Distinct and diverse chromatin-proteomes of ageing mouse organs reveal protein signatures that correlate with physiological functions

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SUMMARY

Temporal molecular changes in ageing mammalian organs are of relevance to disease etiology because many age-related diseases are linked to changes in the transcriptional and epigenetic machinery that regulate gene expression. We performed quantitative proteome analysis of chromatin-enriched protein extracts to investigate the dynamics of the chromatin-proteomes of the mouse brain, heart, lung, kidney, liver, and spleen at 3, 5, 10, and 15 months of age. Each organ exhibited a distinct chromatin-proteome and sets of unique proteins. The brain and spleen chromatin-proteomes were the most extensive, diverse, and heterogenous among the six organs. The spleen chromatin proteome appeared static during the lifespan, presenting a young phenotype that reflects the permanent alertness state and important role of this organ in physiological defense and immunity. We identified a total of 5928 proteins, including 2472 nuclear or chromatin associated proteins across the six mouse organs. Up to 3125 proteins were quantified in each organ demonstrating distinct and organ-specific temporal protein expression timelines and regulation at the post-translational level. Bioinformatics meta-analysis of these chromatin proteomes revealed distinct physiological and ageing-related features for each organ. Our results demonstrate the efficiency of organelle specific proteomics for in vivo studies of a model organism and consolidate the hypothesis that chromatin-associated proteins are involved in distinct and specific physiological functions in ageing organs.
HIGHLIGHTS

1. Quantitative chromatin-proteome analysis during mouse lifespan;
2. Chromatin analysis in vitro and in vivo mouse models;
3. Distinct chromatin proteomes of six organs during mouse lifespan;
4. Correlations between ageing and chromatin regulation in mammalian lifespan.

KEYWORDS

• Ageing
• Proteomics
• Chromatin
• Epigenetic
• Tissue
• Mouse lifespan

DATA:

LINK-1: mESC: ftp://massive.ucsd.edu/MSV000084270/
LINK-2: Mouse Brain Total Lysate: ftp://massive.ucsd.edu/MSV000084279/
LINK-3: Mouse organ chromatin proteomes: ftp://massive.ucsd.edu/MSV000084375/

SUPPLEMENTARY MATERIALS:
ProteinGroup.file (Proteome Tables) in Excel format:

1) Proteome of mouse embryonic stem cell obtained from whole cell lysate and chromatin enriched lysate.
File name: Supplementary material, Table S1

2) Brain proteome whole cell lysate during adult mouse lifespan
File name: Supplementary material, Table S2

3) Chromatin proteomics of six mouse organs over time
File name: Supplementary material, Table S3

4) Uniquely regulated proteins organ-specific during ageing.
File name: Supplementary material, Table S4
INTRODUCTION

Ageing is a natural process resulting in progressive changes of most, if not all, cellular components. Ageing is generally associated with declining biological performance and increased incidence of disease (1). The gene expression apparatus, comprised of the DNA itself, the chromatin environment it is housed in, and the machinery of transcription and translation is profoundly affected by ageing (2) (3). Models of ageing often display similar phenotypes to those undergoing senescence or genome instability, highlighting how integrated the ageing process is with these phenomena (1). Diseases that mimic or accelerate the ageing process, including Hutchinson-Gilford progeria and Werner syndromes, result in molecular changes in nucleosomes and chromatin (4) (5) (6).

In eukaryotes, chromatin includes histone molecules that package the DNA, locally controlling access to the underlying genes by facilitating 'open' or 'closed' states associated with transcriptional activation or repression, respectively (7). In this way, transcription of individual gene products may be regulated in temporal or location-specific manners.

One of the main routes of proteome expansion is dedicated to enzymes that carry out post-translational modifications of proteins. Enzyme catalysed PTMs at distinct amino acid residues regulate or modulate protein structure, interactions, and functions (8).

The mouse, *Mus musculus*, is the most commonly used experimental animal in biomedical research, and serves as a model system for studying human health and disease (9)(10). Mice have a relatively short lifespan, with one adult mouse month equivalent to approximately three human years (11)(12). This allows for maximum lifespan studies to proceed within the timelines of typical research projects, while environmental factors that affect ageing can be controlled (13)(14)(15). Lifespan and health-span are mutually influenced by many genes that can either predispose to age-related diseases or slow the ageing process itself (16).

We recently applied a middle-down proteomics strategy to demonstrate that mouse chromatin undergoes major changes during ageing, specifically that histone H3.3 replaces H3.1 and that the extent of H3 methylation marks at multiple sites is profoundly altered during ageing (17). We here extend these proteomics studies of mouse chromatin to investigate the protein composition of chromatin in multiple mouse organs during ageing.

We hypothesised that a time-course investigation of the dynamic chromatin proteome could reveal distinct molecular differences of mammalian organs and provide new insights into regulatory mechanisms in different organs during ageing.

We studied the progressive chromatin protein expression changes in six mouse organs during ageing by quantitative proteomics by mass spectrometry (graphical abstract).

Among almost 6,000 proteins identified, organ-specific patterns predominated, with age-responsive subsets identified for each organ. We mapped pathway level molecular changes specific to individual organ over time.
Our results demonstrate that the ageing process affects each mouse organ in a distinct manner illustrated by the diversity and heterogeneity of the temporal chromatin proteomes of each organ.
RESULTS

Isolation of chromatin associated proteins from mouse cells and organs.

We aimed to provide a comprehensive overview of the chromatin-enriched proteome in mouse organs and obtain insights into molecular processes involved in growth and ageing.

We initially applied a nuclear protein extraction protocol to the mouse embryonic stem cell (mESC) model, which is a “gold standard” for epigenetics research (Supplementary material, Table S1) (18) (19). Briefly, mESCs were lysed and cellular compartments were isolated by mechanical disruption followed by a high salt gradient separation to obtain cytosolic, nuclear, chromatin and histone fractions (20) (21). These lysates were initially analysed by western blotting using specific protein markers for each cellular compartment to assess the degree of enrichment of chromatin associated proteins (Figure 1, Panel A).

We performed quantitative proteomics by triplicate high-mass-accuracy mass spectrometry analysis of the mESC proteome and the mESC chromatin proteome to assess the enrichment of chromatin associated proteins (Figure 1, panels B and C). Subsequently, we presented the difference in chromatin enzymes detection in Table 1.

We annotated all nuclear or chromatin associated proteins using available database resources (Supplementary material, Figure S1) (22) (23) (24) (25).

Table 1. Number of chromatin enzymes detected in mESC.

| Chromatin Remodelling Complex | Whole cell lysate | Chromatin enriched lysate |
|------------------------------|------------------|--------------------------|
| BHC                          | 2                | 6                        |
| CCR4-NOT                     | 2                | 6                        |
| ING                          | 1                | 15                       |
| INO80                        | 2                | 9                        |
| MEDIATOR                     | 8                | 20                       |
| MLL                          | 15               | 56                       |
| NUA4HAT                      | 17               | 8                        |
| NURD                         | 2                | 35                       |
| NURF                         | 10               | 2                        |
The dynamic range plot was used to assess the measurements of the protein expression across these proteomes. The mESC chromatin fraction was indeed highly enriched for chromatin associated proteins as compared to the whole cell lysate (Figure 1, panels B and C).

We detected major chromatin associated protein complexes, including the Polycomb Repressive Complex 2 (PRC2), Nucleosome Remodelling and Deacetylase (NuRD), BRAF-HDAC complex (BHC), and mixed-lineage leukaemia (MLL) complex (Figure 1, panel B) (22) (23) (25).

In general, we observed an overall increase in the proportion of proteins classified as either "nuclear" (20%) or "chromatin associated" (35%) within the mESC chromatin sample (Figure 1, panel C).

Gene ontology (GO) analysis showed a distinct enrichment of proteins associated with "DNA-protein binding", "histone binding", and "chromatin and nucleosome organisation" within the chromatin sample (Figure 1, panel D). The mESC total lysate sample mainly contained proteins involved in "translational protein", "cell-cell structure organisation" and "ribosomal and ATP processes" (Figure 1, panel D).

We next demonstrated that the chromatin enrichment protocol used for mESCs is applicable to mouse organs. We first extracted chromatin proteins from mouse brain and assessed chromatin enrichment by western blotting using specific protein markers (Supplementary material, Figure S2). We observed enrichment of the chromatin marker histone H3 and reduced level of the cytosolic marker GAPDH expression within the "chromatin fraction" lysate.
Next, we performed quantitative mass spectrometry profiling of the mouse brain proteome and the mouse brain chromatin-enriched proteome. The dynamic range plots demonstrated that the major chromatin binding protein complexes were enriched in the brain chromatin sample, including MLL, NuRD, Polycomb and BHC complexes (Figure 2, panel A, Supplementary material, Table S2).

The mouse brain chromatin enriched fraction contained more than 30% chromatin associated proteins, up from ~8% in the total brain lysate (Figure 2 panel A, bottom). This was accompanied by a large increase in the content of "nuclear proteins" (~20%) (Figure 2, panel A).

We conclude that our chromatin-proteome fraction of mouse brain tissue was highly enriched in chromatin associated and nuclear proteins as compared to the whole brain lysate (Figure 2, panel A).

Quantitative chromatin proteomics of ageing mouse organs.

We performed quantitative chromatin proteomics of six mouse organs to investigate the in vivo dynamics of chromatin during ageing. We isolated chromatin associated proteins from mouse brain, heart, liver, kidney, lung and spleen at time points 3, 5, 10 and 15 months representing the "mature adult mouse lifespan", from the early adult stage (3month), middle aged adult (5 to 10 months) and mature adult (15month) (graphical abstract) (12) (13) (14) (15).

We excluded mice older than 18 months to minimize any age-related changes that might be due to social behavior, physical characteristics of motor function and locomotor activity.

Proteins were identified and quantified by high mass accuracy LC-MS/MS by hybrid quadrupole-orbitrap technology using a peptide-intensity based (label-free) protein quantification strategy (see Materials and Methods, Supplementary material, Table S3).

The proportion of nuclear proteins or chromatin associated proteins ranged from 30% to 60% of all detected proteins and it was similar across all time points for each organ (Figure 2, panel B).

Subsequent data analysis included all identified proteins of each organ and all time points to avoid loss of essential information and to achieve a more detailed characterisation of the chromatin proteomes of mouse organs.

We identified a total of 5928 proteins in the chromatin enriched protein samples across all six mouse organs over time (Figure 2, panel C). Most proteins were identified in the chromatin fractions of mouse brain (3110) and spleen (3125), whereas the lowest number of proteins were identified in the chromatin fraction of mouse heart organ (2051) (Figure 2 panel C). The lung and spleen samples were highly enriched in nuclear proteins and chromatin associated proteins (55-65%), whereas the chromatin enriched heart sample contained 30-35% nuclear/chromatin associated proteins.
The very different morphology and cell type compositions of the mouse organs likely influence the efficiency of the chromatin protein extraction protocol and thereby the detected proteome compositions. Cell-type and cell cycle specific transcriptional activities likely explain some of the observed variation in proteome composition.

Nevertheless, the fraction of proteins classified as either “nuclear” or “chromatin associated” was similar among all mouse organs and time points, demonstrating the high reproducibility and reliability of the experimental approach with a coefficient variation estimated less than 10% among all the samples (Figure 2B).

Overall, we identified a total of 4581 different chromatin associated proteins across all organs, including 2717 different nuclear proteins (Table 2).

