR-mediated regulation of DR-mediated organismal metabolic homeostasis. I also found wide-spread changes of the Hox cluster in the liver and WAT which were especially pronounced and associated with transcriptional changes in four HoxA genes in the WAT, however functional consequences of these changes remain obscure. Late-life dietary switches following chronic AL and DR treatments also induced wide-spread changes in the DNA methylation landscape which were, however, largely uncoupled from transcriptional changes. Thus, opposing the initial hypothesis, DNA methylation could not explain the previously reported transcriptional memory of the WAT (Hahn et al., 2019), but could potentially still contribute to it, as suggested by some persistently methylated and regulated memory candidate genes which included lipogenesis genes *Me1*, *Gpam*, *Acly*, and *Fasn* in the WAT. The same candidates remained stably hypermethylated in the liver, but they were still differentially expressed in response to dietary switches. This suggests different modes of transcriptional and DNA methylation regulation in both tissues. I hypothesize that DNA methylation could be a "regulatory buffer" with the potential to stably suppress regulatory processes, which can however be outweighed by further regulative factors to enable transcriptional changes in response to more sudden changes. Persistent DNA methylation marks then remain as an "artefact" of prior long-term regulation.

The significant DNA methylation changes we found in the WAT in response to chronic DR were more frequent than in the liver (approximately 10 times more DMRs), and for lipogenesis genes, the trends were also opposite to those in the liver (Hahn et al., 2017): lipogenesis was down-regulated in the liver, but up-regulated in the WAT. This may reflect a metabolic shift

from reduced lipogenesis in the liver to increased lipogenesis in the WAT, an interplay that was previously also reported to be associated with the key mediator *Srebp-1c* (Fujii et al., 2017), which was also central to the identified differentially methylated candidates in response to DR in both tissues. Thus, the lipogenesis interplay in both tissues could be regulated at least in part by DNA methylation. In the future, it would be interesting to integrate data correlating circulating lipids with altered gene expression in the WAT to further elucidate how the lipogenesis profile affects global circulating lipid levels, and how DNA methylation might be involved in its regulation. Interestingly, a recent epigenome-wide association study showed that DNA methylation in whole blood was associated with circulating lipid metabolites (Gomez-Alonso et al., 2021). Thus, consistent with its established role in controlling metabolic regulation (Barres & Zierath, 2011; Giannoukakis et al., 1993; Serrano et al., 2020; Waterland et al., 2006), DNA methylation is an important mediator of the regulation of lipogenesis in particular across multiple tissues.

In addition to the WAT and liver, DR was found to mediate the DNA methylation landscape associated with functional consequences in additional tissues, such as blood (Gomez-Alonso et al., 2021; Maegawa et al., 2017), skeletal muscle (Barres et al., 2013), and the brain (Hadad et al., 2018). This suggests that the DNA methylation landscape may mediate DR responses in multiple tissues, highlighting a larger role for this epigenetic mark in whole-body DR regulation. However, it remains largely unclear to what extent DR might also affect the DNA methylation pattern in tissues that are not directly associated with metabolic homeostasis. Investigating how the DNA methylation landscape responds to DR in these tissues could provide insights into (1) the role DNA methylation has in mediating the effects of DR, and (2) give further insights into how DNA methylation landscapes could be established and maintained on at the whole-body level.

A limitation of our study was the lack of WAT DNA methylation data in young animals at the onset time of chronic DR feeding. In the liver study, WGBS data was available and it was shown that DR protects against age-related DNA methylation changes (Hahn et al., 2017). In the WAT, while we observe differences between chronic AL, DR, and late-life dietary onsets, we cannot compare these with the baseline DNA methylation pattern in young animals. This removes an interesting axis of information from which we could have inferred how ageing itself shapes the WAT DNA methylation

landscape, and how the DNA methylation pattern of a dietary onset in young animals compares to a dietary onset in old animals. Due to our findings of the relatively large impact of DR on DNA methylation in the WAT, which were larger than in the liver, it could be interesting to add this axis of young DNA methylation changes in a follow-up study.

