1 **TITLE**

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

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19 SUMMARY

20 Temporal molecular changes in ageing mammalian organs are of relevance to disease
 21 etiology because many age-related diseases are linked to changes in the transcriptional
 22 and epigenetic machinery that regulate gene expression. We performed quantitative
 23 proteome analysis of chromatin-enriched protein extracts to investigate the dynamics of
 24 the chromatin-proteomes of the mouse brain, heart, lung, kidney, liver, and spleen at 3,
 25 5, 10, and 15 months of age. Each organ exhibited a distinct chromatin-proteome and
 26 sets of unique proteins. The brain and spleen chromatin-proteomes were the most
 27 extensive, diverse, and heterogenous among the six organs. The spleen chromatin
 28 proteome appeared static during the lifespan, presenting a young phenotype that
 29 reflects the permanent alertness state and important role of this organ in physiological
 30 defense and immunity. We identified a total of 5928 proteins, including 2472 nuclear or
 31 chromatin associated proteins across the six mouse organs. Up to 3125 proteins were
 32 quantified in each organ demonstrating distinct and organ-specific temporal protein
 33 expression timelines and regulation at the post-translational level. Bioinformatics meta-
 34 analysis of these chromatin proteomes revealed distinct physiological and ageing-
 35 related features for each organ. Our results demonstrate the efficiency of organelle
 36 specific proteomics for *in vivo* studies of a model organism and consolidate the
 37 hypothesis that chromatin-associated proteins are involved in distinct and specific
 38 physiological functions in ageing organs.

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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

82

83 Ageing is a natural process resulting in progressive changes of most, if not all, cellular
84 components. Ageing is generally associated with declining biological performance and
85 increased incidence of disease (1). The gene expression apparatus, comprised of the
86 DNA itself, the chromatin environment it is housed in, and the machinery of
87 transcription and translation is profoundly affected by ageing (2) (3). Models of ageing
88 often display similar phenotypes to those undergoing senescence or genome instability,
89 highlighting how integrated the ageing process is with these phenomena (1). Diseases
90 that mimic or accelerate the ageing process, including Hutchinson-Gilford progeria and
91 Werner syndromes, result in molecular changes in nucleosomes and chromatin (4) (5)
92 (6).

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

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

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

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

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

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

120 Among almost 6,000 proteins identified, organ-specific patterns predominated, with
121 age-responsive subsets identified for each organ. We mapped pathway level molecular
122 changes specific to individual organ over time.

123 Our results demonstrate that the ageing process affects each mouse organ in a distinct
124 manner illustrated by the diversity and heterogeneity of the temporal chromatin
125 proteomes of each organ.

126

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

ORC	1	30
POLYCOMB	7	30
READER	8	33
SAGA	8	8
SET1	3	6
SIN3A	1	26
SWI/SNF	5	43
Other chromatin enzymes	10	80

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148 The dynamic range plot was used to assess the measurements of the protein expression
149 across these proteomes. The mESC chromatin fraction was indeed highly enriched for
150 chromatin associated proteins as compared to the whole cell lysate (Figure 1, panels B
151 and C).

152 We detected major chromatin associated protein complexes, including the Polycomb
153 Repressive Complex 2 (PRC2), Nucleosome Remodelling and Deacetylase (NuRD), BRAF-
154 HDAC complex (BHC), and mixed-lineage leukaemia (MLL) complex (Figure 1, panel B)
155 (22) (23) (25).

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

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

164 We next demonstrated that the chromatin enrichment protocol used for mESCs is
165 applicable to mouse organs. We first extracted chromatin proteins from mouse brain
166 and assessed chromatin enrichment by western blotting using specific protein markers
167 (Supplementary material, Figure S2). We observed enrichment of the chromatin marker
168 histone H3 and reduced level of the cytosolic marker GAPDH expression within the
169 “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.

Chromatin proteomes during mouse lifespan							
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							
	Shared proteins		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)

270 (22) (23) (25) (27) (28). Many of these proteins are expressed during cell fate
271 commitment (22) (29).

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

274 The mouse brain chromatin proteome contained 793 unique proteins, constituting the
275 largest set of unique proteins among the six organs (Figure 4, Panel A). Approximately
276 24% of these proteins (188) were classified as chromatin associated proteins or nuclear
277 proteins (Figure 4, Panel A, below) (Table 3). They included protein and histone
278 methyltransferase enzymes (HNMT, SAP30L, CARM1, SETD7, SUV39H2) and histone
279 deacetylase enzymes (CHD5, IRF2BP1, MAPK10, MEF2D, MACROD2) that are mainly
280 involved in transcriptional gene silencing (22) (23) (25) (30).

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

284 The spleen is an organ with the innate capacity to regenerate (31). It acts as a filter for
285 blood and it controls the blood-borne immune response (32). We detected several
286 epigenetic complexes such as BHC, ING, MLL, NURD, ORC, PCG, SIN3A, SAGA, SWI/SNF.
287 Also we detected chromatin “reader” enzymes not yet assigned to a specific chromatin
288 remodelling complex (KDM3B, KDM2A, PHF23, CHD1L, UHRF2, MORC3, BRD9,
289 ZMYND11, BAZ1A). These proteins recognise single post-translational histone marks or
290 combinations of histone marks and histone variants to direct a particular transcriptional
291 outcome (22) (23) (25) (30).