Table 2. Number of proteins detected across six mouse organs and four time points and their annotated subcellular location. Detected number of unique proteins across all organs are shown for each subcellular location.

| Organ | Brain | Heart | Liver | Kidney | Lung | Spleen | Unique proteins across all organs |
|-------|-------|-------|-------|--------|------|--------|----------------------------------|
| Chromatin | 936   | 457   | 648   | 714    | 713  | 1113   | 1542                             |
| Nuclear  | 615   | 323   | 355   | 426    | 442  | 556    | 930                              |
| Other    | 1559  | 1272  | 1483  | 1465   | 1477 | 1457   | 3456                             |

Proteomics reveals organ-specific protein profiles during ageing.

We assessed the entire mouse organ chromatin enriched proteome dataset using principle component analysis (PCA) (Figure 3, Panel A). The time points (age of mouse at organ harvest) were well separated from one another for each organ, such that the replicates for each time point were more closely clustered to one another than to replicates of other time points. More strikingly, however, was the observation that the origin of organ was the fundamental discriminating factor for the overall clustering of samples: each organ formed its distinct cluster made up of sub-clusters comprising the different ages of the organ samples (Figure 3, Panel A).

Pearson correlation analysis (Supplementary material, Figure S3) demonstrated reproducibility of biological replicates and confirms the robustness of our biochemical and proteomics methodology. To further characterise similarities between ageing organ and proteome expression profiles, we performed clustering of Pearson correlation coefficients (Figure 3, Panel B). This showed the consistency and reproducibility of
analysis of three biological replicates at all time points, and revealed distinct organ proteome profiles. The brain derived proteome exhibited poor correlation to all other organs. Kidney, liver, heart and lung samples exhibited protein expression profiles, which were slightly positively correlated. Spleen displayed slightly positive correlation with kidney and lung and poor correlation to brain, liver and heart.

Overall, our PCA and Pearson correlation analyses demonstrate that each mouse tissue exhibits a distinct and ageing-related chromatin-proteome profile (Figure 3).

Quantitative proteomics define biological changes in the ageing process.

To define ageing signatures across mouse lifespan in each organ, we performed a comprehensive functional analysis of the proteomics datasets using an UpSetR plot to investigate ageing markers (Figure 4, Panel A) [26].

Briefly, overlapping organ proteome was performed to estimate the number of co-occurrence proteins shared and determined degree of similarity between datasets and subsequently, identify unique organ-specific feature. Then, the dataset undergo to Gene Ontology analysis to explore the relationship between chromatin and ageing.

A “core” proteome of 863 proteins was identified in all six organs during mouse lifespan across all four time points, including 289 shared chromatin binding proteins and 157 shared nuclear proteins (Figure 4, Panel A, Table 3).

Table 3. “Core” proteins shared in all six mouse organs and over time were sorted to their cell compartments.

| Chromatin proteome during mouse lifespan | Unique proteins |
|-----------------------------------------|-----------------|
|                                | Core | Brain | Heart | Liver | Kidney | Lung | Spleen |
| Chromatin | 289  | 119   | 18    | 27    | 22     | 17   | 242    |
| Nuclear   | 157  | 69    | 21    | 33    | 16     | 15   | 54     |
| Other     | 417  | 605   | 175   | 329   | 196    | 211  | 286    |
| Total     | 863  | 793   | 214   | 389   | 234    | 243  | 582    |

The core chromatin proteome contained proteins shared across all tissue during mouse lifespan. These proteins were associated with the major transcriptional/epigenetic chromatin complexes such as BHC (GTF2I), MLL (ACTB, RUVBL2, DPY30, WDR82, PPP1CA, SEPT9, PPP1CC, PPP1CB, SNX2, LPP, DDX6), NuA4HAT (RUVBL1), NURD (CHD4, TRIM28, CSNK2A1), NURF (SMARCA5), PcG (RBBP4, RBBP7), SAGA (FGG), SIN3A (MECP2, SFPQ, PA2G4) and SWI/SNF (SMARCC2, YWHAB, H2AFY, RAC1, EIF4B)
Many of these proteins are expressed during cell fate commitment (22) (29).

For each of the six organs we detected from 214 to 793 proteins that were unique to that organ, i.e. proteins not detected in other organs (Figure 4, Panel A and Table 3).

The mouse brain chromatin proteome contained 793 unique proteins, constituting the largest set of unique proteins among the six organs (Figure 4, Panel A). Approximately 24% of these proteins (188) were classified as chromatin associated proteins or nuclear methyltransferase enzymes (HNMT, SAP30L, CARM1, SETD7, SUV39H2) and histone deacetylase enzymes (CHD5, IRF2BP1, MAPK10, MEF2D, MACROD2) that are mainly involved in transcriptional gene silencing (22) (23) (25) (30).

The spleen chromatin preparation contained 582 unique proteins, ~50% of which are chromatin associated proteins or nuclear proteins, strongly suggesting a distinctive chromatin proteome profile for this organ (Figure 4, Panel A, below) (Table 3).

The spleen is an organ with the innate capacity to regenerate (31). It acts as a filter for blood and it controls the blood-borne immune response (32). We detected several epigenetic complexes such as BHC, ING, MLL, NURD, ORC, PCG, SIN3A, SAGA, SWI/SNF. Also we detected chromatin “reader” enzymes not yet assigned to a specific chromatin remodelling complex (KDM3B, KDM2A, PHF23, CHD1L, UHRF2, MOC3, BRD9, ZMYND11, BAZ1A). These proteins recognise single post-translational histone marks or combinations of histone marks and histone variants to direct a particular transcriptional outcome (22) (23) (25) (30).

This result is in line with our above observations suggesting spleens were highly enriched in epigenetic markers and therefore are a good model to study chromatin remodelling complexes. Ageing effects are difficult to distinguish in spleen tissue.

The relatively large numbers of unique proteins of each organ likely reflects the inherent features of the individual organs, the diversity of cell types and physiology (Figure 4, Panel A).

GO analysis of the “core” 863 proteins revealed a large number of “chromatin associated” or “nuclear” proteins. The GO output was enriched for categories related to mouse ageing, such as: “oxidation-reduction process”, “ageing”, “regulator of cell cycle”, and “stress response” (Figure 4, Panel B) (33) (34) (35) (36) (37).

Subsequently, each category of the core proteome was further broken down into its constituent parts to create a map of the shared ageing-related molecular network of the six mouse organs (Figure 4, Panel B). Our aim was to identify novel biological features and associate them with ageing-related pathways and annotations.

GO classification of the unique proteins present in each organ proteome, suggested distinctive molecular signs of ageing in each organ (Figure 4, Panel C). We observed distinctive organ-specific categories, “age-classes and age-development” and categories that reflected their organ source (Figure 4, Panel C). For instance, unique GO term
categories were associated with each organ, such as: “Nervous system development” and “chemical synaptic transmission” related to the brain; “Cardiac myofibril assembly” and “adult heart development” were attributed to the heart; “Steroid metabolic process” and “liver development” were distinctive to the liver; “Transport” and “sodium ion transport” categories related to the kidney, and “Angiogenesis” and “respiratory gaseous exchange” were present in the lung.

These results confirm that many chromatin-proteins found in individual organs likely confer organ-specific functions (Figure 4, Panel C).

Taken together, our proteomics analysis showed a robust enrichment of chromatin associated proteins in mouse organs as confirmed by GO term analysis. We reported a significant enrichment of age-related proteome features, including a large class of protein annotations associated with the core chromatin environment present in all organs.

Distinct organ ageing profiles are defined by unique protein expression patterns.

We hypothesised that different mammalian organs have ageing-dependent and distinct expression profiles of characteristic chromatin associated proteins.

We employed a temporal analysis of the overall dataset which included chromatin associated proteins, nuclear proteins and unassigned proteins, to uncover common features that may contribute to the chromatin environment during ageing.

We used the rank products test to identify proteins that exhibited significant abundance changes during ageing (supplementary material, Figure S5, Panel D) (38) (39). We call these “differentially regulated proteins” (Figure 5).

We then retrieved those unique regulated proteins that were specifically detected in only one organ, in two organs or three organs (UpSet plot) (Figure 5, Panel A). The majority of uniquely regulated proteins were indeed specific to one organ.

We subjected the organ-specific uniquely regulated proteins to hierarchical clustering based on their expression changes and depicted them as heatmaps for each organ (three replicates per time point) (Figure 5, Panel B). This allowed us to compare how the relative expression of unique chromatin associated proteins changed over time in each organ, from early to late time points (3 to 15 months) (Figure 5, Panel B) (Supplementary material, Table S4).

The hierarchical clustering shows that the number of up and down-regulated proteins is similar in each organ. Further, assigned cell compartments of the “unique differentially regulated proteins” are shown as sidebars (Figure 5, Panel B) (supplementary material, Table S4). A large number of non-annotated “unique differentially regulated proteins” show a similar quantitative behaviour across all organs. We hypothesised that these proteins may represent a useful list of candidates that may regulate gene expression by been transiently recruited to chromatin at distinct time points during ageing.
Brain, heart and kidney showed mainly gradual changes of protein levels over time. Lung and liver show a more dramatic change of protein expression between 5 and 10 months of age (Figure 5, Panel B, supplementary material Figure S5, Panel E). We detected few significant protein abundance changes in the spleen. The spleen fraction was highly enriched in chromatin associated and nuclear proteins (approx. 60%) and contained many unique proteins (Figure 3 and 4). Thus, the spleen seems to continuously exhibit a young phenotype that may be due to the constitutively active role of the spleen in maintaining immune functions, red blood cell turnover and microbial defense (40). The spleen contains multiple cell populations capable of supporting immune responses, which may indicate the presence of self-renewal cell types that are “age-less” (31).

In summary, we identified a large number of unique differentially regulated proteins in the chromatin enriched proteomes of mouse organs. The abundance of these proteins changes dramatically during ageing from month 3 to month 15, across all organs, except for spleen.

Next, we explored potential functional links between chromatin proteome dynamics and ageing. We investigated all known chromatin associated proteins that were identified among the “unique differentially regulated proteins”.

The majority of chromatin modifying enzymes belonging to a given multiprotein complex, exhibited similar expression profiles over time within a specific organ (Figure 5, Panel C). For instance, the MLL subunits WDR82, CTR9 and WDR61 were down-regulated in liver during ageing. Components of same chromatin modifying complexes were detected in several organs, albeit not by the same subunits and with opposite temporal expression profiles. NuRD subunit HDAC2, was down-regulated in liver whereas NuRD subunits GATA2AD and TRIM28 were up-regulated in kidney.

This is consistent with the highly dynamic nature and spatio-temporal regulation of chromatin remodelling complexes. Some protein subunits are only present in a complex at distinct time-points to provide a unique function or feature (41).

A series of “Reader” enzymes were up-regulated in brain (GLYR1, BAZ1B) and spleen (BRD3, BRD7), whereas other “Reader” enzymes were down-regulated in liver (DPF2, BRD2, CHD2) (Figure 4C).

Next, we queried the “Human Ageing Genomic Resources” and “GenAge machine learning databank” using our complete list of “unique regulated proteins” that are not yet assigned to chromatin or nuclear environment, to demonstrate the ability of our mouse organ proteomics approach to detect known human ageing biomarkers (supplementary material Figure S5, Panel F) (42)(43).

