

Accurate Quantification of More Than 4000 Mouse Tissue Proteins Reveals Minimal Proteome Changes During Aging*[§]

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The biological process of aging is believed to be the result of an accumulation of cellular damage to biomolecules. Although there are numerous studies addressing mutation frequencies, morphological or transcriptional changes in aging mammalian tissues, few have measured global changes at the protein level. Here, we present an in depth proteomic analysis of three brain regions as well as heart and kidney in mice aged 5 or 26 months, using stable isotope labeling of whole animals (SILAC mouse) and high resolution mass spectrometry. In the frontal cortex and hippocampal regions of the brain, more than 4200 proteins were quantitatively compared between age groups. Proteome differences between individual mice were observable within and between age groups. However, mean protein abundance changes of more than twofold between young and old mice were detected in less than 1% of all proteins and very few of these were statistically significant. Similar outcomes were obtained when comparing cerebellum, heart, and kidney between age groups. Thus, unexpectedly, our results indicate that aging-related effects on the tissue proteome composition at the bulk level are only minor and that protein homeostasis remains functional up to a relatively high age. *Molecular & Cellular Proteomics* 10: 10.1074/mcp.M110.004523, 1–7, 2011.

Aging in higher organisms is a multifactorial process. It is commonly believed that lifespan is restricted because of the accumulation of cellular damage, ultimately interfering with crucial biological functions. In mammals, hallmarks of aging tissues include declining rates of self renewal capability and accumulating damages to DNA, proteins, and lipids (1, 2). Senescence in mice has been associated with transcriptional deregulation and an increased mutational burden. Interestingly, different tissues are not affected to the same extent - for example, mutation rates appear to be lower in brain (3–5). Aging-related changes in gene expression in the mouse brain have already been investigated by microarrays (6–9),

however, it is important to study the impact of senescence directly at the protein level to include the effects of post-transcriptional events such as translational regulation or altered protein degradation.

In recent years, great progress has been made in the field of high resolution mass spectrometry (MS)-based proteomics, now allowing for accurate identification of thousands of proteins (10–13). Since MS is not inherently quantitative, the majority of quantification methods rely on the simultaneous comparison of signal intensities between two or more samples during a single analysis following stable isotope coded labeling of peptides (14–16). For proteomic analysis of tissue samples, chemical labeling strategies such as the isotope-coded affinity tag (ICAT) (17) and the isobaric tag for relative and absolute quantification (iTRAQ) (18) have been widely applied. As an alternative, metabolic protein labeling approaches of mammalian model organisms *in vivo* have been described (19, 20). For example, full incorporation of ¹⁵N into the proteome of rats has been achieved by an isotope-pure diet (21). Arguably the most accurate method of protein quantitation by MS is stable isotope labeling with amino acids in cell culture (SILAC)¹ in which only heavy isotope containing derivatives of specific amino acids are used (22). This concept has recently been extended to mice to allow for quantitative comparison of tissue samples from *in vivo* experiments (23).

To date, few proteomics studies have investigated aging in mammalian tissues. Effects of senescence on the left rat heart ventricle was addressed using two-dimensional gel electrophoresis or iTRAQ labeling and matrix-assisted laser desorption/ionization (MALDI)-based quantitative mass spectrometry in which differential expression of metabolic enzymes, structural and antioxidant proteins were reported (24–26). Very recently, Mao *et al.* published a two-dimensional gel-based time course analysis of aging mouse brain. The authors suggest that aging is associated with a reduction in abundance of proteasomal subunits and an accumulation of non-functional proteins (27). In general, the depth and reliability of quantification of the above proteome studies was low because of technical limitations of the methods used.

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¹ The abbreviations used are: SILAC, stable isotope labeling by amino acids in cell culture; LTQ, linear trap quadrupole; MS/MS, tandem mass spectrometry.

Here, we took advantage of the SILAC mouse technology and high resolution MS to study global effects of aging in mammalian tissues at the protein level. Using this technology, we compared the proteomes of each of four female C57BL/6JN mice aged 5 or 26 months and obtained highly accurate quantification over a broad range of tissues.

EXPERIMENTAL PROCEDURES

Mouse Tissues—Female C57BL/6JN mice aged 5 or 26 months, fed *ad libitum*, were obtained from the Aged Rodent Colonies (National Institute of Aging, Bethesda, MD). Animals were starved overnight and sacrificed by cervical dislocation. Brains were immediately removed and placed in ice cold PBS. The frontal cortex region was isolated by removal of olfactory tracts and cutting 1 mm posterior to the bregma. Subsequently, hippocampus and cerebellum were collected. Remaining corpses were perfused by injection of PBS into the heart before removal of kidneys and heart. All tissues and organs were washed in cold PBS and shock frozen in liquid nitrogen. For SILAC labeled standards, animals were fed for four generations with a diet containing exclusively $^{13}\text{C}_6$ lysine as previously described (23). Corresponding tissues were obtained from two 12-month-old females with 97.9% incorporation rate of heavy lysine. SILAC tissues were pooled from both animals before processing. All tissue samples were stored at -80°C until use.