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

295

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

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

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

307 GO classification of the unique proteins present in each organ proteome, suggested
308 distinctive molecular signs of ageing in each organ (Figure 4, Panel C). We observed
309 distinctive organ-specific categories, “age-classes and age-development” and categories
310 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.

350 Brain, heart and kidney showed mainly gradual changes of protein levels over time.
351 Lung and liver show a more dramatic change of protein expression between 5 and 10
352 months of age (Figure 5, Panel B, supplementary material Figure S5, Panel E). We
353 detected few significant protein abundance changes in the spleen. The spleen fraction
354 was highly enriched in chromatin associated and nuclear proteins (approx. 60%) and
355 contained many unique proteins (Figure 3 and 4). Thus, the spleen seems to
356 continuously exhibit a young phenotype that may be due to the constitutively active role
357 of the spleen in maintaining immune functions, red blood cell turnover and microbial
358 defense (40). The spleen contains multiple cell populations capable of supporting
359 immune responses, which may indicate the presence of self-renewal cell types that are
360 “age-less” (31).

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

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

368 The majority of chromatin modifying enzymes belonging to a given multiprotein
369 complex, exhibited similar expression profiles over time within a specific organ (Figure
370 5, Panel C). For instance, the MLL subunits WDR82, CTR9 and WDR61 were down-
371 regulated in liver during ageing. Components of same chromatin modifying complexes
372 were detected in several organs, albeit not by the same subunits and with opposite
373 temporal expression profiles. NuRD subunit HDAC2, was down-regulated in liver
374 whereas NuRD subunits GATA2AD and TRIM28 were up-regulated in kidney.

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

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

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

386 We identified a series of human protein biomarker candidates for ageing. The brain
387 protein IREB2 is associated with Alzheimer’s disease, whereas the brain protein MAOB
388 is associated with both Alzheimer’s and Parkinson’s diseases. The heart proteins ADD3,
389 PTGIS, and COL1A2 are candidates for hypertension and myocardial infarction. The liver
390 proteins INSR, PTPN1, and ENPP1 are associated with diabetes mellitus type 2 and
391 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

(Figure 7, Panel A). 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 15months.

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

591 complex involving these two proteins is implicated in maintaining heterochromatin
592 integrity and promote gene silencing during mouse lifespan.

593 Reversible acetylation of histones plays a critical role in transcriptional regulation in
594 eukaryotic cells. We detected reduced levels of histone H3 acetylation during ageing in
595 mouse liver. The opposite trend was observed in ageing heart, i.e. an increase in histone
596 H3 acetylation. Two families of deacylase enzymes were identified: the histone
597 deacetylases, HDACs, and the Sir family protein (Silent Information Regulator)-like
598 family of NAD-dependent deacylases, or sirtuins (62). Both enzyme families play a
599 major role in gene regulation by modifying the histone acetylation/acylation landscape
600 in response to external stimuli and specific environmental stress conditions, such as
601 oxidative stress (63). In mammals, the brain and heart have the greatest oxygen demand
602 for their ATP dependent processes. Nearly all cellular processes of cardiomyocytes are
603 driven by ATP-dependent pathways (64). SIRT3 plays an important role in maintaining
604 basal ATP levels and regulated energy production in mouse embryonic fibroblasts (64)
605 (65).

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

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

623 The proteasome is a complex proteolytic machine formed by the assembly of several
624 subunits (69). The ubiquitin-proteasome system (UPS) is the primary selective
625 degradation system in eukaryotic cells, localised both in the nuclei and cytoplasm
626 compartment, which is required for the turnover of soluble proteins (70). The UPS is
627 mainly implicated in protein degradation in response to the regulation of several
628 processes including the maintenance of cellular quality control, transcription, cell cycle
629 progression, DNA repair, receptor-mediated endocytosis, cell stress response, and
630 apoptosis (71). Before a protein is degraded, it is first flagged for destruction by the
631 ubiquitin conjugation system, which ultimately results in the attachment of a
632 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 × 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 × 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 × g, 1 h at 4 °C). After centrifugation the supernatant was snap frozen.

752

753 Immunoblotting

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

763

764 Protein sample preparation for mass spectrometry

765 Protein concentration was measured by the Bradford assay. 50µg of of chromatin lysate
766 was precipitated with 4 volumes of acetone and re-suspended in 20µl 6M Urea/2M
767 Thiourea in 50mM ABC. Cysteines were reduced with 1mM DTT for 30 minutes and
768 alkylated with 5mM iodoacetamide for 30 minutes. 500ng LysC (Wako) was added and
769 proteins were digested at room temperature for 3 hours. Samples were diluted 1:4 with
770 50mM ABC and 1µg trypsin (Sigma) and incubating overnight at 37 °C. Digestion was
771 quenched by addition of formic acid (FA) to a final concentration of ~1%, and the
772 resulting peptide mixture was centrifuged at 5000 × g for 15 min. Peptides in the
773 supernatant were desalted by stage tipping with C18 material (84) and eluted by 50%
774 ACN, 0.1% FA in water and dried in a SpeedVac. Samples were stored at -80 °C until
775 further use.