An interesting and surprising finding of our study was a strong, significant DNA methylation pattern change spanning all four Hox clusters in the WAT, and albeit weaker, the HoxA cluster in the liver. This signal was furthermore associated with the differential expression of four HoxA genes in the WAT: *Hoxa5*, *Hoxa7*, *Hoxa10* and *Hoxa11*. Hox genes are transcription factors known especially for their evolutionary conserved role in the complex mediation of developmental processes, including throughout the embryonic phase of most bilaterian animals (Pearson et al., 2005). Due to their primary role in developmental processes their role in the ageing process remains largely understudied. However, following embryogenesis, several Hox genes were already found to be continually expressed in several tissues of the body with their role spanning stem cell associated functions including tissue homeostasis and tissue repair processes (Hubert & Wellik, 2023). For example, mural *Hoxa9* was observed to have increased activity in aged skeletal muscle tissue which was found to play an inhibiting role in muscle tissue regeneration. *Hoxa9* inhibition in aged tissue ameliorated tissue regeneration by activating developmental pathways in muscle stem cells, whereas its knockout in young mice mimicked age-associated impairment in stem cell function (Schwörer et al., 2016). In another study, several Hox genes were found to differentially methylated in human skeletal muscle tissue, and Hox gene hypermethylation was linked to a putative decline in cellular differentiation due to decreased expression HOXB1 and HOXA3 in particular, which could be counteracted with increased physical activity (Turner et al., 2020). In line with their regulating role in stem cells, misregulation of Hox genes is also commonly associated with the formation of cancer, highlighting them as potential targets for tumour suppression (Feng et al., 2021). These findings indicate wide-spread roles for Hox genes also in adult and aged individuals. In the WAT specifically, the expression pattern of Hox genes is determining the fat pad identity (Procino & Cillo, 2013; Yamamoto et al., 2010). In human adipocytes originating from visceral and subcutaneous depots, depot-specific Hox gene expression was associated with different DNA methylation landscapes on Hox clusters (Bradford et al., 2019), indicating that depot-specific expression differences could be

associated with DNA methylation. We found that chronic DR also induced large-scale changes in the DNA methylation landscape in the gonadal WAT across all Hox clusters. While the function of this pattern change remains obscure, we find that in the gonadal adipose tissue, Hox genes are primarily expressed in adipose mesenchymal stem cells and epithelial cells (Schaum et al., 2018), which could indicate that their role is associated with tissue differentiation functionality. Supporting this and in line with our findings, the potentially most interesting candidate *Hoxa5* (which we found to be up-regulated in response to DR) was found to be inhibited by high-fat diet in younger mice, likely by DNA methylation induced silencing (Parrillo et al., 2016), and its down-regulation was associated with reduced activation of the *Fatty acid binding protein 4* (*Fabp4*) adipocyte differentiation mediator (W. Cao et al., 2018). In consequence, its DR-mediated up-regulation could promote adipocyte differentiation also in aged mouse WAT. However, this would only explain pattern changes of *Hoxa5*, but not the big methylation landscape changes across all Hox clusters. In future, it will be of interest to observe how methylation changes of these genes could potentially alter Hox gene expression, and which downstream consequences this could have. Transcriptional changes should be assessed either in higher resolution (due to low general expression level of Hox genes), or on the level of single cells as they are not uniquely expressed in all adipose cell types.

Epigenetic patterns in particular, but also the transcriptional landscape can be influenced by spatial and temporal effects even in the same tissue section (Titus et al., 2017), and such effects could also bias results in our analysis which are based on bulk tissue sequencing. While (reference-free) deconvolution approaches are principally available to resolve the contribution of cell types to the bulk DNA methylation signal, it is questionable if they could provide additional information for our data specifically *ad hoc*, as sequencing depth was rather shallow, i.e. we merged samples and then binned CpGs, while common deconvolution algorithms require single-CpG resolution (Houseman et al., 2016). To properly resolve cell-type biases, the experiment could be repeated in a design specifically dedicated to deconvolute the DNA methylation landscape, while single cell sequencing could give further insights into the cellular origin of transcriptional effects. Using such approaches, we could identify in more detail which cell types are responsible for the transcriptional and DNA methylation pattern interactions that we observe in bulk.