Lysate Preparation and Protein Digestion—Tissues were blended with an Ultra-Turrax disperser (IKA, Staufen, Germany) in 150 mM Tris/HCl pH 8, 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride at 4°C . Hearts were ground in the frozen state in a mortar before homogenization. Immediately following homogenization, SDS was added to a final concentration of 4% (w/w) and samples were incubated at 95°C for 3 min. For shearing of DNA, samples were subjected to treatment with a Bioruptor ultra sonication bath (Diagenode, Liège, Belgium) at high energy setting for 10 min. Samples were heated again for 3 min at 95°C and clarified by centrifugation for 10 min at $20,000 \times g$. Protein content was determined using the BCA Protein Assay Kit (Thermo, Rockford, IL) according to the manufacturer's instructions. For frontal cortex and hippocampus, 100 μg protein from each animal were mixed with an identical amount of the corresponding SILAC labeled standard. For cerebellum and kidney, lysates were pooled within the young or old animal groups before mixing with SILAC standard. Reduction of disulfide bridges was achieved by addition of dithiothreitol to a final concentration of 0.1 M followed by incubation at 75°C for 5 min. Further processing for in-solution digestion was performed using the previously described filter-aided sample preparation method (28) with Microcon YM-30 devices (Millipore, Billerica, MA), but with the following minor modifications: Alkylation was carried out with 2-chloroacetamide instead of 2-iodoacetamide and proteins were digested exclusively with endoproteinase LysC (Wako Bioproducts, Richmond, VA) in 2 M urea, 25 mM Tris/HCl, pH 8 overnight at room temperature. Obtained peptides were acidified with trifluoroacetic acid and desalted via C_{18} solid phase extraction cartridges (3M, St. Paul, MN). Peptide mixtures were measured both directly and following fractionation into six fractions via strong anion exchange chromatography according to published procedures (29).

Mass Spectrometry—Liquid chromatography (LC)-MS experiments were essentially performed as described previously (30, 31). Briefly, reversed phase separation of peptides was performed using an Easy nLC nanoflow HPLC system (Proxeon Biosystems, Odense, Denmark now Thermo Fisher Scientific). Peptide mixtures were loaded onto a column with 15 cm length and $75\ \mu\text{m}$ inner diameter, packed in-house with RepoSil-Pur C_{18} -AQ $3\ \mu\text{m}$ resin (Dr. Maisch, Ammerbuch-Entringen, Germany) at $0.7\ \mu\text{m}/\text{min}$. Peptides were then eluted in fraction-

optimized nonlinear gradient from 3% to 60% acetonitrile in 0.5% acetic acid over a duration of 200 min. Eluting peptides were electrosprayed online via a nanospray ion source (Proxeon Biosystems) at a voltage of 2.2 kV into an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), except for unfractionated frontal cortex samples, which were acquired using an LTQ-FT mass spectrometer (Thermo Fisher Scientific). Survey scans were performed in the Orbitrap analyzer at a resolution of 60,000 at target values of 1,000,000 ions and maximum allowed fill times of 1 s over a mass range between m/z 350–1750. The 10 most intense peaks were subjected to fragmentation via collision induced dissociation in the LTQ. For each scan, 5000 ions were accumulated over a maximum allowed fill time of 250 ms and fragmented by wideband activation. Exclusion of precursor ion masses over a time window of 150 s was used to suppress repeated fragmentation of peaks. In all MS experiments except for the measurements of the hippocampus proteomes, internal lock mass recalibration was disabled and an Active Background Ion Repression Device (ABIRD, ESI Source Solutions, Woburn, MA) was used to increase the signal to noise ratio.