776 Mass spectrometry analysis

777 Samples were redissolved in 50 µl of Trifluoroacetic acid 0.1% (vol/vol) in water, as
778 buffer A, and sonicated for 1 minute and centrifuged for 15 minutes at 15000 × g.
779 Analysis was carried out on an Ultimate 3000 RSLCnano HPLC system connected to a
780 mass accuracy high resolution mass spectrometry, Q Exactive HF (ThermoFisher). The
781 MS instrument was controlled by Xcalibur software (ThermoFisher). The nano-
782 electrospray ion source (ThermoFisher) was used with a spray voltage of 2.2 kV. The ion
783 transfer tube temperature was 275 °C. Samples were loaded on a cartridge pre-column
784 PepMap 100 5*0.3mm (ThermoFisher) in 2% ACN, 98% H₂O, 0.1% TFA at 10ml/min,
785 and then separated either with an easy Spray C18 or a 75mm* 50cm 2mm PepMap 100
786 column (ThermoFisher). Separation was done in a linear gradient of ACN, 0.1% FA
787 (buffer B) in H₂O, 0.1% FA (buffer A, from 4% to 32% B in 2h) at 0.25ml/min at 45oC.
788 To avoid sample carryover between different samples, both pre-column and column
789 were washed with 3*10 minute gradients from 2% to 95% B (3 minutes at 2%B – 3
790 minutes from 2% to 95% B – 3 minutes at 95% B – 1 minute from 95% to 2% B). MS
791 analysis was done in DDA mode with 1 MS1 scan, followed by 20 dependent MS2 scans.
792 MS1 parameters were 120,000 resolution, 3e6 AGC target, maximum IT 100ms, with a
793 scan range of 300 to 2000m/z. MS2 parameters were: 15,000 resolution; 2e5 AGC
794 target; maximum IT 15ms; isolation window 1.2m/z; isolation offset 0 m/z; fixed first
795 mass 110 m/z; (N) CE 30; minimum AGC 8e3; exclude unassigned and 1; 6-8 charges;
796 preferred peptide match; exclude isotopes, and; dynamic exclusion was set to 40s. All
797 mass spectrometry raw data were deposited in MassIVE
798 (<https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp>) with accession number
799 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 QI 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)

936 ABSTRACT FIGURE

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

942 **FIGURE 1. Strategy for the enrichment of chromatin associated proteins in mouse** 943 **embryonic stem cells.**

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

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

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

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

963

964 **FIGURE 2. Comparative and quantitative proteomics of chromatin protein in** 965 **mouse organs.**

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

974 B) High resolution LC-MS/MS chromatin proteome harvested from 3, 5, 10, and 15
975 month old mice. Evaluation of the amount of chromatin associated proteins and nuclear
976 protein among different organs (heart, liver, lung, kidney and spleen). The relative
977 abundances were quantified based on the total ion current. Obtained quantitative
978 results were used to calculate the relative abundances of distinct chromatin associated
979 and nuclear proteins in each organ, where the sum of all total ion current intensities was
980 considered as 100%. Legend colour indicates blue chromatin associated protein, green
981 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).

1072 SUPPLEMENTARY FIGURE

1073 SUPPLEMENTARY FIGURE 1 (S1). Comparison of annotated chromatin-associated 1074 proteins from the most recent depository protein libraries relative to 1075 chromatin studies

1076 UpSet plot measurement the amount of chromatin associated proteins detected in
1077 different studies (22) (23) (24) (25). Unique proteins related to their sources are
1078 highlighted by different colours while the multiple intersection nodes highlighted in
1079 black display shared proteins between organs.

1080 SUPPLEMENTARY FIGURE 2 (S2). Evaluation of the enrichment of chromatin 1081 associated proteins in mouse organs.

1082 Brain organ fractionation was measured by western blot showing separation between
1083 nuclear and relative chromatin fraction in mouse brain. The heterochromatin marker
1084 HP1BP3 and histone H2A variant H2A2.1 shown lysate was enrichment. The GAPDH and
1085 H3 antibodies were used as control respectively whole cell lysate and chromatin
1086 fraction.

1087 SUPPLEMENTARY FIGURE 3 (S3). Evaluated the robustness of LC-MS/MS strategy

1088 The biological reproducibility of each organ proteome over time is displayed as a scatter
1089 plot and measured by the Pearson correlation coefficient. Red dots indicate high
1090 reproducibility and blue dots indicate low reproducibility.
1091

1092 SUPPLEMENTARY FIGURE 5 (S4). Evaluation of protein expression changed in 1093 mouse organ over time.

1094 A) Workflow of the proteome data analysis.

1095 B) Box plots of protein expression profile before and after normalisation across six
1096 organs (brain, heart, kidney, lung, liver, and spleen) over time. Colour subgroups
1097 represent each mouse adult lifespan point, being 3, 5, 10, and 15 months, respectively.
1098 The square black line underneath each box plot represents a technical replicate of each
1099 biological set.