We identified a series of human protein biomarker candidates for ageing. The brain protein IREB2 is associated with Alzheimer’s disease, whereas the brain protein MAOB is associated with both Alzheimer’s and Parkinson’s diseases. The heart proteins ADD3, PTGIS, and COL1A2 are candidates for hypertension and myocardial infarction. The liver proteins INSR, PTPN1, and ENPP1 are associated with diabetes mellitus type 2 and obesity. Lung protein CYP2E1 is related to lung adenocarcinoma and MMP9 is associated
with lung neoplasms. In the spleen, KLK1 protein is a biomarker candidate for hypertension.

**Functional analysis of chromatin-enriched proteomes of ageing mouse organs**

Next, we applied Gene Ontology (GO) analysis to characterise all “unique differentially regulated proteins” detected in each organ (Figure 6).

We listed the overall common pathways and processes that were found by quantitative chromatin proteomics to be differentially regulated during ageing across all the organs. We sorted the annotated features by their relative GO term category and separated them by their main family source (Figure 6, left panel).

During adult mouse lifespan, we observed several ageing stress response pathways and categories associated to regulate chromatin architecture leads to effect the cell structure conformation.

By listing every single category, we could describe the biological profile and pathways affected by the age-related protein expression responses present in all organs (Figure 6).

Subsequently, the differentially regulated proteins were sorted by their organ source and subject to further GO Term analysis to distinguish unique organ related processes from those pathways associated with ageing (Figure 6, center panel).

We report a high proportion of uniquely annotated categories for each mouse organ. For instance, the highest unique changes observed in the brain were relative to “gene expression” and “ageing/development”; in the heart and kidney significant changes were observed relative to “structure organization and biogenesis”; the liver showed changes across the “gene expression” and “structure organization and biogenesis” categories; the lung showed the highest unique changes in the “ageing/development” category, and; relative changes in the spleen were detected at the “gene expression” level.

These results are in line with our above observations suggesting a unique ageing response from each organ as evidenced by distinct dynamic changes of chromatin associated proteins.

We further interrogated the list of unique annotated organ categories to highlight distinct and significant temporal pathway profiles among the up- and down-regulated proteins in each organ to reveal the most distinctive regulated features (Figure 6, right panel).

In the mouse brain tissue, proteins involved in pathways such as “chromosome organisation” and “histone modification” were strongly up-regulated, while those involved in the regulation of “neuron projection regeneration” were down-regulated. In mouse heart tissue, up-regulated protein pathways included “oxidoreductase activity” and “regulation of response to stress”, while down-regulated pathways included...
“cardiovascular system development” and “chromosome organisation”. In the kidneys, proteins involved in pathways related to changes in chromatin conformation were both going up and going down during the mouse lifespan. In the liver, we observed that down-regulated proteins were associated with “chromosome organisation” and “histone modification”, while “oxidation-reduction process” pathways were up-regulated. In the lungs we noticed proteins associated with “apoptotic process” and “programmed cell death” were up-regulated, while pathways related to “muscle organisation and reassemble” were down-regulated.

Similarly, several different processes were altered in the other organs, except for the spleen that did not show many significant changes during ageing. In line with previous data, little pathway-level changes were observed in the spleen, especially in the down-regulated proteins, possibly indicating the important role of this organ in removing old red blood cells and microbes which seem, from our data, to not be affected by ageing. For this reason the spleen was not considered for further analysis.

Overall, using GO term analysis we dissected the biological features of the chromatin proteomes of organs in the context of mouse lifespan. By breaking down common and unique regulated functional categories we surveyed ageing-related pathways and improved gene-annotation enrichment analyses.

In conclusion, we identified and measured distinct and extensive protein abundance changes during ageing, specifically in early to mature adult mouse lifespan. A large number of differentially expressed proteins are unique for each organ as defined by specific GO Term categories. This demonstrated that the ageing process affects each mouse organ differently.

**Characterization of regulated molecular networks in ageing mouse organs.**

We looked in more detail at the most significant regulated organ-specific up-regulated and down-regulated protein categories during mouse ageing (Figure 6, right panel).

We used protein-protein interaction (PPI) data, from the STRING database to map the network of chromatin associated protein belonging to the most significant GO categories of each organ (44) (45) (Figure 7, Panel A).

Subsequently, we combined the protein interaction networks with the quantitative dataset (Figure 7, Panel A). By integrating protein-protein interactions and protein expression we derived co-interaction and co-expression networks to improve our understanding of biological mechanisms involved in ageing.

Finally, we attempted to confirm independently, by western blot, the observations noted in our wider dataset, specifically to the co-expression network generated (Figure 7, Panel B).

**Brain:** The majority of up-regulated proteins of the ageing brain belonged to the category “chromosome organisation”, including histones and histone binding enzymes
We noticed a strong sub-network of PPIs between two histone variants and histone binding enzymes: Macro-H2A2.1, Macro-H2A2.2, and HP1BP3. 

Macro-H2A2.1, Macro-H2A2.2, and HP1BP3 accumulated during mouse brain ageing (Figure 7, Panel B). Macro-H2A2.1, Macro-H2A2.2, and HP1BP3 are mammalian heterochromatin components that are highly expressed in adult mouse brains (46) (47) (48) (49). All three proteins increased in expression during organ development and ageing. These proteins were not detected in any other mouse organ in this proteomics study (data not shown). Macro-H2A2.1 is an epigenetic marker whose major function is to maintain nuclear organisation and heterochromatin architecture (46). HP1BP3 is a heterochromatin marker protein that recognises the histone mark H3K9me3 and promotes transcriptional repression (48). HP1BP3 loss-of-function is associated with cognitive impairment suggesting a role for this protein in establishing or maintaining cognitive functions (49). We also confirmed the expression of other heterochromatin markers including RING1b (50) and RNF20 (51) used as protein controls to monitor our strategy (Figure 7, Panel B). These results suggest a link between regulation of heterochromatin components and accumulation of histone variants during the ageing of brain tissue in adult mammals.

Heart: GO analysis of the regulated proteins of ageing heart tissue indicated up-regulation of “oxidoreductase activity” (Figure 7, Panel A). A chromatin associated protein of the sirtuin family (SIRT5) is a member of this category. Sirtuins are histone deacylases that play an important role in age-related pathological conditions such as cancer and the deregulation of metabolism (52) (53) (54). Sirtuin proteins are mostly annotated as mitochondrial proteins, but they can translocate further into the nucleus or other cell compartment (55) (56) (57). Our results suggest that activation of specific members of the sirtuin family and their translocation to the nucleus is involved in the ageing process (55) (58).

SIRT5 and SIRT3 were detected in our chromatin-enriched proteome dataset of ageing mouse heart. Both SIRT3 and SIRT5 expression levels increased during mouse lifespan, with SIRT5 being up-regulated from 3 to 15 months.

The abundance change of SIRT3 levels was less pronounced. This data was confirmed by western blotting (Figure 7, Panel B). SIRT5 is a histone deacylase that removes malonyl, succinyl, and glutaryl groups from histones. The ageing-dependent increase in histone H3 acetylation observed in our proteomics study and by western blotting (Figure 7, Panel B) is consistent with the fact that SIRT5 has no deacylases activity towards histone H3.

Liver: We observed drastically down-regulated “chromosome organisation” in the liver during ageing (Figure 7, Panel A). This category contained histone acylation/acetylation-related chromatin remodelling enzymes associated with different chromatin modifying complexes. Examples include KAT2A (either ATAC or SAGA complex), CHD2 (NuRD), HDAC2 (CoREST, NuRD, SWI/SNF, Sin3A-like), WDR82 (COMPASS), and DNMT1 (ACF) (22) (25) (Figure 7, Panel B, liver).

Western blot analysis confirmed that the protein expression levels of CHD2 and KAT2A are significantly reduced at 15 months (Figure 7, Panel B), which leads to lower histone
acylation levels. We indeed observed decreased levels of global histone H3 acetylation at this time point (Figure 7, Panel B), which contrasts with what we observe in the heart tissue. These results confirm our previously published data on decreased H3 acetylation (H3K14, K23, K27) in liver tissue during ageing (17), and that there is a decreased activity of histone acylation in the liver at late stages of ageing.

**Kidney:** The up-regulated "chromosomal part" category in ageing kidney tissue included components of the SCF-type E3 ubiquitin-protein ligase family (FBXO41, RNF13, NEDD4, TRIM28) and the proteasome subunits PSMD14 and PSME2. (Figure 7, Panel A). The mechanistic links between proteasome activity and ageing are well established (59) (60).

The proteasome is a large self-compartmentalised protease complex that recognizes, unfolds, and destroys ubiquitylated substrates (60).

The protein expression levels of FBXO41 and NEDD4 increased gradually during kidney ageing, while the signal intensity for PSMD14 and PSME2 was more pronounced at the latest time point (15 months) (Figure 7, Panel B). Thus, ageing kidney increases E3 ubiquitin-protein ligases, enhance the ubiquitylated substrates and stimulate proteasome abundance and activity.

**Lungs:** Many down-regulated proteins of ageing lungs were involved in processes such as "muscle organisation and reassembly", and the top GO Term encompasses an array of myosin motor proteins (Figure 7, Panel A). The down-regulated "myosin complex" GO category included several proteins belonging to the myosin family, following the observations that human muscle ageing is accompanied by changes in expression of myosins (61). We did not perform any immunoblotting validation of these myosin proteins due to the high sequence similarity among myosin subunits and the lack of highly specific antibodies against them.

These observations suggested that ageing might influence the lung cell structure through alteration of the higher-order chromatin architecture.

**Spleen:** Only few differentially expressed proteins were observed in the spleen and they did not allow for useful GO analysis and protein network analysis.
DISCUSSION

We implemented a comprehensive high mass accuracy mass spectrometry based proteomics strategy to monitor changes in the chromatin-enriched proteomes of six mouse organs over a time course that mimics adult development and ageing, from the early adult to the mature adult stage.

Many age-related diseases are linked to changes in the transcriptional and epigenetic machinery that regulate gene expression.

We focused on the changes in the expression of proteins that mediate transcription, including DNA-binding proteins and chromatin modifiers such as “writers”, “erasers” and “readers” (29) (30). Chromatin modifiers add, remove or recognise particular post-translational modifications (PTMs) of proteins associated with the alteration of chromatin architecture, and ultimately involved in the regulation of gene expression (8).

Over 2000 proteins were quantified in each organ, generating a useful resource for researchers investigating mammalian development and ageing.

We identified distinct and organ-specific unique ageing features associated with each organ. We observed unique chromatin modifiers that were expressed and accumulated differently during ageing, leading to changes in the chromatin architecture, including changes in expression of heterochromatin markers, histone deacetylases, ubiquitin-protein ligase, histone acetylation enzymes, and myosin complex in brain, heart, kidney, liver, and lung respectively.

Brain and spleen displayed the largest and most diverse and heterogenous chromatin-proteomes. The brain is arguably the most complex organ of a mammal. Brain chromatin structure and function is sustained by a large set of chromatin-associated and nuclear proteins, that also exhibit temporal dynamics of expression during mouse lifespan as demonstrated here. The spleen chromatin proteome was rather constant during lifespan whilst large and diverse, which may reflect the physiological role of the spleen for continuous maintenance of important immune and defense activities of the organism.

We demonstrated progressive changes of chromatin associated protein expression in response to ageing. Also, the specific nature of the organ was more of a significant discrimination factor, and subsequently distinct proteome profiles in response to ageing were observed. For instance, we noticed over the mouse lifespan, strongly up-regulated chromatin associated proteins relate to distinctive pathways involved in “oxidation-reduction response”, “response to oxidation stress” and “nucleosome assembly”, as well as signals that promote apoptosis processes. Conversely, chromatin associated proteins strongly down-regulated, are related to “muscle organization and reassembly” and “histone-modifying enzymes” associated with chromatin assembly and organisation.