In WAT, a late-life ALDR switch only rarely induced persistent DNA methylation patterns that overlapped with genes of the transcriptional AL memory. However, these rare candidates included four lipogenesis genes (*Me1*, *Acly*, *Gpam* and *Fasn*), all of which were also among candidates for methylation-associated regulation in response to chronic DR. While it is possible that the AL dietary memory of AL is partially influenced by such persistent DNA methylation marks, it is likely that it is established and maintained by other processes that could store information about the previous diet. In this line, our group has already investigated the effects of late-life dietary switches at the single-cell level (Nathalie Jauré) and at the microbiome level (Carolina Monzó), which could be factors that together contribute to the AL feeding memory. Other information carriers could be, for example, other epigenetic markers of the WAT such as histone modifications of which histone methylation in particular is known to regulate transcription also in the longer-term (Mews et al., 2014; Puri et al., 2015). Another possible axis of information storage could be the regulatory contribution initiated by other tissues, for example via the constant release of circulating factors that could mediate WAT transcriptional programs (Carmean et al., 2014). However, identifying such patterns would be challenging as circulating factors could originate from multiple sources throughout the body.

Late-life dietary ALDR and DRAL switches mainly induced changes in DNA methylation in the liver and WAT that did not correlate with transcriptional changes in the corresponding genes. We hypothesised that this could be due to an asynchrony in the rate of adaptation of DNA methylation and the transcriptome in response to a dietary change. For example, transcriptional changes could be induced rapidly in response to dietary changes, as suggested by findings which demonstrated a rapid transcriptional response to DR already in the time frame of up to 72 hours in the fly (Whitaker et al., 2014), and two days in mouse tissues including the liver (Selman et al., 2006). In contrast, DNA methylation pattern changes could then follow up later, and potentially also in orchestration with prior transcriptional changes, as for example observed in the contest of infection-induced DNA methylation changes occurring after transcriptional responses, but in association with the binding of transcription factors to specific loci (Pacis et al., 2019). Thus, while transcriptional regulation and DNA methylation changes are associated, they would not be resolved on the same time scale. Adding to this complexity, the alteration of DNA methylation changes furthermore is highly variable: for example, not every locus is altered at the same rate

by demethylation processes, and DNA methylation alterations can also be dependent on the interaction with further epigenetic marks such as histone modifications (de Mendoza et al., 2022) about which we do not have information. To further elucidate the dynamics of how quickly DNA methylation and transcription change in response to a cue, data needs to be collected at time points closer to the introduction of a dietary change. It may also be interesting to integrate DNA methylation data with epigenetic data at the histone level, as both are known to jointly coordinate gene expression (Du et al., 2015; Rose & Klose, 2014).

4.2 DR and its influence on gene expression correlation

Ageing induces a functional decline in most tissues, which ultimately compromises the homeostasis of the whole organism (López-Otín et al., 2023; Priest & Tontonoz, 2019; Tahara et al., 2017). For example, ageing is associated with the formation of metabolic diseases (Bonomini et al., 2015), which may result from, but also contribute to, functional impairment of metabolically active tissues such as the WAT (Burhans et al., 2019), renal tissue (Slee, 2012), and the liver (H. Ren et al., 2019). Other related factors contributing to the age-related loss of homeostasis are chronic inflammatory processes affecting one or more tissues (Franceschi et al., 2018), and the age-related deterioration of the circadian rhythm (Acosta-Rodríguez et al., 2021; Hood & Amir, 2017). Ultimately, all of these processes lead to age-related changes in the downstream gene expression profiles of each individual tissue, manifesting, for example, in changes of metabolic programmes (Hahn et al., 2017; Saxton & Sabatini, 2017), the increase in immune gene expression that may reflect immune cell infiltration (Mogilenko et al., 2022; Schaum et al., 2020), or perturbations in the rhythmic expression of circadian genes (Wolff et al., 2023). We hypothesized that gene expression correlation could be an indicator of such inter-tissue coordination processes, and could thus both be affected by age and DR.

In the second project (project B), I therefore investigated the gene expression correlation between tissues of the *Tabula muris senis* dataset (Schaum et al., 2020) in the context of ageing and DR. Correlation of gene expression was positively biased across most tissues, with significant shifts in 36 (around 26%) tissue pairs of *Tabula muris senis*. I found that frequently correlated genes were sufficient to cluster tissues into functionally groups