1100 C) Volcano plots of differentially regulated proteins in mouse brain using PolySTest
1101 statistical analysis tool. Specific enrichments for each protein were calculated by Rack
1102 test. Adjusted q-values were calculated to correct for multiple testing ($-\log_{10} \text{qValue} <$
1103 0.1 cutoff).

1104

1105 SUPPLEMENTARY FIGURE 5 (S5). Distinct organ ageing profiles are defined by 1106 unique differentially protein expression.

1107 D) Volcano plots of differentially regulated proteins across six organs (brain, heart,
1108 kidney, lung, liver and spleen) between the early (3 months) and the late (15 months)
1109 adult mouse lifespan. Expressed protein significantly changed highlighted in red and
1110 green show respectively down-regulation and up-regulation during mouse lifespan.
1111 Specific enrichments for each protein were calculated by Rack test. Adjusted q-values
1112 were calculated to correct for multiple testing ($-\log_{10} \text{qValue} < 0.1$ cutoff)

1113 E) Co-expression cluster profile, extrapolated from the hierarchical clustering heatmap,
1114 shows the “unique differentially expressed protein” trend changed over time in each
1115 organ.

1116 F) Meta-data analysis reported the quantitative changed expression profile of human
1117 ageing biomarker candidates obtained from the “Human Ageing Genomic Resources”
1118 and “GenAge machine learning databank”.

1119

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1121 REFERENCE

- 1122 1. Oberdoerffer, P., and Sinclair, D. A. (2007) The role of nuclear architecture in
1123 genomic instability and ageing. *Nat. Rev. Mol. Cell Biol.* 8, 692–702
- 1124 2. Busuttil, R., Bahar, R., and Vijg, J. (2007) Genome dynamics and transcriptional
1125 deregulation in aging. *Neuroscience* 145, 1341–1347
- 1126 3. Edifizi, D., and Schumacher, B. (2015) Genome Instability in Development and
1127 Aging: Insights from Nucleotide Excision Repair in Humans, Mice, and Worms.
1128 *Biomolecules* 5, 1855–69
- 1129 4. Arancio, W., Pizzolanti, G., Genovese, S. I., Pitrone, M., and Giordano, C. (2014)
1130 Epigenetic Involvement in Hutchinson-Gilford Progeria Syndrome: A Mini-
1131 Review. *Gerontology* 60, 197–203
- 1132 5. Burtner, C. R., and Kennedy, B. K. (2010) Progeria syndromes and ageing: what is
1133 the connection? *Nat. Rev. Mol. Cell Biol.* 11, 567–578
- 1134 6. Feser, J., and Tyler, J. (2011) Chromatin structure as a mediator of aging. *FEBS*
1135 *Lett.* 585, 2041–2048
- 1136 7. Laugesen, A., and Helin, K. (2014) Chromatin Repressive Complexes in Stem Cells,
1137 Development, and Cancer. *Cell Stem Cell* 14, 735–751
- 1138 8. Santos, A. L., and Lindner, A. B. (2017) Protein Posttranslational Modifications:
1139 Roles in Aging and Age-Related Disease. *Oxid. Med. Cell. Longev.* 2017,
- 1140 9. Phifer-Rixey, M., and Nachman, M. W. (2015) Insights into mammalian biology
1141 from the wild house mouse *Mus musculus*. *Elife* 2015, 1–13
- 1142 10. Fontana, L., and Partridge, L. (2015) Promoting Health and Longevity through
1143 Diet: From Model Organisms to Humans. *Cell* 161, 106–118
- 1144 11. Sengupta, P. (2013) The Laboratory Rat: Relating Its Age With Human's. *Int. J.*
1145 *Prev. Med.* 4, 624–30
- 1146 12. Dutta, S., and Sengupta, P. (2016) Men and mice: Relating their ages. *Life Sci.* 152,
1147 244–248
- 1148 13. Shoji, H., and Miyakawa, T. (2019) Age-related behavioral changes from young to
1149 old age in male mice of a C57BL/6J strain maintained under a genetic stability
1150 program. *Neuropsychopharmacol. Reports* 39, 100–118
- 1151 14. Delgado-morales, R. (2017) *Neuroepigenomics in Aging and Disease*, 2730-6216
- 1152 15. Shoji, H., Takao, K., Hattori, S., and Miyakawa, T. (2016) Age-related changes in
1153 behavior in C57BL/6J mice from young adulthood to middle age. *Mol. Brain*, 9
- 1154 16. Murabito, J. M., Yuan, R., and Lunetta, K. L. (2012) The Search for Longevity and
1155 Healthy Aging Genes: Insights From Epidemiological Studies and Samples of
1156 Long-Lived Individuals. *Journals Gerontol. Ser. A Biol. Sci. Med. Sci.* 67A, 470–479
- 1157 17. Tvardovskiy, A., Schwämmle, V., Kempf, S. J., Rogowska-Wrzesinska, A., and
1158 Jensen, O. N. (2017) Accumulation of histone variant H3.3 with age is associated
1159 with profound changes in the histone methylation landscape. *Nucleic Acids Res.*
1160 33, 5005–5020