Our study of chromatin-enriched proteomes demonstrated that Macro-H2A2 accumulates in the mouse brain during ageing. The epigenetic regulator HP1BP3 accumulates at a similar rate and very likely interacts with macro-H2A2. Both macro-H2A2 and HP1BP3 are highly expressed in the adult mouse brain and we suggest that a
complex involving these two proteins is implicated in maintaining heterochromatin integrity and promote gene silencing during mouse lifespan.

Reversible acetylation of histones plays a critical role in transcriptional regulation in eukaryotic cells. We detected reduced levels of histone H3 acetylation during ageing in mouse liver. The opposite trend was observed in ageing heart, i.e. an increase in histone H3 acetylation. Two families of deacetylase enzymes were identified: the histone deacetylases, HDACs, and the Sir family protein (Silent Information Regulator)-like family of NAD-dependent deacylases, or sirtuins (62). Both enzyme families play a major role in gene regulation by modifying the histone acetylation/acylation landscape in response to external stimuli and specific environmental stress conditions, such as oxidative stress (63). In mammals, the brain and heart have the greatest oxygen demand for their ATP dependent processes. Nearly all cellular processes of cardiomyocytes are driven by ATP-dependent pathways (64). SIRT3 plays an important role in maintaining basal ATP levels and regulated energy production in mouse embryonic fibroblasts (64) (65).

Sirtuin proteins are mostly annotated as mitochondrial proteins, but they can translocate further into the nucleus or other cell compartment (55) (56) (57). The translocation of sirtuins through different cell departments remains unclear. Here, we speculate that the roles of SIRT3 and SIRT5 in heart are essential as they compensate for age-related cellular dysfunction by controlling the levels of histone acylation marks. They may thereby promote the expression of proteins required for DNA repair to prevent cardiac hypertrophy in response to oxidative stress (64).

The SAGA complex is a multi-subunit histone modifying complex. KAT2A is a SAGA component in mammals, containing both a HAT domain and a bromodomain and extended N-terminal domain which confers the ability to acetylate mononucleosomal H3 (66). KAT2A is required for normal development in mice (67) (68). Furthermore, KAT2A level expression decreases during cell differentiation (67), suggesting the down-regulation of histone acetylation is connected with reducing activation of gene expression of target genes which promote self-renewal and pluripotency state during ageing. Overall, our experiments suggest a connection between the roles of two epigenetic enzymes CHD2 and KAT2A, whereby their mutual protein expression is associated with liver differentiation during mouse lifespan.

The proteasome is a complex proteolytic machine formed by the assembly of several subunits (69). The ubiquitin-proteasome system (UPS) is the primary selective degradation system in eukaryotic cells, localised both in the nuclei and cytoplasm compartment, which is required for the turnover of soluble proteins (70). The UPS is mainly implicated in protein degradation in response to the regulation of several processes including the maintenance of cellular quality control, transcription, cell cycle progression, DNA repair, receptor-mediated endocytosis, cell stress response, and apoptosis (71). Before a protein is degraded, it is first flagged for destruction by the ubiquitin conjugation system, which ultimately results in the attachment of a polyubiquitin chain on the target protein (72) (73).
Ubiquitin and the proteasome have been implicated in processes as diverse as the control of transcription, the response to DNA damage, the regulation of chromatin structure and function, and the export of RNAs from the nucleus (60).

We found an increase in the levels of two proteasome subunits in ageing kidney organ consistent with increased E3-ubiquitin ligase activity. Increased expression of FBXO41, a subunit of the SCF E3 ubiquitin ligase complex, correlates with NEDD4 expression. Recent reports suggested a decline of proteasome function related to senescence observed in several mammalian tissues and human cells (74) (75) (76) (77). During the ageing process, dysfunction of the ubiquitination machinery or the proteolytic activity may occurred, lead to proteasome failure, which is linked to several age-related human diseases (70) (78). We therefore, speculate that an increase expression E3-ubiquitination ligase activity may compensate for the proteasome activity, during ageing in kidneys.

Not all functions of the actin and actin-related proteins in the complexes are yet clear: it is known that they play important roles in maintaining the stability of the proteins, possibly by bridging subunits and recruiting the complexes to chromatin. In line with previous analysis, the majority of down-regulated “chromatin associated proteins” in lung tissue were assigned to the categories: “myosin complex”, “chromatin organisation” and “histone modification”. The most significant sub-network corresponds to “muscle organisation and reassembly” category and is related to the myosin family. This is in accordance with recent reports where the change of protein expression of particular myosin subsets implied a human ageing response (79) (80) The presence of actomyosin-like protein in the chromatin environment raises questions about the role of actin-like protein such as myosin in nuclear and chromatin processes (81).

In the spleen we detected a large number of chromatin associated proteins but only a fraction of these proteins were differentially regulated during ageing. Thus, the spleen chromatin proteome exhibits a “young” or “age-less” phenotype Consequently, only a few pathway-level changes were observed, possibly indicating the important role of this organ in maintaining immune functions, removing old red blood cells and microbes which seem, from our data, not be affected by ageing and consistent with our hypothesis that the time-course changes in protein expression distinctly affect each organ. We observed the largest numbers of chromatin remodelling proteins in the spleen, which suggest that this organ is may provide a good model for epigenetic studies.

Overall, our findings using high mass resolution LC-MS/MS suggest a new approach to investigate the dynamic chromatin protein environment during the lifespan of an organism. We provide a high quality and robust dataset of protein expression changes in mouse organs during the ageing process. The dataset shows that in vivo models can describe how the dynamic changes of chromatin associated protein may alternatively promote or repress gene expression during ageing, also reflecting some physiological features of the organ.

Our study adds novel details to mouse biology and chromatin dynamics of organs, and it complements previous attempts to identify biomarkers for mouse lifespan (82) (83). Walther et al. reported the bulk proteins abundance are less prone to change in organs
such as the brain, heart, and kidney obtained from mice aged 5 or 26 months. They reported only few proteins that exhibited statistically significant expression changes during ageing (82). Thus, bulk proteome analysis of mammalian organs has limitations, whereas organelle specific proteomics, as presented here for chromatin, is a more viable strategy to reveal molecular details of important biological processes, such as ageing and chromatin regulation.

Using a rat model (83), Glass et al studied alterations of gene expression to identify a putative mammalian ageing signature. Unfortunately, our chromatin proteomics strategy does not readily compare to the study by Glass et al, as only 3 out of the 7 time points are in common between the studies. We observed common ageing signatures (trends) such as cell stress response and transcriptional alterations changing at late stage of adult rodent lifespan.

We see a consistent overlap between our results and a list of human ageing-related biomarker candidates of the “Human Ageing Genomic Resources” and “GenAge machine learning databank” (supplementary material Figure 5, Panel F) (42) (43). Our differentially expressed candidate mouse proteins behaved just as human ageing biomarkers or ageing-related human proteins that promote disease.

Whereas our study provides novel features and details of molecular ageing processes in mammals, it does not provide mechanistic details of the protein-mediated ageing process in chromatin. Quantitative proteomics is an important tool for further studies of chromatin dynamics and the emerging field of high-sensitivity single-cell proteomics will assist in revealing features of organ function in health, ageing, and disease using limited sample amounts. The experimental protocols used in the present study provides a foundation for a more detailed interrogation of chromatin biology by functional proteomics. The data resource associated with this study provides a framework for generating novel hypotheses aimed at revealing the molecular features of ageing and at developing novel approaches to mitigate age-related ailments.
MATERIALS AND METHODS

Animals and organ collection

Male C57BL/6J mice were obtained from a study approved by the Danish Animal Ethics Inspectorate (J.nr. 2011/561-1950). Wild-type mice were bred in the Biomedical Laboratory, University of Southern Denmark, under a 12h/12h light/dark cycle (lights on at 6:30 am). Food and water were available ad libitum. Mice were sacrificed by cervical dislocation at the ages of 3, 5, 10, and 15 months. Liver, kidneys, brain, heart, spleen and lungs were excised, rinsed in ice-cold phosphate-buffered saline (PBS), and immediately snap frozen. Organs were stored at -80°C until further processing.

Isolation of chromatin lysate in mouse embryonic cells (ESCs)

Mouse embryonic cell pellets were washed in PBS and resuspended in lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, complete protease inhibitor w/o EDTA (Roche), 0.5mM DTT). The lysates were incubated for 15 min on ice and cell membranes disrupted mechanically by syringing 5 times with 21G narrow gauge needle and sonicating 3 Å~ for 2 s at high power. Lysates were incubated on ice for another 15 min and cleared by centrifugation at 14,000 rpm 4 °C 30 min. To harvest the nuclear fraction, lysates were resuspended in an equal volume of Nuclear Buffer (120 mM NaCl, 20 mM HEPES pH 7.9, 0.2 mM EDTA, 1.5 mM MgCl2, 20% glycerol, complete protease inhibitor w/o EDTA (Roche), 0.5mM DTT) and dounced 20 times with tight pestle type B. Lysates were incubated for 45 min rotating to dissociated chromatin-bound proteins and pre-cleared by centrifugation at 14,000 rpm 4°C for 30 min. Subsequently, nuclear pellets were lysed in Buffer C containing protease inhibitors (420mM NaCl, 20mM Hepes pH 7.9, 20% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, complete protease inhibitor w/o EDTA (Roche), 0.5mM DTT). Lysates were incubated for 1h rotating at 4 °C, in the presence of 250 U/mL benzonase nuclease, to form dissociated chromatin-bound proteins and pre-cleared by centrifugation (20000 x g, 1 h at 4 °C). After centrifugation the supernatant was snap frozen.

Isolation of chromatin lysate in mouse organ

Organ samples were homogenised on ice in a homogenisation buffer (2.2 M sucrose, 10mM Hepes/ KOH pH 7.6, 15mM KCl, 2mM EDTA, 0.15mM Spermine, 0.5mM Spermidine, 1mM DTT, and complete protease inhibitor w/o EDTA (Roche), 0.5mM PMSF and phosphatase inhibitor (PhosSTOP, Roche) using a "loose" type pestle tissue. The solution was stacked over with a cushion buffer (2.05 M sucrose, 10mM Hepes/ KOH pH 7.6, 15mM KCl, 2mM EDTA, 0.15mM Spermine, 0.5mM Spermidine, 1mM DTT, complete protease inhibitor w/o EDTA (Roche), 0.5mM PMSF) in a ultracentrifuge tube and cleared by centrifugation (20000 x g, 1 h at 4 °C). Pellet containing nuclei was washed twice with 1ml Dulbecco PBS (3000 g, 5 minutes at 4 °C). To harvest the nuclear fraction, lysates were subsequently re-suspended in Buffer C (420mM NaCl, 20mM Hepes pH 7.9, 20 % v/v glycerol, 2mM MgCl2, 0.2mM EDTA, 0.1 % NP40, complete protease inhibitor w/o EDTA (Roche), 0.5mM DTT) and dounced 20 times with a “tight” pestle. Lysates were incubated for 1h rotating at 4 °C, in the presence of 250 U/mL benzonase nuclease, to form dissociated chromatin-bound proteins and pre-cleared by centrifugation (20000 x g, 1 h at 4 °C). After centrifugation the supernatant was snap frozen.
Electrophoresis

Protein concentration was measured by the Bradford assay. Approximately 30 μg protein lysates were separated on SDS–PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat milk or 5% BSA at room temperature for 1 hour and incubated overnight with diluted primary antibody at 4 °C. Membranes were then washed and incubated with HRP-conjugated goat-anti-rabbit or mouse IgG secondary antibody for 1 hour at room temperature. Membrane was incubated with enhanced chemiluminescence reagents (Thermo Scientific) followed by exposure to X-ray films. Immunoblotting was performed using the antibodies and conditions listed in Supplementary material Table S5.