of adipose tissues and metabolically active tissues. Thereby, transcriptional correlation was most frequent in circadian genes, protein folding genes, and metabolic genes. Thus, gene expression correlation between tissues is a potential indicator of the coordination of functional processes across tissues. With age, gene expression correlation between tissues decreased globally, which could also be recapitulated in the independent GTEx dataset and a newly generated RNA-seq dataset of mouse liver and kidney. In *Tabula muris senis*, age-related loss of expression correlation was most frequent in circadian and proteostasis genes, but also genes with more broad impact on downstream expression regulation such as *Btaf1* or *Cirbp*. In contrast, genes associated with tissue inflammation and innate immune response gained expression correlation with age. Because the transcriptional profile of kidney and liver were showing the strongest expression correlation aside from adipose tissues, and are DR-responsive tissues, I next asked for the effect of DR on the age-related expression correlation changes in both tissues. I found that DR attenuated the age-associated correlation decline in the liver and kidney especially for circadian genes which benefits circadian alignment and thereby also several downstream processes that contribute to organismal homeostasis. Furthermore, expression correlation was generally increased in DR as compared to AL, which could be explained by less expression variance in DR as compared to AL at least in aged tissue. Finally, DR established an age-persistent and DR-specific expression profile of several longevity pathway related genes, including *Foxo1*, *Foxo3*, *Klf13* and *Klf15*. Thus, DR promotes a generally tighter transcriptional coordination of both tissues across age, ameliorating expression correlation of circadian genes and longevity pathway related genes which could contribute towards the age-ameliorating effects of DR.

In *Tabula muris senis*, we observed both the strongest correlation signal, and the most frequent age-related correlation loss across tissues in genes associated with (1) the circadian rhythm across all tissues, (2) associated with proteostasis across metabolically active tissues, and (3) in genes that displayed age-related correlation loss consistently across multiple tissue pairs. The circadian correlation signal of well-correlated targets across tissues included most of the key members of the central genomic circadian clock (Takahashi, 2017), highlighting expression correlation is an interesting novel tool to study such whole-body coordinated processes and their age-related decline. Thereby, expression correlation of genes likely reflects the cell-autonomous circadian rhythm that is intrinsically well

synchronized across most tissues (Mohawk et al., 2012), and the age-related decline circadian rhythm entrainment capability (Acosta-Rodríguez et al., 2022; Tahara et al., 2017). The proteostasis signal is shaped by a manifold of chaperones and co-chaperones proteins that are associated with heat shock protein families *Hsp70*, *Hsp90*, and *Hsp40*, whereas specifically *Hsp90* associated genes lose gene expression correlation with ageing. While we cannot state how loss of expression correlation affects functionality of the dense heat shock protein network, the observed correlation loss at least suggests a loss of coordinated expression of heat shock proteins in at least one tissue as compared to others in the dataset. In turn, an impairment in protein folding capability was associated with a manifold of systemic diseases (Herczenik & Gebbink, 2008; Moreno-Gonzalez & Soto, 2011), and *Hsp90* associated expression was directly linked to formation of neurodegenerative diseases (Brehme et al., 2014), cell survival (Rodina et al., 2016), and increased mural lifespan (Fuhrmann-Stroissnigg et al., 2017). Loss of their expression correlation could be an indicator for age-related loss of tight regulation in individual tissues as compared to others with age-related functional consequences which could be an interesting aspect of follow-up research. Finally, we some genes lost correlation with age across multiple tissue pairs, highlighting them as potential targets that are affected by the ageing process on a more systemic scale. Aside from circadian and protein folding genes, genes with frequent correlation loss included *Btaf1*, *Ide*, and *Map3k6*. Both *Ide* and *Map3k6* are thereby at least associated with the ageing process especially through their link to nutrient sensing pathways: MAP3K6 was observed to suppress autophagy likely by activating mTOR (Guo et al., 2015), and *Ide* is directly involved in insulin regulation through its degrading function and was implicated in the formation of age-related pathologies (Kochkina et al., 2015; Qiu & Folstein, 2006). A role for *Btaf1* in ageing was not yet suggested, however, it has the potential to affect broad systemic processes through its interaction with TBP (Akhtar & Veenstra, 2011; De Graaf et al., 2010; Klejman et al., 2004). In summary, although their putative connection to age-related processes, *Map3k6*, *Ide*, and especially *Btaf1* remain to be largely unstudied in the context of ageing *per se*. As our expression correlation based approach picked all three targets up as top genes with potential regulative decline, and all three targets genes have the potential to regulate downstream signalling, we propose them as novel targets contributing to age-related functional decline.