- 1161 18. Tobin, S. C., and Kim, K. (2012) Generating pluripotent stem cells: differential
1162 epigenetic changes during cellular reprogramming. *FEBS Lett.* 586, 2874–81
- 1163 19. Takahashi, S., Kobayashi, S., and Hiratani, I. (2018) Epigenetic differences
1164 between naïve and primed pluripotent stem cells. *Cell. Mol. Life Sci.* 75, 1191–
1165 1203
- 1166 20. Streubel, G., Fitzpatrick, D. J., Oliviero, G., Scelfo, A., Moran, B., Das, S., Munawar, N.,
1167 Watson, A., Wynne, K., Negri, G. L., Dillon, E. T., Jammula, S., Hokamp, K., O'Connor,
1168 D. P., Pasini, D., Cagney, G., and Bracken, A. P. (2017) Fam60a defines a variant
1169 Sin3a-Hdac complex in embryonic stem cells required for self-renewal. *EMBO J.*
1170 36, 2216–2232
- 1171 21. Herrmann, C., Avgousti, D. C., and Weitzman, M. D. (2017) Differential Salt
1172 Fractionation of Nuclei to Analyze Chromatin-associated Proteins from Cultured
1173 Mammalian Cells. *Bio-protocol* 7,
- 1174 22. Medvedeva, Y. A., Lennartsson, A., Ehsani, R., Kulakovskiy, I. V., Vorontsov, I. E.,
1175 Panahandeh, P., Khimulya, G., Kasukawa, T., and Drabløs, F. (2015) EpiFactors: A
1176 comprehensive database of human epigenetic factors and complexes. *Database*
1177 2015, 1–10
- 1178 23. Mierlo, G., Wester, R. A., and Marks, H. (2019) A Mass Spectrometry Survey of
1179 Chromatin-Associated Proteins in Pluripotency and Early Lineage Commitment.
1180 *Proteomics* 19, 1900047
- 1181 24. Christoforou, A., Mulvey, C. M., Breckels, L. M., Geladaki, A., Hurrell, T., Hayward, P.
1182 C., Naake, T., Gatto, L., Viner, R., Arias, A. M., and Lilley, K. S. (2016) A draft map of
1183 the mouse pluripotent stem cell spatial proteome. *Nat. Commun.* 7, 9992
- 1184 25. Xu, Y., Zhang, S., Lin, S., Guo, Y., Deng, W., Zhang, Y., and Xue, Y. (2016) OUP
1185 accepted manuscript. *Nucleic Acids Res.* 45, D264–D270
- 1186 26. Conway, J. R., Lex, A., and Gehlenborg, N. (2017) UpSetR: an R package for the
1187 visualization of intersecting sets and their properties. *Bioinformatics* 33, 2938–
1188 2940
- 1189 27. Spruijt, C. G., Luijsterburg, M. S., Menafrà, R., Stunnenberg, H. G., Van Attikum, H.,
1190 Correspondence, M. V., Lindeboom, R. G. H., Jansen, P. W. T. C., Ram, R. E.,
1191 Baltissen, M. P., Wiegant, W. W., Voelker-Albert, M. C., Matarese, F., Mensinga, A.,
1192 Poser, I., Vos, H. R., and Vermeulen, M. (2016) ZMYND8 Co-localizes with NuRD on
1193 Target Genes and Regulates Poly(ADP-Ribose)-Dependent Recruitment of
1194 GATAD2A/NuRD to Sites of DNA Damage. *CellReports* 17, 783–798
- 1195 28. Varier, R. A., Carrillo de Santa Pau, E., van der Groep, P., Lindeboom, R. G. H.,
1196 Matarese, F., Mensinga, A., Smits, A. H., Edupuganti, R. R., Baltissen, M. P., Jansen,
1197 P. W. T. C., Ter Hoeve, N., van Weely, D. R., Poser, I., van Diest, P. J., Stunnenberg, H.
1198 G., and Vermeulen, M. (2016) Recruitment of the Mammalian Histone-modifying
1199 EMSY Complex to Target Genes Is Regulated by ZNF131. *J. Biol. Chem.* 291, 7313–
1200 24
- 1201 29. Signolet, J., and Hendrich, B. (2015) The function of chromatin modifiers in
1202 lineage commitment and cell fate specification. *FEBS J.* 282, 1692–1702
- 1203 30. Hyun, K., Jeon, J., Park, K., and Kim, J. (2017) Writing, erasing and reading histone
1204 lysine methylations. *Exp. Mol. Med.* 49