Protein sample preparation for mass spectrometry

Protein samples were re-disolved in 50 μl of Trifluoroacetic acid 0.1% (vol/vol) in water, as buffer A, and sonicated for 1 minute and centrifuged for 15 minutes at 15000 × g. Analysis was carried out on an Ultimate 3000 RSLCnano HPLC system connected to a mass accuracy high resolution mass spectrometry, Q Exactive HF (ThermoFisher). The MS instrument was controlled by Xcalibur software (ThermoFisher). The nanoelectrospray ion source (ThermoFisher) was used with a spray voltage of 2.2 kV. The ion transfer tube temperature was 275 °C. Samples were loaded on a cartridge pre-column PepMap 100 5’0.3mm (ThermoFisher) in 2% ACN, 98% H2O, 0.1% TFA at 10ml/min, and then separated either with an easy Spray C18 or a 75mm* 50cm 2mm PepMap 100 column (ThermoFisher). Separation was done in a linear gradient of ACN, 0.1% FA (buffer B) in H2O, 0.1% FA (buffer A, from 4% to 32% B in 2h) at 0.25ml/min at 450C. To avoid sample carryover between different samples, both pre-column and column were washed with 3*10 minute gradients from 2% to 95% B (3 minutes at 2%B – 3 minutes from 2% to 95% B – 3 minutes at 95% B – 1 minute from 95% to 2% B). MS analysis was done in DDA mode with 1 MS1 scan, followed by 20 dependent MS2 scans. MS1 parameters were 120,000 resolution, 3e6 AGC target, maximum IT 100ms, with a scan range of 300 to 2000m/z. MS2 parameters were: 15,000 resolution; 2e5 AGC target; maximum IT 15ms; isolation window 1.2m/z; isolation offset 0 m/z; fixed first mass 110 m/z; (N) CE 30; minimum AGC 8e3; exclude unassigned and 1; 6-8 charges; preferred peptide match; exclude isotopes, and; dynamic exclusion was set to 40s. All mass spectrometry raw data were deposited in MassIVE (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp) with accession number MSV000084270, MSV000084279, MSV000084375.
Data processing

A combination of Progenesis QI v2.2 (Waters) and Proteome Discoverer v2.1.0.81 (Thermo Scientific) was used to estimate the relative protein abundance of protein and peptide using in label-free approach. Thermo Raw MS files were imported into Progenesis QI v2.2 (Waters) and the match-between-runs feature was enabled. Subsequently, the matching and alignment time window were performed according to m/z and retention time enable using default settings. Filtering only ions with a charge state of up to +4 was considered. The aligned ion intensity map was carried out using default Peak Peaking settings and no further imputation analysis were performed. The aligned ion intensity map was exported in .pepXml files and imported into Proteome Discoverer v2.1.0.81 (Thermo Scientific) for further protein and peptide identification and searched against the SwissProt mouse reference database by using an in-house MASCOT server (v2.5.1, Matrix Science Ltd, London, UK). Database searches were performed with the following parameters: Fixed Mod: cysteine carbamidomethylation; Variable Mods: methionine oxidation; Trypsin/P digest enzyme (maximum 2 missed cleavages); Precursor and fragment mass tolerance was set to 10ppm and 0.8Da respectively. Identified peptides and proteins were filtered using a False Discovery Rate (FDR) set at 1% and a Peptide Spectrum Match (PSM) set at 1%. Subsequently, the MS/MS and ion abundance search was exported in .mgf files and import into Progenesis Q1 v2.2 to perform peptide and protein normalisation and relative quantitation using respectively a label-free analysis Hi-N/3 summarization method and ion abundance normalisation default method (85) (86). Due to the large proteome scale, each organ were analyzed separately to maintain the reliability of the statistical analysis and to prevent potential homogenization of the protein expression differences that occurred during the mouse’s lifespan. Protein group database are listed in the Supplementary material Table S3.

Quantitative analysis and interpretation

Data analysis was performed in R.

Statistical tests based on the rank product test were carried out to quantify the dynamic protein expressions changing during the mouse lifespan (38)(39). False discovery rates were calculated to correct for multiple testing (87).

Hierarchical heatmap clustering based on Euclidean distance was performed using Perseus software (1.6.0) (88) Z-scores were calculated by subtracting the mean of protein abundance values in all samples and dividing by the standard deviation. Protein group tables are listed in the Supplementary material Table S4.

Gene ontology (GO) analysis of differentially expressed proteins was performed using the DAVID online tool (v6.8) to obtain the biological processes (BPs) and pathways from the enriched chromatin proteome organs during ageing. FDR 1% was set as the minimum threshold value. To generate a detailed dataset, which provides information on the location and the topology of the protein in the cell, the data was sorted according to the Uniprot (SwissProt) subcellular location library. In particular, the subcellular location section was upgraded with the most recent depository protein libraries relative to chromatin studies (22) (23) (24) (25).
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AUTHOR CONTRIBUTIONS

G.O., A.R.W. and O.N.J. conceived and designed the study. G.O. carried out the experimental work, analysed the data, and drafted the manuscript; G. O and S.K performed mass spectrometry experiments. V.S supervised the bioinformatics study with assistance from G.O and A.R-W. O.N.J and A.R-W supervised the study. O.N.J and G.O edited and completed the manuscript with input from all co-authors.

COMPETING INTERESTS

The Authors declare no competing interest

FOOTNOTES

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ABBREVIATIONS

Ada2a-containing complex (ATAC)
ATP-utilizing chromatin assembly and remodelling factor protein complex (ACF)
Biological process (BP)
BRAF-HDAC complex (BHC)
Complex Proteins Associated with Set1 (COMPASS)
Core histone macro-H2A.1 (H2A2.1)
Core histone macro-H2A.2 (H2A2.2)
Gene Ontology (GO)
Heterochromatin associated protein (HP1BP3)
Histone (H3)
Liquid chromatography-tandem mass spectrometry (LC-MS/MS)
Mixed lineage leukemia (MLL)
Mixed-lineage leukaemia complex (MLL)
Mouse embryonic stem cell (mESC)
Nucleosome Remodelling Deacetylase (NuRD)
Polycomb group proteins (PcG)
Polycomb Repressive Complex 2 (PRC2)
Post-translational modification (PTM)
Principle Component Analysis (PCA)
Protein-protein interaction (PPI)
Silent Information Regulator-like family (Sir)
Spt-Ada-Gcn5 acetyltransferase (SAGA)
Ubiquitin-proteasome system (UPS)
ABSTRACT

Quantitative proteomics strategy for the enrichment of chromatin associated proteins from six mouse organs harvested from an ageing model. Following chromatin extraction, proteins were analysed using high resolution quantitative mass spectrometry couple with biochemistry and bioinformatics analysis to identify unique differentially regulated proteins and pathways.

FIGURE 1. Strategy for the enrichment of chromatin associated proteins in mouse embryonic stem cells.

A) Western blot analysis of mouse embryonic cell fractionation. The cytoplasmic, nuclei, chromatin and histone compartments are probed (respectively) by GAPDH, Laminin A/C, SUZ12 and H3 antibodies.

B) Dynamic Range plot of mouse embryonic cell fractionation. Epigenetic subunits associated with chromatin remodelling complexes are listed and sorted by their abundance (Log2 MS intensity) among the two datasets: chromatin proteome lysate and whole cell lysate.

C) Evaluation of the enrichment of chromatin associated proteins in mouse embryonic cell fractionation. The proportion of chromatin associated and nuclear proteins in each organ is shown. Legend colour indicates blue chromatin associated protein, green nuclear proteins, and yellow protein associated with other cellular components. The relative abundances were quantified based on the total ion current. Obtained quantitative results were used to calculate the relative abundances of distinct chromatin associated and nuclear proteins corresponding to each cell compartment, where the sum of all total ion current intensities was considered as 100%.

D) Hierarchical clustering heatmap of mouse embryonic cell fractionation. Chromatin fractionation and total lysate proteomes are compared. Significantly enriched GO term BP (Biological Process) associated with nuclear and chromatin environments are enriched in the chromatin fraction lysate.

FIGURE 2. Comparative and quantitative proteomics of chromatin protein in mouse organs.

A) High resolution LC-MS/MS brain proteome harvested during mouse lifespan. Dynamic Range plot of the brain tissue fractionation obtained from a mouse. Chromatin lysate and whole total lysate proteomes are compared. Epigenetic subunits associated with chromatin remodelling complexes are listed and sorted by specific abundance (Log2 MS Intensity) in both proteomes datasets. Legend colour indicates blue chromatin associated protein, green nuclear proteins, and yellow protein associated with other cellular components. The continuous line box indicates the total lysate proteome, and the dashed line box indicates the chromatin fractions proteome.

B) High resolution LC-MS/MS chromatin proteome harvested from 3, 5, 10, and 15 month old mice. Evaluation of the amount of chromatin associated proteins and nuclear protein among different organs (heart, liver, lung, kidney and spleen). The relative abundances were quantified based on the total ion current. Obtained quantitative results were used to calculate the relative abundances of distinct chromatin associated and nuclear proteins in each organ, where the sum of all total ion current intensities was considered as 100%. Legend colour indicates blue chromatin associated protein, green nuclear proteins, and yellow protein associated with other cellular components. The
continuous line box indicates total lysate proteome, and the dashed line box indicates the chromatin fractions proteome.

C) Histogram showing the number of proteins identified across six organs over time.

FIGURE 3. High Resolution Mass Spectrometry indicates a distinct organ ageing profile.

A) Principle Component Analysis (PCA) of the dataset. Each data point represents a single replicate (n=3). Colour subgroups represent each mouse lifespan point, being 3, 5, 10 and 15 months, respectively. The square grey colour behind each replicate highlights a distinct separation between each organ during mouse lifespan.

B) Pearson correlation coefficient showing the relationship between the different enriched chromatin proteome organs and ageing. The positive correlation coefficient is displayed in red and reduced values are shown in bright blue and white.

FIGURE 4. Common and distinct organ ageing profiles during mouse lifespan.

A) Overlap of proteome sets across six organs, using an UpSet plot. The number of common (orange) and unique (red) organ-specific proteins detected are shown, while various inter-organ combinations are displayed in black. In the bottom of the panel, the proportion of chromatin associated proteins and nuclear proteins present for the “core” proteome and for the unique organ-specific profile are shown. The relative abundances were quantified based on the total ion current. Obtained quantitative results were used to calculate the relative abundances of distinct chromatin associated and nuclear proteins in each organ, where the sum of all total ion current intensities was considered as 100%. Legend colour indicates blue chromatin associated protein, green nuclear proteins, and yellow protein associated with other cellular components.

B) Gene Ontology analysis (Biological Processes) of the core proteome (863) was performed using DAVID GO term analysis. The right panel indicates the most significant GO term categories. The left panel shows multi sub-annotations corresponding to each category. Dot size represents the logarithm of the P-Value assigned to the detected category, while dot colour represents the number of proteins in the pathway.