Data Analysis—Raw data consisting of 154 liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) files are deposited at Tranche and are freely available upon publication. Hash keys are provided in the [Supplemental Materials](#) section. Raw data was analyzed using the MaxQuant software environment, version 1.1.0.25. Retention time dependent mass recalibration was applied and peak lists were searched against a database containing all 56,729 entries from the International Protein Index mouse protein database version 3.68 and 255 frequently observed contaminants as well as reversed sequences of all entries. Searches were performed with the following settings: Precursor and fragment ion peaks were searched with an initial mass tolerance of 7 ppm and 0.5 Th, respectively. Enzyme specificity was set to LysC, additionally allowing cleavage between lysine and proline. Up to two missed cleavages were allowed and only peptides with at least six amino acids in length were considered. Carbamidomethylcysteine was set as a fixed modification whereas oxidation on methionine was set as a variable modification. Up to two missed cleavages were allowed. Precursor masses of already identified peptides were further searched within a 3 min time window in chromatograms derived from corresponding and adjacent peptide fractions (“match between runs” option in MaxQuant). For reliability estimation of peptide identifications, the posterior error probability for each top scoring hit was calculated. This metric is based on the tandem MS (MS/MS) score but additionally takes into account peptide length dependent histograms of forward and reverse hits to assess the probability of a false identification using the Bayes theorem (described in detail in (32)). Using a decoy database strategy (33), peptide identifications were accepted based on their posterior error probability until less than 1% reverse hits were retained in the list. Accepted peptide sequences were subsequently assembled into proteins in ascending order of their posterior error probability up to false discovery rate of 1% at the protein level. For successful protein identifications, at least two peptides and one peptide with a unique peptide sequence were required. If no unique peptide sequence to a single database entry was identified, the resulting protein identification was reported as an indistinguishable “protein group.” Protein quantifications were based on the median SILAC ratios of at least two peptides (two valid “ratio counts”) in each biological sample. Quantification of SILAC pairs was performed by MaxQuant with standard settings (32). Briefly, centroids of isotope clusters in the intensity- m/z plain were detected over multiple full scans and the median intensity ratios were used for the calculation of SILAC ratios.

For histogram representations, only proteins with at least one quantification per age group were considered. Mean normalized protein ratios between sample and SILAC standard were then calculated

within age groups and histograms were plotted using the R statistical programming language (34). For calculation of correlation coefficients and principal component analyses, datasets were filtered for entries with valid quantifications in all experiments (see above). Principal component analyses were performed using the Gene Expression Similarity Investigation Suite (Genesis) (35) following mean centering of expression data. Two-sided t-tests were carried out after filtering for proteins with at least three out of four quantifications under the assumption of unequal variance between age groups. Significance thresholds were then calculated via a permutation-based false discovery rate estimation of 1% (36). For comparison of microarray and proteome studies, median intensities for probes corresponding to Uniprot annotations of identified protein groups were calculated before statistical analyses analogous to the proteome data.

RESULTS

A Proteomic Screen to Detect Proteomic Changes With Aging in Mice—To quantify age-related proteome changes in mouse tissue, we used the SILAC technology *in vivo* and coupled it to high-resolution LC-MS/MS (Fig. 1). We labeled mice with a diet containing exclusively $^{13}\text{C}_6$ lysine over several generations and obtained virtually complete incorporation. To exclude potential effects because of the SILAC diet, we performed all experiments in a “spike-in” format, using the tissue from the SILAC mice as a common internal standard for all experiments. This design also allows comparison between multiple animals within and between each age group. For each experiment, equal amounts of protein sample from biological replicates and the corresponding SILAC standard were mixed, digested in solution with endoproteinase LysC using the filter-aided sample preparation method and peptides were fractionated via strong anion exchange chromatography (28, 29). These peptide fractions as well as unfractionated peptide preparations from each experiment were then analyzed by LC-MS/MS on a hybrid high resolution linear ion trap Orbitrap instrument (Fig. 1). Hippocampal and frontal cortex regions from the brains of four young and four old animals were processed individually, resulting in eight individual quantitative proteomes. We extended the study to cerebellum, kidney, and heart muscle but in contrast to the previous experiments, lysates within age groups were pooled for each of these three tissues.

The combined dataset over all five tissues comprises 154 LC-MS/MS experiments with 4 h gradients, during which more than 4.5 million MS/MS scans were acquired. The observed average absolute mass deviation for the corresponding precursor ions was 450 ppb. Following analysis with the MaxQuant software environment (32), more than 60% of the MS/MS scans were unambiguously identified. This led to 44,737 identified nonredundant peptide sequences and 5619 proteins (or protein groups) at a protein false discovery rate of less than 1%.

The Aging Tissue Proteomes of Frontal Cortex and Hippocampus—In frontal cortex and hippocampus, expression data for more than 4200 proteins of each four young and old mice were acquired individually. Of this subset of proteins, approximately two thirds were expressed and detected in at