- 1205 31. Holdsworth, R. J. (1991) Regeneration of the spleen and splenic
1206 autotransplantation. *Br. J. Surg.* 78, 270–278
- 1207 32. Tan, J. K. H., and Watanabe, T. (2018) Determinants of postnatal spleen tissue
1208 regeneration and organogenesis. *npj Regen. Med.* 3, 3–6
- 1209 33. Go, Y.-M., and Jones, D. P. (2017) Redox theory of aging: implications for health
1210 and disease. *Clin. Sci. (Lond).* 131, 1669–1688
- 1211 34. Haigis, M. C., and Yankner, B. A. (2010) The aging stress response. *Mol. Cell* 40,
1212 333–44
- 1213 35. Epel, E. S., and Lithgow, G. J. (2014) Stress biology and aging mechanisms: toward
1214 understanding the deep connection between adaptation to stress and longevity. *J.*
1215 *Gerontol. A Biol. Sci. Med. Sci.* 69 Suppl 1, S10–6
- 1216 36. Postnikoff, S. D. L., and Harkness, T. A. A. (2012) Mechanistic insights into aging,
1217 cell-cycle progression, and stress response. *Front. Physiol.* 3, 183
- 1218 37. Chandler, H., and Peters, G. (2013) Stressing the cell cycle in senescence and
1219 aging. *Curr. Opin. Cell Biol.* 25, 765–771
- 1220 38. Koziol, J. A. (2010) Comments on the Rank Product Method for Analyzing
1221 Replicated Experiments. *FEBS Lett.* 584, 941
- 1222 39. Rank products: a simple, yet powerful, new method to detect differentially
1223 regulated genes in replicated microarray experiments (2004) *FEBS Lett.* 573, 83–
1224 92
- 1225 40. Turner, V. M., and Mabbott, N. A. (2017) Influence of ageing on the
1226 microarchitecture of the spleen and lymph nodes. *Biogerontology* 18, 723–738
- 1227 41. Oliviero, G., Brien, G. L., Watson, A., Streubel, G., Jerman, E., Andrews, D., Doyle, B.,
1228 Munawar, N., Wynne, K., Crean, J., Bracken, A. P., and Cagney, G. (2016) Dynamic
1229 protein interactions of the Polycomb Repressive Complex 2 during differentiation
1230 of pluripotent cells. *Mol. Cell. Proteomics*, mcp.M116.062240
- 1231 42. Tacutu, R., Craig, T., Budovsky, A., Wuttke, D., Lehmann, G., Taranukha, D., Costa, J.,
1232 Fraifeld, V. E., and De Magalhães, J. P. (2013) Human Ageing Genomic Resources:
1233 Integrated databases and tools for the biology and genetics of ageing. *Nucleic*
1234 *Acids Res.* 41, 1027–1033
- 1235 43. Kerepesi, C., Daróczy, B., Sturm, Á., Vellai, T., and Benczúr, A. (2018) Prediction
1236 and characterization of human ageing-related proteins by using machine
1237 learning. *Sci. Rep.* 8, 1–13
- 1238 44. Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J.,
1239 Simonovic, M., Roth, A., Santos, A., Tsafou, K. P., Kuhn, M., Bork, P., Jensen, L. J., and
1240 von Mering, C. (2015) STRING v10: protein–protein interaction networks,
1241 integrated over the tree of life. *Nucleic Acids Res.* 43, D447–D452
- 1242 45. Szklarczyk, D., Morris, J. H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., Santos, A.,
1243 Doncheva, N. T., Roth, A., Bork, P., Jensen, L. J., and von Mering, C. (2017) The
1244 STRING database in 2017: quality-controlled protein-protein association
1245 networks, made broadly accessible. *Nucleic Acids Res.* 45, D362–D368
- 1246 46. Douet, J., Corujo, D., Malinverni, R., Renauld, J., Sansoni, V., Posavec Marjanović, M.,