Unfortunately, due to technical aspects of the GTEx dataset, we could

not repeat our functional and candidate gene analysis in GTEx to confirm the above-mentioned functional findings on the ageing axis in the mouse datasets. These aspects include that (1) samples of GTEx were generally imbalanced in sample number whereas we only had a low number of samples in young and old conditions, (2) available tissues with high frequency were not included in the tissue sets of *Tabula muris senis* (e.g. skin or sun-exposed skin) which limited comparability, and (3) samples of the dataset are intrinsically pre-grouped in age-groups of ten years to ensure privacy of participant identity, which limits resolution in the data. However, aside from ageing, we could observe that similar genes were contributing to the correlation signal in GTEx and Tabula muris senis. In future, we will investigate this further and optimize our functional analysis workflow to potentially get more insights into the GTEx dataset and correlation changes in human.

In the present study, we limited our analysis to bulk RNA-seq data. However, our gene expression correlation is only a snapshot view of the similarity of regulatory states across tissues. As such, gene expression correlation suggests a regulatory coordination of transcriptional processes between tissues, but misses most of their causes and can only infer their consequences. For example, gene expression correlation in circadian genes and their loss with age is not surprising, as discussed before. However, gene expression correlation could also be driven by circulating factors, such as metabolites (Jang et al., 2019) or hormones (Hiller-Sturmhöfel & Bartke, 1998), that induce similar transcriptional programmes across multiple tissues. We currently miss this axis due to a lack of corresponding data. In future experiments, it would be therefore interesting to generate and integrate "paired" metabolomic data with RNA-seq data to further elucidate how a set of circulating factors might mediate transcriptional programmes across the whole body, and how such a profile changes with ageing or in DR.

Next, bulk RNA-seq only summarizes the transcriptional events in the total of all cells of the isolated tissue, as tissue-specific gene expression profiles are also partly reflecting its cellular composition (X. Wang et al., 2019). Ageing is known to alter tissue composition of individual tissues (Almanzar et al., 2020). Hypothetically, a change in age-related tissue composition could be a source of age-related loss off gene expression correlation, for example when the cellular proportion of one cell type driving the majority of the signal is decreasing. On the other hand, it could also contribute to

an age-related increase in correlation, for example through an infiltration of immune cells into aged tissues (Mogilenko et al., 2022) which was also reported in mice of the Tabula muris single-cell dataset (Almanzar et al., 2020) and is in line with the age-related increase in correlation of innate immune genes that we observed. Interestingly, age-related changes in cell type composition were also affected by late-onset caloric restriction, whereas caloric restriction rescued age-related effects in tissues of aged rats (S. Ma et al., 2020). Differences in the correlation signals of AL and DR could also be affected by such differences, for example if DR helps to maintain cell type composition in tissues throughout an organism's life. Unfortunately, while corresponding single-cell datasets could help to resolve such biases, they still are rare and shallow especially as compared to bulk RNA-seq data.

We observed that DR, as opposed to AL, generally increased the whole-genome expression correlation irrespective of functional associations. With age, DR then ameliorated the age-related maintenance of correlation. These findings suggest that DR could induce a tighter regulation of similar expression profiles in both tissues that is also maintained better throughout age, which is in line with the systemic impact of DR in the ageing process. Importantly, especially genes associated with the circadian rhythm were showing an DR-mediated maintenance of expression correlation, which could be linked to an improvement of the circadian expression pattern promoted by timed feeding which is intrinsic to DR. This is in line with findings of a recent study which found that circadian alignment contributes to increased health- and lifespan (Acosta-Rodríguez et al., 2022). Thus, expression correlation of circadian genes could be a reflection of similar circadian alignment. Importantly, the amelioration of circadian processes can effect a plethora of downstream functions (Patel et al., 2015) in synchrony in both tissues, thus potentially contributing to the global ameliorated expression correlation signal. To further elucidate the mechanism of how expression correlation is maintained with ageing, it may be interesting to also test the effects of other treatments. For example, it would be interesting to test whether treatments that are known to promote ageing such as high-fat diet (Hariri & Thibault, 2010; Honma et al., 2012) would also induce an accelerated loss of gene expression correlation, increase inflammation-related gene expression correlation, or if they would trigger alternative pathways independent of AL and DR. Alternatively, studying expression correlations in DR mimetic models, such as rapamycin treatment or IRS1 knockout

mice (Cole et al., 2017; Page et al., 2018), could provide further insight into how expression patterns in individual tissues respond to such cues in a coordinated manner, and what this could imply in terms of their function.