C) Gene Ontology analysis (Biological Processes) of the unique proteins present in each organ proteome. The dot size represents the significant P-Value assigned to the detected category, while the dot colour represents the number of proteins correspondent to the source pathway.

FIGURE 5. Identification of unique changes in organ protein profiles affected by ageing.

A) UpSet plot measurement the amount of nuclear and relative chromatin associated proteins differentially expressed during mouse lifespan across all organs. Unique chromatin associated proteins related to their organ sources are highlighted by an orange bar. The multiple intersection nodes highlighted in black display shared proteins between organs.
B) Hierarchical clustering heatmap of the “unique differentially expressed protein” related to each organ among the early (3 months) and the late (15 months) ageing stages. Green and red represent increased and decreased expression (respectively) during mouse lifespan. Side bar heatmap legend colour indicates blue chromatin associated protein, green nuclear proteins, and yellow protein associated with other cellular components.

C) Extrapolation of the quantitative expression profile of epigenetic subunits associated with chromatin remodeling complexes. Green and red represent increased and decreased expression (respectively) during mouse lifespan.

FIGURE 6. Identification of differentially regulated organ-specific profiles map to ageing, epigenetic, and other pathways.

A) Dot plot of functional annotation Gene Ontology analysis of the biological process (BP) showing the enrichment pathway terms among ageing. GO categories are sorted by four group labels: gene expression, ageing/development, cellular metabolic process, and structure organisation and biogenesis. The left panel indicates the significant top 30 annotation categories shared between all organs and changing during mouse lifespan. The centre panel showing distinctive ageing-pathway related to their organ sources. The right panel showing the enrichment pathway terms that changed during ageing among all organs. Red and green highlights represent protein down-regulation and up-regulation between early (3 months) and late (15 months) ageing stages. The dot colour represents the significant P-Value of the pathway.

FIGURE 7. Distinct organ ageing profiles are defined by unique chromatin-associated proteins.

A) Protein interaction modules (obtained from the STRING database) are shown for Gene Ontology pathways found to be significantly up- or down-regulated in five organs. Each chord corresponds to a protein-protein interaction while the STRING interaction score is indicated by colour (red for high confidence). The quantitative differentially protein expression during mouse lifespan between 3 and 15 months is shown on the outer circle on a grey-black intensity scale.

(B) Biochemical validation of four protein module responses to ageing identified using chromatin associated proteomics. Organ lysates (from brain, heart, liver and kidney) were immunoblotted with the indicated antibodies. The bar above the blots, corresponded to the quantitative protein expression levels determined in our proteomics experiments over time (green/red scale up- or down-regulated, respectively).
SUPPLEMENTARY FIGURE 1 (S1). Comparison of annotated chromatin-associated proteins from the most recent depository protein libraries relative to chromatin studies

UpSet plot measurement the amount of chromatin associated proteins detected in different studies (22) (23) (24) (25). Unique proteins related to their sources are highlighted by different colours while the multiple intersection nodes highlighted in black display shared proteins between organs.

SUPPLEMENTARY FIGURE 2 (S2). Evaluation of the enrichment of chromatin associated proteins in mouse organs.

Brain organ fractionation was measured by western blot showing separation between nuclear and relative chromatin fraction in mouse brain. The heterochromatin marker HP1BP3 and histone H2A variant H2A2.1 shown lysate was enrichment. The GAPDH and H3 antibodies were used as control respectively whole cell lysate and chromatin fraction.

SUPPLEMENTARY FIGURE 3 (S3). Evaluated the robustness of LC-MS/MS strategy

The biological reproducibility of each organ proteome over time is displayed as a scatter plot and measured by the Pearson correlation coefficient. Red dots indicate high reproducibility and blue dots indicate low reproducibility.

SUPPLEMENTARY FIGURE 5 (S4). Evaluation of protein expression changed in mouse organ over time.

A) Workflow of the proteome data analysis.

B) Box plots of protein expression profile before and after normalisation across six organs (brain, heart, kidney, lung, liver, and spleen) over time. Colour subgroups represent each mouse adult lifespan point, being 3, 5, 10, and 15 months, respectively. The square black line underneath each box plot represents a technical replicate of each biological set.

C) Volcano plots of differentially regulated proteins in mouse brain using PolySTest statistical analysis tool. Specific enrichments for each protein were calculated by Rack test. Adjusted q-values were calculated to correct for multiple testing (-log10 qValue < 0.1 cutoff).

SUPPLEMENTARY FIGURE 5 (S5). Distinct organ ageing profiles are defined by unique differentially protein expression.

D) Volcano plots of differentially regulated proteins across six organs (brain, heart, kidney, lung, liver and spleen) between the early (3 months) and the late (15 months) adult mouse lifespan. Expressed protein significantly changed highlighted in red and green show respectively down-regulation and up-regulation during mouse lifespan. Specific enrichments for each protein were calculated by Rack test. Adjusted q-values were calculated to correct for multiple testing (-log10 qValue < 0.1 cutoff)
E) Co-expression cluster profile, extrapolated from the hierarchical clustering heatmap, shows the “unique differentially expressed protein” trend changed over time in each organ.

F) Meta-data analysis reported the quantitative changed expression profile of human ageing biomarker candidates obtained from the “Human Ageing Genomic Resources” and “GenAge machine learning databank”.

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REFERENCE

1. Oberdoerffer, P., and Sinclair, D. A. (2007) The role of nuclear architecture in genomic instability and ageing. Nat. Rev. Mol. Cell Biol. 8, 692–702

2. Busuttil, R., Bahar, R., and Vijg, J. (2007) Genome dynamics and transcriptional deregulation in aging. Neuroscience 145, 1341–1347

3. Edifizi, D., and Schumacher, B. (2015) Genome Instability in Development and Aging: Insights from Nucleotide Excision Repair in Humans, Mice, and Worms. Biomolecules 5, 1855–69

4. Arancio, W., Pizzolanti, G., Genovese, S. I., Pitrone, M., and Giordano, C. (2014) Epigenetic Involvement in Hutchinson-Gilford Progeria Syndrome: A Mini-Review. Gerontology 60, 197–203

5. Burtner, C. R., and Kennedy, B. K. (2010) Progeria syndromes and ageing: what is the connection? Nat. Rev. Mol. Cell Biol. 11, 567–578

6. Feser, J., and Tyler, J. (2011) Chromatin structure as a mediator of aging. FEBS Lett. 585, 2041–2048

7. Laugesen, A., and Helin, K. (2014) Chromatin Repressive Complexes in Stem Cells, Development, and Cancer. Cell Stem Cell 14, 735–751

8. Santos, A. L., and Lindner, A. B. (2017) Protein Posttranslational Modifications: Roles in Aging and Age-Related Disease. Oxid. Med. Cell. Longev. 2017,

9. Phifer-Rixey, M., and Nachman, M. W. (2015) Insights into mammalian biology from the wild house mouse Mus musculus. Elife 2015, 1–13

10. Fontana, L., and Partridge, L. (2015) Promoting Health and Longevity through Diet: From Model Organisms to Humans. Cell 161, 106–118

11. Sengupta, P. (2013) The Laboratory Rat: Relating Its Age With Human’s. Int. J. Prev. Med. 4, 624–30

12. Dutta, S., and Sengupta, P. (2016) Men and mice: Relating their ages. Life Sci. 152, 244–248

13. Shoji, H., and Miyakawa, T. (2019) Age-related behavioral changes from young to old age in male mice of a C57BL/6J strain maintained under a genetic stability program. Neuropsychopharmacol. Reports 39, 100–118

14. Delgado-morales, R. (2017) Neuroepigenomics in Aging and Disease, 2730-6216

15. Shoji, H., Takao, K., Hattori, S., and Miyakawa, T. (2016) Age-related changes in behavior in C57BL/6J mice from young adulthood to middle age. Mol. Brain, 9

16. Murabito, J. M., Yuan, R., and Lunetta, K. L. (2012) The Search for Longevity and Healthy Aging Genes: Insights From Epidemiological Studies and Samples of Long-Lived Individuals. Journals Gerontol. Ser. A Biol. Sci. Med. Sci. 67A, 470–479

17. Tvardovskiy, A., Schwämmle, V., Kempf, S. J., Rogowska-Wrzesinska, A., and Jensen, O. N. (2017) Accumulation of histone variant H3.3 with age is associated with profound changes in the histone methylation landscape. Nucleic Acids Res. 33, 5005–5020
18. Tobin, S. C., and Kim, K. (2012) Generating pluripotent stem cells: differential epigenetic changes during cellular reprogramming. *FEBS Lett.* 586, 2874–81

19. Takahashi, S., Kobayashi, S., and Hiratani, I. (2018) Epigenetic differences between naïve and primed pluripotent stem cells. *Cell. Mol. Life Sci.* 75, 1191–1203

20. Streubel, G., Fitzpatrick, D. J., Oliviero, G., Scelfo, A., Moran, B., Das, S., Munawar, N., Watson, A., Wynne, K., Negri, G. L., Dillon, E. T., Jammula, S., Hokamp, K., O’Connor, D. P., Pasini, D., Cagney, G., and Bracken, A. P. (2017) Fam60a defines a variant Sin3a-Hdac complex in embryonic stem cells required for self-renewal. *EMBO J.* 36, 2216–2232

21. Herrmann, C., Avgousti, D. C., and Weitzman, M. D. (2017) Differential Salt Fractionation of Nuclei to Analyze Chromatin-associated Proteins from Cultured Mammalian Cells. *Bio-protocol 7,*

22. Medvedeva, Y. A., Lennartsson, A., Ehsani, R., Kulakovskiy, I. V., Vorontsov, I. E., Panahandeh, P., Khimulya, G., Kasukawa, T., and Drabløs, F. (2015) EpiFactors: A comprehensive database of human epigenetic factors and complexes. *Database 2015,* 1–10

23. Mierlo, G., Wester, R. A., and Marks, H. (2019) A Mass Spectrometry Survey of Chromatin-Associated Proteins in Pluripotency and Early Lineage Commitment. *Proteomics* 19, 1900047

24. Christoforou, A., Mulvey, C. M., Breckels, L. M., Geladaki, A., Hurrell, T., Hayward, P. C., Naake, T., Gatto, L., Viner, R., Arias, A. M., and Lilley, K. S. (2016) A draft map of the mouse pluripotent stem cell spatial proteome. *Nat. Commun.* 7, 9992

25. Xu, Y., Zhang, S., Lin, S., Guo, Y., Deng, W., Zhang, Y., and Xue, Y. (2016) OUP accepted manuscript. *Nucleic Acids Res.* 45, D264–D270

26. Conway, J. R., Lex, A., and Gehlenborg, N. (2017) UpSetR: an R package for the visualization of intersecting sets and their properties. *Bioinformatics* 33, 2938–2940

27. Spruijt, C. G., Luijsterburg, M. S., Menafra, R., Stunnenberg, H. G., Van Attikum, H., Correspondence, M. V., Lindeboom, R. G. H., Jansen, P. W. T. C., Ram, R. E., Baltissen, M. P., Wiegant, W. W., Voelker-Albert, M. C., Matarese, F., Mensinga, A., Poser, I., Vos, H. R., and Vermeulen, M. (2016) ZMYND8 Co-localizes with NuRD on Target Genes and Regulates Poly(ADP-Ribose)-Dependent Recruitment of GATAD2A/NuRD to Sites of DNA Damage. *Cell Reports* 17, 783–798