- 1247 Cantariño, N., Valero, V., Mongelard, F., Bouvet, P., Imhof, A., Thiry, M., and
1248 Buschbeck, M. (2017) MacroH2A histone variants maintain nuclear organization
1249 and heterochromatin architecture. *J. Cell Sci.* 130, 1570–1582
- 1250 47. Barrero, M. J., Sese, B., Martí, M., and Belmonte, J. C. I. (2013) Macro histone
1251 variants are critical for the differentiation of human pluripotent cells. *J. Biol.*
1252 *Chem.* 288, 16110–16116
- 1253 48. Garfinkel, B. P., Melamed-Book, N., Anuka, E., Bustin, M., and Orly, J. (2015)
1254 HP1BP3 is a novel histone H1 related protein with essential roles in viability and
1255 growth. *Nucleic Acids Res.* 43, 2074–2090
- 1256 49. Garfinkel, B. P., Arad, S., Neuner, S. M., Netser, S., Wagner, S., Kaczorowski, C. C.,
1257 Rosen, C. J., Gal, M., Soreq, H., and Orly, J. (2016) HP1BP3 expression determines
1258 maternal behavior and offspring survival. *Genes, Brain Behav.* 15, 678–688
- 1259 50. Saksouk, N., Simboeck, E., Déjardin, J., Song, I., Sullivan, B., and Plotnikova, O.
1260 (2015) Constitutive heterochromatin formation and transcription in mammals.
1261 *Epigenetics Chromatin* 8, 3
- 1262 51. Kim, J., Hake, S. B., and Roeder, R. G. (2005) The Human Homolog of Yeast BRE1
1263 Functions as a Transcriptional Coactivator through Direct Activator Interactions.
1264 *Mol. Cell* 20, 759–770
- 1265 52. Gillette, T. G., and Hill, J. A. (2015) Readers, writers, and erasers: chromatin as the
1266 whiteboard of heart disease. *Circ. Res.* 116, 1245–53
- 1267 53. Mei, Z., Zhang, X., Yi, J., Huang, J., He, J., and Tao, Y. (2016) Sirtuins in metabolism,
1268 DNA repair and cancer. *J. Exp. Clin. Cancer Res.* 35, 1–14
- 1269 54. MASSÉ, S., SEVAPTSIDIS, E., PARSON, I. D., and DOWNAR, E. (1991) A Three-
1270 Dimensional Display for Cardiac Activation Mapping. *Pacing Clin. Electrophysiol.*
1271 14, 538–545
- 1272 55. Kupis, W., Pałyga, J., Tomal, E., and Niewiadomska, E. (2016) The role of sirtuins
1273 in cellular homeostasis. *J. Physiol. Biochem.* 72, 371–380
- 1274 56. Vaquero, A. (2009) The conserved role of sirtuins in chromatin regulation. *Int. J.*
1275 *Dev. Biol.* 53, 303–322
- 1276 57. Li, M., Valsakumar, V., Poorey, K., Bekiranov, S., and Smith, J. S. (2013) Genome-
1277 wide analysis of functional sirtuin chromatin targets in yeast. *Genome Biol.* 14,
1278 R48
- 1279 58. Rajendran, R., Garva, R., Krstic-Demonacos, M., and Demonacos, C. (2011)
1280 Sirtuins: Molecular traffic lights in the crossroad of oxidative stress, chromatin
1281 remodeling, and transcription. *J. Biomed. Biotechnol.* 2011
- 1282 59. Saez, I., and Vilchez, D. (2014) The Mechanistic Links Between Proteasome
1283 Activity, Aging and Age-related Diseases. *Curr. Genomics* 15, 38–51
- 1284 60. McCann, T. S., and Tansey, W. P. (2014) Functions of the proteasome on
1285 chromatin. *Biomolecules* 4, 1026–1044
- 1286 61. Murgia, M., Toniolo, L., Nagaraj, N., Ciciliot, S., Vindigni, V., Schiaffino, S., Reggiani,
1287 C., and Mann, M. (2017) Single Muscle Fiber Proteomics Reveals Fiber-Type-
1288 Specific Features of Human Muscle Aging. *Cell Rep.* 19, 2396–2409

- 1289 62. Grozinger, C. M., Schreiber, S. L., Sternglanz, R., Xu, R.-L., Broach, J. R., Starai, V. J.,
1290 Avalos, J. L., Escalante-Semerena, J. C., Grubmeyer, C., Wolberger, C., and al., et
1291 (2002) Deacetylase enzymes: biological functions and the use of small-molecule
1292 inhibitors. *Chem. Biol.* 9, 3–16
- 1293 63. Drazic, A., Myklebust, L. M., Ree, R., and Arnesen, T. (2016) The world of protein
1294 acetylation. *Biochim. Biophys. Acta - Proteins Proteomics* 1864, 1372–1401
- 1295 64. Hu, D.-X., Liu, X.-B., Song, W.-C., and Wang, J.-A. (2016) Roles of SIRT3 in heart
1296 failure: from bench to bedside. *J. Zhejiang Univ. Sci. B* 17, 821–830
- 1297 65. Ahn, B.-H., Kim, H.-S., Song, S., Lee, I. H., Liu, J., Vassilopoulos, A., Deng, C.-X., and
1298 Finkel, T. (2008) A role for the mitochondrial deacetylase Sirt3 in regulating
1299 energy homeostasis. *Proc. Natl. Acad. Sci. U. S. A.* 105, 14447–52
- 1300 66. Gamper, A. M., Kim, J., and Roeder, R. G. (2009) The STAGA Subunit ADA2b Is an
1301 Important Regulator of Human GCN5 Catalysis. *Mol. Cell. Biol.* 29, 266–280
- 1302 67. Xu, W., Edmondson, D. G., Evrard, Y. A., Wakamiya, M., Behringer, R. R., and Roth,
1303 S. Y. (2000) Loss of Gcn5l2 leads to increased apoptosis and mesodermal defects
1304 during mouse development. *Nat. Genet.* 26, 229–232
- 1305 68. Lin, W., Srajer, G., Evrard, Y. A., Phan, H. M., Furuta, Y., and Dent, S. Y. R. (2007)
1306 Developmental potential of Gcn5^{-/-} embryonic stem cells in vivo and in vitro.
1307 *Dev. Dyn.* 236, 1547–1557
- 1308 69. Finley, D. (2009) Recognition and Processing of Ubiquitin-Protein Conjugates by
1309 the Proteasome. *Annu. Rev. Biochem.* 78, 477–513
- 1310 70. Schmidt, M., and Finley, D. (2014) Regulation of proteasome activity in health and
1311 disease. *Biochim. Biophys. Acta - Mol. Cell Res.* 1843, 13–25
- 1312 71. Lecker, S. H., Goldberg, A. L., and Mitch, W. E. (2006) Protein degradation by the
1313 ubiquitin-proteasome pathway in normal and disease states. *J. Am. Soc. Nephrol.*
1314 17, 1807–19
- 1315 72. Tanaka, K. (2009) The proteasome: overview of structure and functions. *Proc. Jpn.*
1316 *Acad. Ser. B. Phys. Biol. Sci.* 85, 12–36
- 1317 73. Adams, J. (2003) The proteasome: structure, function, and role in the cell. *Cancer*
1318 *Treat. Rev.* 29 Suppl 1, 3–9
- 1319 74. Bulteau, A. L., Petropoulos, I., and Friguet, B. (2000) Age-related alterations of
1320 proteasome structure and function in aging epidermis. *Exp. Gerontol.* 35, 767–77
- 1321 75. Carrard, G., Dieu, M., Raes, M., Toussaint, O., and Friguet, B. (2003) Impact of
1322 ageing on proteasome structure and function in human lymphocytes. *Int. J.*
1323 *Biochem. Cell Biol.* 35, 728–39
- 1324 76. Petropoulos, I., Conconi, M., Wang, X., Hoenel, B., Brégégère, F., Milner, Y., and
1325 Friguet, B. (2000) Increase of Oxidatively Modified Protein Is Associated With a
1326 Decrease of Proteasome Activity and Content in Aging Epidermal Cells. *J. Gerontol.*
1327 *Biol. Sci. Am.* 55, 220–227
- 1328 77. Bardag-Gorce, F., Farout, L., Veyrat-Durebex, C., Briand, Y., and Briand, M. (1999)
1329 Changes in 20S proteasome activity during ageing of the LOU rat. *Mol. Biol. Rep.*
1330 26, 89–93