In future, it will be interesting to investigate the effect of DR on the correlation of further tissues, for example the adipose tissues. As discussed in the first chapter of this thesis the adipose tissue is a key mediator of DR and is associated with the liver and other tissues to mediate lipid and energy metabolism (Gooley, 2016; Oishi & Manabe, 2020). Furthermore, the adipose tissue is an endocrine organ (Carmean et al., 2014) with the potential to influence the regulation of other tissues. Therefore, understanding how its expression correlates with age and under DR could provide further insight into how it responds to environmental influences in coordination with other tissues.

In summary, we have shown that gene expression correlations measured on RNA sequencing-based data can provide insight into how gene expression is coordinated between tissues in response to ageing and DR. We demonstrated that our correlation-based approach to analysing data could both confirm known ageing trends, but also reveal novel target genes that could contribute to age-related functional loss. Of note, bulk RNA-seq has already become a standard technique in many biological experiments to investigate the regulatory effects of environmental cues on tissues in many publicly available databases such as NCBI SRA (Leinonen et al., 2011). Thus, analysing the correlation of transcriptional profiles potentially opens up such databases for a re-examination with a different set of biological questions in mind. Unfortunately, most experimental designs are set up to meet the requirements of studying differential expression (Deshpande et al., 2023), and therefore focus on single tissues, include only unpaired tissues, or are limited in sample size. Suitable datasets for correlation-based approaches are still rare, which has limited us to exploring the *Tabula muris senis* (Schaum et al., 2020) and validating in the GTEx (Lonsdale et al., 2013) datasets. However, the generation of large-scale expression atlases of multiple tissues under different treatments is becoming more common, and already small changes in the experimental design could make high-throughput datasets amendable to correlation-based analyses, ultimately increasing the analytical potential of the data generated data with little or no downside.

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Icon and graphical element sources: Figures 1.1 and 1.2 have been created using BioRender. Tissue icons (WAT, liver, kidney) from Figures 2.3, 2.4, 2.5, S2.2, S2.5, 3.1, S3.10, and S3.15 are taken from BioRender. Mouse and human icons in Figure 3.3 are taken from BioRender.

7 Erklärungen

Erklärung zur Dissertation
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8 Curriculum vitae



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Nationality: German

Work Experience

since December 2022	**Bioinformatician**, CRC1310: Predictability in Evolution University of Cologne
since March 2019	**Ph.D. candidate**, Groups Prof. Dr. Partridge, Prof. Dr. Beyer MPI for Biology of Ageing, Köln
October 2018 to February 2019	**Research Assistant**, Group Prof. Dr. Bornberg-Bauer Institute of Evolution and Biodiversity, Münster
August 2017 to December 2017	**Research Assistant**, Group Dr. Böhmer Institute of Plant Biology and Biotechnology, Münster
May 2015 to April 2016	**Student Assistant**, Group Prof. Dr. Kudla Institute of Plant Biology and Biotechnology, Münster

Academic Education

October 2016 to September 2018	Study: **M.Sc. Biotechnology** with final grade: 1.1 at Westfälische Wilhelms-Universität Münster
Thesis	*"Reading frame extensions as possible source for de novo domain emergence"* in the group of Prof. Dr. Bornberg-Bauer
October 2013 to September 2016	Study: **B.Sc. Biological Sciences** with final grade: 1.6 at Westfälische Wilhelms-Universität Münster
Thesis:	*"Quantitative Phosphoproteome analysis after stimulation in real microgravity identified MGP and ISTL8 as candidates in early gravitropic signaling"* in the group of Prof. Dr. Kudla / Dr. Böhmer
August 2004 to June 2013	Qualification: **German Abitur** with final grade: 1.0 at the Freiherr-vom-Stein Gymnasium Recklinghausen

Publications

Gadalla D.S., Braun M., Böhmer M. (2018). Gravitropism in Higher Plants: Cellular Aspects. In: Gravitational Biology I. SpringerBriefs in Space Life Sciences. Springer, Cham.

Srivastava A., Lu J., **Gadalla D.S.**, Hendrich O., Grönke S., Partridge L. (2022). The role of GCN2 kinase in mediating the effects of amino acids on longevity and feeding behaviour in Drosophila. Frontiers in Aging 3:944466.