28. Varier, R. A., Carrillo de Santa Pau, E., van der Groep, P., Lindeboom, R. G. H., Matarese, F., Mensinga, A., Smits, A. H., Edupuganti, R. R., Baltissen, M. P., Jansen, P. W. T. C., Ter Hoeve, N., van Weely, D. R., Poser, I., van Diest, P. J., Stunnenberg, H. G., and Vermeulen, M. (2016) Recruitment of the Mammalian Histone-modifying EMSY Complex to Target Genes Is Regulated by ZNF131. *J. Biol. Chem.* 291, 7313–24

29. Signolet, J., and Hendrich, B. (2015) The function of chromatin modifiers in lineage commitment and cell fate specification. *FEBS J.* 282, 1692–1702

30. Hyun, K., Jeon, J., Park, K., and Kim, J. (2017) Writing, erasing and reading histone lysine methylations. *Exp. Mol. Med.* 49
31. Holdsworth, R. J. (1991) Regeneration of the spleen and splenic autotransplantation. *Br. J. Surg.* 78, 270–278

32. Tan, J. K. H., and Watanabe, T. (2018) Determinants of postnatal spleen tissue regeneration and organogenesis. *npj Regen. Med.* 3, 3–6

33. Go, Y.-M., and Jones, D. P. (2017) Redox theory of aging: implications for health and disease. *Clin. Sci. (Lond.)* 131, 1669–1688

34. Haigis, M. C., and Yankner, B. A. (2010) The aging stress response. *Mol. Cell* 40, 333–44

35. Epel, E. S., and Lithgow, G. J. (2014) Stress biology and aging mechanisms: toward understanding the deep connection between adaptation to stress and longevity. *J. Gerontol. A. Biol. Sci. Med. Sci.* 69 Suppl 1, S10–6

36. Postnikoff, S. D. L., and Harkness, T. A. A. (2012) Mechanistic insights into aging, cell-cycle progression, and stress response. *Front. Physiol.* 3, 183

37. Chandler, H., and Peters, G. (2013) Stressing the cell cycle in senescence and aging. *Curr. Opin. Cell Biol.* 25, 765–771

38. Koziol, J. A. (2010) Comments on the Rank Product Method for Analyzing Replicated Experiments. *FEBS Lett.* 584, 941

39. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments (2004) *FEBS Lett.* 573, 83–92

40. Turner, V. M., and Mabbott, N. A. (2017) Influence of ageing on the microarchitecture of the spleen and lymph nodes. *Biogerontology* 18, 723–738

41. Oliviero, G., Brien, G. L., Watson, A., Streubel, G., Jerman, E., Andrews, D., Doyle, B., Munawar, N., Wynne, K., Crean, J., Bracken, A. P., and Cagney, G. (2016) Dynamic protein interactions of the Polycomb Repressive Complex 2 during differentiation of pluripotent cells. *Mol. Cell. Proteomics*, mcp.M116.062240

42. Tacutu, R., Craig, T., Budovsky, A., Wuttke, D., Lehmann, G., Taranukha, D., Costa, J., Fraifeld, V. E., and De Magalhães, J. P. (2013) Human Ageing Genomic Resources: Integrated databases and tools for the biology and genetics of ageing. *Nucleic Acids Res.* 41, 1027–1033

43. Kerepesi, C., Daróczy, B., Sturm, Á., Vellai, T., and Benczúr, A. (2018) Prediction and characterization of human ageing-related proteins by using machine learning. *Sci. Rep.* 8, 1–13

44. Szklarczyk, D., Franceschini, A., Wyder, S., Forsslund, K., Heller, D., Huerta-Cepas, J., Simonovic, M., Roth, A., Santos, A., Tsafou, K. P., Kuhn, M., Bork, P., Jensen, L. J., and von Mering, C. (2015) STRING v10: protein–protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* 43, D447–D452

45. Szklarczyk, D., Morris, J. H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., Santos, A., Doncheva, N. T., Roth, A., Bork, P., Jensen, L. J., and von Mering, C. (2017) The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. *Nucleic Acids Res.* 45, D362–D368

46. Douet, J., Corujo, D., Malinverni, R., Renaud, J., Sansoni, V., Posavec Marjanović, M.
Cantariño, N., Valero, V., Mongelard, F., Bouvet, P., Imhof, A., Thiry, M., and Buschbeck, M. (2017) MacroH2A histone variants maintain nuclear organization and heterochromatin architecture. *J. Cell Sci.* 130, 1570–1582

Barrero, M. J., Sese, B., Martí, M., and Belmonte, J. C. I. (2013) Macro histone variants are critical for the differentiation of human pluripotent cells. *J. Biol. Chem.* 288, 16110–16116

Garfinkel, B. P., Melamed-Book, N., Anuka, E., Bustin, M., and Orly, J. (2015) HP1BP3 is a novel histone H1 related protein with essential roles in viability and growth. *Nucleic Acids Res.* 43, 2074–2090

Garfinkel, B. P., Arad, S., Neuner, S. M., Netser, S., Wagner, S., Kaczorowski, C. C., Rosen, C. J., Gal, M., Soreq, H., and Orly, J. (2016) HP1BP3 expression determines maternal behavior and offspring survival. *Genes, Brain Behav.* 15, 678–688

Saksouk, N., Simboeck, E., Déjardin, J., Song, I., Sullivan, B., and Plotnikova, O. (2015) Constitutive heterochromatin formation and transcription in mammals. *Epigenetics Chromatin* 8, 3

Kim, J., Hake, S. B., and Roeder, R. G. (2005) The Human Homolog of Yeast BRE1 Functions as a Transcriptional Coactivator through Direct Activator Interactions. *Mol. Cell* 20, 759–770

Gillette, T. G., and Hill, J. A. (2015) Readers, writers, and erasers: chromatin as the whiteboard of heart disease. *Circ. Res.* 116, 1245–53

Mei, Z., Zhang, X., Yi, J., Huang, J., He, J., and Tao, Y. (2016) Sirtuins in metabolism, DNA repair and cancer. *J. Exp. Clin. Cancer Res.* 35, 1–14

MASSÉ, S., SEVAPTSIDIS, E., PARSON, I. D., and DOWNAR, E. (1991) A Three-Dimensional Display for Cardiac Activation Mapping. *Pacing Clin. Electrophysiol.* 14, 538–545

Kupis, W., Pałyga, J., Tomal, E., and Niewiadomska, E. (2016) The role of sirtuins in cellular homeostasis. *J. Physiol. Biochem.* 72, 371–380

Vaquero, A. (2009) The conserved role of sirtuins in chromatin regulation. *Int. J. Dev. Biol.* 53, 303–322

Li, M., Valsakumar, V., Poorey, K., Bekiranov, S., and Smith, J. S. (2013) Genome-wide analysis of functional sirtuin chromatin targets in yeast. *Genome Biol.* 14, R48

Rajendran, R., Garva, R., Krstic-Demonacos, M., and Demonacos, C. (2011) Sirtuins: Molecular traffic lights in the crossroad of oxidative stress, chromatin remodeling, and transcription. *J. Biomed. Biotechnol.* 2011

Saez, I., and Vilchez, D. (2014) The Mechanistic Links Between Proteasome Activity, Aging and Agerelated Diseases. *Curr. Genomics* 15, 38–51

McCann, T. S., and Tansey, W. P. (2014) Functions of the proteasome on chromatin. *Biomolecules* 4, 1026–1044

Murgia, M., Tonio, L., Nagaraj, N., Cicioli, S., Vindigni, V., Schiaffino, S., Reggiani, C., and Mann, M. (2017) Single Muscle Fiber Proteomics Reveals Fiber-Type-Specific Features of Human Muscle Aging. *Cell Rep.* 19, 2396–2409
62. Grozinger, C. M., Schreiber, S. L., Sternglanz, R., Xu, R.-L., Broach, J. R., Starai, V. J., Avalos, J. L., Escalante-Semerena, J. C., Grubmeyer, C., Wolberger, C., and al., et (2002) Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. *Chem. Biol.* 9, 3–16

63. Drazic, A., Myklebust, L. M., Ree, R., and Arnesen, T. (2016) The world of protein acetylation. *Biochim. Biophys. Acta - Proteins Proteomics* 1864, 1372–1401

64. Hu, D.-X., Liu, X.-B., Song, W.-C., and Wang, J.-A. (2016) Roles of SIRT3 in heart failure: from bench to bedside. *J. Zhejiang Univ. Sci. B* 17, 821–830

65. Ahn, B.-H., Kim, H.-S., Song, S., Lee, I. H., Liu, J., Vassilopoulos, A., Deng, C.-X., and Finkel, T. (2008) A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis. *Proc. Natl. Acad. Sci. U. S. A.* 105, 14447–52

66. Gamper, A. M., Kim, J., and Roeder, R. G. (2009) The STAGA Subunit ADA2b Is an Important Regulator of Human GCN5 Catalysis. *Mol. Cell. Biol.* 29, 266–280

67. Xu, W., Edmondson, D. G., Evrard, Y. A., Wakamiya, M., Behringer, R. R., and Roth, S. Y. (2000) Loss of Gcn5l2 leads to increased apoptosis and mesodermal defects during mouse development. *Nat. Genet.* 26, 229–232

68. Lin, W., Srajer, G., Evrard, Y. A., Phan, H. M., Furuta, Y., and Dent, S. Y. R. (2007) Developmental potential of Gcn5−/− embryonic stem cells in vivo and in vitro. *Dev. Dyn.* 236, 1547–1557

69. Finley, D. (2009) Recognition and Processing of Ubiquitin-Protein Conjugates by the Proteasome. *Annu. Rev. Biochem.* 78, 477–513

70. Schmidt, M., and Finley, D. (2014) Regulation of proteasome activity in health and disease. *Biochim. Biophys. Acta - Mol. Cell Res.* 1843, 13–25

71. Lecker, S. H., Goldberg, A. L., and Mitch, W. E. (2006) Protein degradation by the ubiquitin-proteasome pathway in normal and disease states. *J. Am. Soc. Nephrol.* 17, 1807–19

72. Tanaka, K. (2009) The proteasome: overview of structure and functions. *Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci.* 85, 12–36

73. Adams, J. (2003) The proteasome: structure, function, and role in the cell. *Cancer Treat. Rev.* 29 Suppl 1, 3–9

74. Bulteau, A. L., Petropoulos, I., and Friguet, B. (2000) Age-related alterations of proteasome structure and function in aging epidermis. *Exp. Gerontol.* 35, 767–77

75. Carrard, G., Dieu, M., Raes, M., Toussaint, O., and Friguet, B. (2003) Impact of ageing on proteasome structure and function in human lymphocytes. *Int. J. Biochem. Cell Biol.* 35, 728–39

76. Petropoulos, I., Conconi, M., Wang, X., Hoenel, B., Brégégère, F., Milner, Y., and Friguet, B. (2000) Increase of Oxidatively Modified Protein Is Associated With a Decrease of Proteasome Activity and Content in Aging Epidermal Cells. *J. Gerontol. Biol. Sci. Am.* 55, 220–227

77. Bardag-Gorce, F., Farout, L., Veyrat-Durex, C., Briand, Y., and Briand, M. (1999) Changes in 20S proteasome activity during ageing of the LOU rat. *Mol. Biol. Rep.* 26, 89–93
Chondrogianni, N., and Gonos, E. S. (2010) Proteasome function determines cellular homeostasis and the rate of aging. Ado. Exp. Med. Biol. 694, 38–46