- 1331 78. Chondrogianni, N., and Gonos, E. S. (2010) Proteasome function determines
1332 cellular homeostasis and the rate of aging. *Adv. Exp. Med. Biol.* 694, 38–46
- 1333 79. Murgia, M., Toniolo, L., Nagaraj, N., Ciciliot, S., Vindigni, V., Schiaffino, S., Reggiani,
1334 C., and Mann, M. (2017) Single Muscle Fiber Proteomics Reveals Fiber-Type-
1335 Specific Features of Human Muscle Aging. *Cell Rep.* 19, 2396–2409
- 1336 80. Lang, F., Khaghani, S., Türk, C., Wiederstein, J. L., Hölper, S., Pillar, T., Nogara, L.,
1337 Blaauw, B., Günther, S., Müller, S., Braun, T., and Krüger, M. (2018) Single Muscle
1338 Fiber Proteomics Reveals Distinct Protein Changes in Slow and Fast Fibers during
1339 Muscle Atrophy. *J. Proteome Res.* 17, 3333–3347
- 1340 81. Farrants, A. K. Ö. (2008) Chromatin remodelling and actin organisation. *FEBS Lett.*
1341 582, 2041–2050
- 1342 82. Walther, D. M., and Mann, M. (2011) Accurate quantification of more than 4000
1343 mouse tissue proteins reveals minimal proteome changes during aging. *Mol. Cell.*
1344 *Proteomics* 10,
- 1345 83. Shavlakadze, T., Morris, M., Fang, J., Wang, S. X., Zhu, J., Zhou, W., Tse, H. W.,
1346 Mondragon-Gonzalez, R., Roma, G., and Glass, D. J. (2019) Age-Related Gene
1347 Expression Signature in Rats Demonstrate Early, Late, and Linear Transcriptional
1348 Changes from Multiple Tissues. *Cell Rep.* 28, 3263–3273.e3
- 1349 84. Rappsilber, J., Ishihama, Y., and Mann, M. (2003) Stop and Go Extraction Tips for
1350 Matrix-Assisted Laser Desorption/Ionization, Nanoelectrospray, and LC/MS
1351 Sample Pretreatment in Proteomics. *Anal. Chem.* 75, 663–670
- 1352 85. Silva, J. C., Gorenstein, M. V., Li, G.-Z., Vissers, J. P. C., and Geromanos, S. J. (2006)
1353 Absolute Quantification of Proteins by LCMS^E. *Mol. Cell. Proteomics* 5, 144–156
- 1354 86. Ferguson, R. E., Carroll, H. P., Harris, A., Maher, E. R., Selby, P. J., and Banks, R. E.
1355 (2005) Housekeeping proteins: A preliminary study illustrating some limitations
1356 as useful references in protein expression studies. *Proteomics* 5, 566–571
- 1357 87. Korthauer, K., Kimes, P. K., Duvallet, C., Reyes, A., Subramanian, A., Teng, M.,
1358 Shukla, C., Alm, E. J., and Hicks, S. C. (2019) A practical guide to methods
1359 controlling false discoveries in computational biology. *Genome Biol.* 20, 118
- 1360 88. Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., and
1361 Cox, J. (2016) The Perseus computational platform for comprehensive analysis of
1362 (prote)omics data. *Nat. Methods*, 13(9):731-40

1363

Tissue isolation

Chromatin extraction

Quantitative proteomics (LC-MS/MS)

Protein digestion

Data analysis

Mouse Proteome

Uni ProtKB
database

Chromatin
proteome
&
Statistical
analysis

Regulated proteins

FIGURE 2

A

B

C

A

FIGURE 4

A

B

C

FIGURE 5

A

B

C

FIGURE 6

FIGURE 7

A

B

S1

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)

S2

BRAIN

S3

A

C

B

D

E

F

Human ageing-related proteins