Murgia, M., Toniolo, L., Nagaraj, N., Ciciliot, S., Vindigni, V., Schiaffino, S., Reggiani, C., and Mann, M. (2017) Single Muscle Fiber Proteomics Reveals Fiber-Type-Specific Features of Human Muscle Aging. Cell Rep. 19, 2396–2409

Lang, F., Khaghani, S., Türk, C., Wiederstein, J. L., Hölper, S., Piller, T., Nogara, L., Blauw, B., Günther, S., Müller, S., Braun, T., and Krüger, M. (2018) Single Muscle Fiber Proteomics Reveals Distinct Protein Changes in Slow and Fast Fibers during Muscle Atrophy. J. Proteome Res. 17, 3333–3347

Farrants, A. K. Ó. (2008) Chromatin remodelling and actin organisation. FEBS Lett. 582, 2041–2050

Walther, D. M., and Mann, M. (2011) Accurate quantification of more than 4000 mouse tissue proteins reveals minimal proteome changes during aging. Mol. Cell. Proteomics 10,

Shavlakadze, T., Morris, M., Fang, J., Wang, S. X., Zhu, J., Zhou, W., Tse, H. W., Mondragon-Gonzalez, R., Roma, G., and Glass, D. J. (2019) Age-Related Gene Expression Signature in Rats Demonstrate Early, Late, and Linear Transcriptional Changes from Multiple Tissues. Cell Rep. 28, 3263–3273.e3

Rappsilber, J., Ishihama, Y., and Mann, M. (2003) Stop and Go Extraction Tips for Matrix-Assisted Laser Desorption/Ionization, Nanoelectrospray, and LC/MS Sample Pretreatment in Proteomics. Anal. Chem. 75, 663–670

Silva, J. C., Gorenstein, M. V., Li, G.-Z., Vissers, J. P. C., and Geromanos, S. J. (2006) Absolute Quantification of Proteins by LC/MS E. Mol. Cell. Proteomics 5, 144–156

Ferguson, R. E., Carroll, H. P., Harris, A., Maher, E. R., Selby, P. J., and Banks, R. E. (2005) Housekeeping proteins: A preliminary study illustrating some limitations as useful references in protein expression studies. Proteomics 5, 566–571

Korthauer, K., Kimes, P. K., Duvallet, C., Reyes, A., Subramanian, A., Teng, M., Shukla, C., Alm, E. J., and Hicks, S. C. (2019) A practical guide to methods controlling false discoveries in computational biology. Genome Biol. 20, 118

Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., and Cox, J. (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat. Methods, 13(9):731–40

81.

Farrant, A. K. Ó. (2008) Chromatin remodelling and actin organisation. FEBS Lett. 582, 2041–2050

82.

Walther, D. M., and Mann, M. (2011) Accurate quantification of more than 4000 mouse tissue proteins reveals minimal proteome changes during aging. Mol. Cell. Proteomics 10,

83.

Shavlakadze, T., Morris, M., Fang, J., Wang, S. X., Zhu, J., Zhou, W., Tse, H. W., Mondragon-Gonzalez, R., Roma, G., and Glass, D. J. (2019) Age-Related Gene Expression Signature in Rats Demonstrate Early, Late, and Linear Transcriptional Changes from Multiple Tissues. Cell Rep. 28, 3263–3273.e3

84.

Rappsilber, J., Ishihama, Y., and Mann, M. (2003) Stop and Go Extraction Tips for Matrix-Assisted Laser Desorption/Ionization, Nanoelectrospray, and LC/MS Sample Pretreatment in Proteomics. Anal. Chem. 75, 663–670

85.

Silva, J. C., Gorenstein, M. V., Li, G.-Z., Vissers, J. P. C., and Geromanos, S. J. (2006) Absolute Quantification of Proteins by LC/MS E. Mol. Cell. Proteomics 5, 144–156

86.

Ferguson, R. E., Carroll, H. P., Harris, A., Maher, E. R., Selby, P. J., and Banks, R. E. (2005) Housekeeping proteins: A preliminary study illustrating some limitations as useful references in protein expression studies. Proteomics 5, 566–571

87.

Korthauer, K., Kimes, P. K., Duvallet, C., Reyes, A., Subramanian, A., Teng, M., Shukla, C., Alm, E. J., and Hicks, S. C. (2019) A practical guide to methods controlling false discoveries in computational biology. Genome Biol. 20, 118

88.

Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., and Cox, J. (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat. Methods, 13(9):731–40
**Tissue isolation**
- C57BL/6J
- TIME (months) 3, 5, 10, 15
- Organ types: BRAIN, HEART, LIVER, KIDNEY, LUNG, SPLEEN

**Chromatin extraction**
- Tissue lysing (Homogenizer, dounce)
- Nuclei extraction (high-salt)
- Chromatin Lysate

**Quantitative proteomics (LC-MS/MS)**
- Protein digestion
- Q Exactive HF
- m/z Relative Abundance

**Data analysis**
- Mouse Proteome
- UniProtKB database
- Chromatin proteome & Statistical analysis
- Regulated proteins
FIGURE 3

A

Component 2 (17.3%)

Component 1 (34%)

Age, months

3 5 10 15

BRAIN
HEART
LIVER
SPLEEN
KIDNEY
LUNG

B

Biological replicates:

1 2 3

Pearson correlation coefficient

BRAIN SPLEEN KIDNEY LIVER HEART LUNG
**FIGURE 4**

**A**

- Unique proteins
- Common proteins
- Other intersections

**B**

**BP: GO TERM**

- Common proteins (863)
- oxidation–reduction process
- Aging
- regulation of cell death
- chromatin
- cell cycle
- translation
- system development
- anatomical structure morphogenesis
- regulation of transcription, DNA–templated
- response to stress
- transport

**C**

**Unique tissue proteins**

- BRAIN
- HEART
- LIVER
- KIDNEY
- oxidation–reduction process
- steroid metabolic process
- liver development
- transport
- negative regulation of stress-activated MAP kinase signaling pathway
- glucose homeostasis
- ATP synthesis coupled proton transport
- MBP synthesis
- phospholipid biosynthetic process
- protein glycosylation

- LUNG
- oxidation–reduction process
- immune system process
- regulation of cell death
- chromatin
- cell cycle
- translation
- system development
- anatomical structure morphogenesis
- regulation of transcription, DNA–templated
- response to stress
- transport

- SPLEEN
- immune system process
- nervous system development
- intracellular signal transduction
- phosphatidylinositol signaling system
- phospholipid biosynthetic process
- protein glycosylation

- HEART
- DNA repair
- innate immune response
- double-strand break repair via homologous recombination
- regulation of transcription, DNA–templated
- double-strand break repair
FIGURE 5

A

Intersection Size

Unique Regulated proteins

B

BRAIN (Age, months)

HEART (Age, months)

KIDNEY (Age, months)

LIVER (Age, months)

SPLEEN (Age, months)

C

Epigenetic Tissue Chromatin Complex

Up-regulation (3 to 15 months)

Down-regulation (3 to 15 months)
## FIGURE 6

### Unique changes in protein expression profile present in each tissue

#### BRAIN
- **Gene Expression**
  - chromatin assembly or disassembly
  - DNA packaging
  - DNA replication-dependent nucleosome assembly
  - mRNP processing
  - nuclear transport
  - negative regulation of gene expression, epigenetic cell aging
  - negative regulation of gene expression, epigenetic cell aging

#### LIVER
- **Gene Expression**
  - chromatin organization involved in regulation of transcription
  - DNA replication-dependent nucleosome assembly
  - histone H3-K79 methylation

#### HEART
- **Gene Expression**
  - cardiac muscle contractile protein transport
  - cardiac muscle development
  - cardiac muscle differentiation

#### LUNG
- **Gene Expression**
  - fibronectin
  - lung development
  - lung epithelium development

#### KIDNEY
- **Gene Expression**
  - actin filament-based process
  - cell death response to endoplasmic reticulum stress
  - cell death response to endoplasmic reticulum stress

#### SPLEEN
- **Gene Expression**
  - serine hydrolase activity

### Common changes in protein expression profile across all tissues

- Cytoskeleton organization and biogenesis
- DNA packaging
- DNA replication-dependent nucleosome assembly
- Gene expression
- Histone modification
- Protein binding
- Protein complex assembly
- Protein-DNA complex assembly
- Regulation of transcription, DNA-templated
- Translation

### Distinctive regulated changes in protein expression profile present in each tissue

#### BRAIN
- Regulation of translation, ribosome biogenesis
- Regulation of translation, ribosome biogenesis

#### LIVER
- Regulation of translation, ribosome biogenesis
- Regulation of translation, ribosome biogenesis

#### HEART
- Regulation of muscle contraction
- Regulation of muscle contraction

#### LUNG
- Regulation of muscle contraction
- Regulation of muscle contraction

#### KIDNEY
- Regulation of muscle contraction
- Regulation of muscle contraction

#### SPLEEN
- Regulation of muscle contraction
- Regulation of muscle contraction
This study
EpiFactors
WERAM
A. Christoforou et al
G.VanMierlo et al

Library:
Chromatin Associated Protein:
- different eukaryote species (2016)
- mouse embryonic stem cells (2015)
- different human cell lines (2015)
- in this study (2020)
- different mouse embryonic stem cells (2019)

Unique Protein
Shared Protein

Intersection Size
S2

BRAIN

| Age (months) | Total lysate | Chromatin lysate |
|--------------|--------------|------------------|
| 3            | H2A2.1       |                  |
| 15           | H2A2.1       |                  |
| 3            | GAPDH        |                  |
| 15           |               |                  |
| 3            | HP1BP3       |                  |
| 15           |               |                  |
| 3            | H3           |                  |
| 15           |               |                  |

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S3
**A**

LC-MS/MS

Label-free strategy

RAW data

Protein Data Analysis

Ageing-Organ data analysis

Analysis of ageing trends in adult mouse lifespan

**Progenosis Ql (.pepXml)**

Proteome Discoverer (.mgf)

(FDR) 1%

(PSM) 1%

Normalisation

Relative protein abundance

Fold change calculation

(PolySTest tool)

(Rank product tests)

(-log10 qValue < 0.1 cutoff)

**B**

BRAIN

Normalised Protein abundance

Raw Protein abundance

3 5 10 15

Log2 (MS Intensity)

HEART

Normalised Protein abundance

Raw Protein abundance

3 5 10 15

Log2 (MS Intensity)

LIVER

Normalised Protein abundance

Raw Protein abundance

3 5 10 15

Log2 (MS Intensity)

KIDNEY

Normalised Protein abundance

Raw Protein abundance

3 5 10 15

Log2 (MS Intensity)

LUNG

Normalised Protein abundance

Raw Protein abundance

3 5 10 15

Log2 (MS Intensity)

SPLEEN

Normalised Protein abundance

Raw Protein abundance

3 5 10 15

Log2 (MS Intensity)

**C**

PolySTest

limma

Miss test

rank products

permutation test

t-test

**Analysis of ageing trends in adult mouse lifespan**

**Relative protein abundance**

**Data Analysis**

Progenesis QI (.pepXml)

Proteome Discoverer (.mgf)

Normalisation

**Fold change calculation**

(PolySTest tool)

(Rank product tests)

(-log10 qValue < 0.1 cutoff)

(FDR) 1%

(PSM) 1%

**Age, months**

3 5 10 15