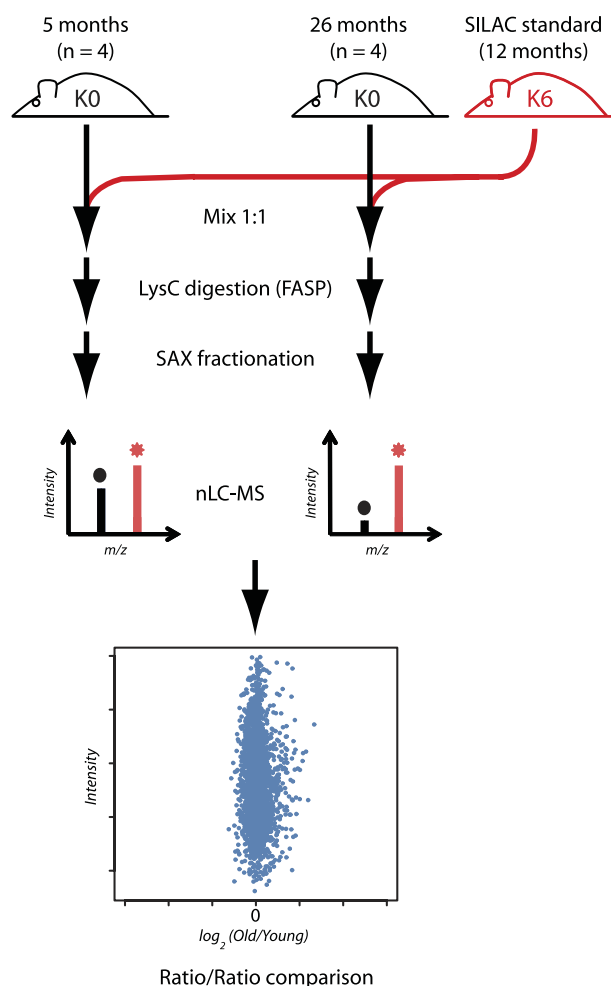


FIG. 1. Schematic of the workflow. Tissue lysates from each of four mice aged 5 or 26 months were analyzed in separate experiments and mixed each time with an identical standard derived from SILAC mice aged 12 months. Proteins were digested using the filter-aided sample preparation method (FASP) and fractionated before analysis by high resolution mass spectrometry. Proteins were quantified by dividing the individual SILAC peptide ratios of the proteomes to be compared (“ratio of ratios”).

least three out of four biological replicate experiments per age group (77 or 61% in frontal cortex or hippocampus, respectively, [Supplemental Tables 1 and 2](#)).

For protein quantification, our study employed an internal standard, which was added to all biological samples of a given tissue, to serve as a fixed reference point for all observed peptide ratios. To determine the quantitative reliability of this data set in more detail, we first extracted the typical number of quantification events per protein. In the above data set of a young animal it turned out to be 18 events on average (median of 8). This is because of the high redundancy of peptide based quantifications and compares favorably to two-dimensional gel experiments in which typically only one quantification event per protein is obtained in each individual sample. As a consequence, when comparing proteomes of

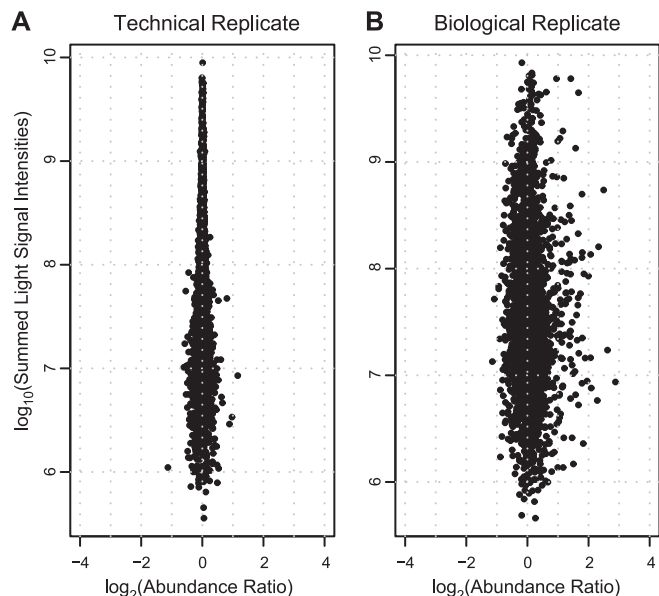


FIG. 2. Intensity-ratio distribution of protein abundances in hippocampus using an internal SILAC standard for quantitation between samples. *A*, Comparison between two technical replicates of a lysate derived from the same animal. *B*, Comparison between an old and a young animal.

two technical replicate experiments virtually all proteins were detected in equal quantities, demonstrating the usefulness of the method. This “ratio-of-ratios” distribution was narrow, with more than 99% of the proteins showing an apparent fold change of less than a factor of two (Fig 2A). In contrast, a much broader abundance ratio distribution was observed when comparing two different biological samples (Fig. 2B). The figure also shows that the few outlier proteins, on the most part, have a higher abundance in the aged animals. However, most these changes are not statistically significant (see below). Next, we investigated the mean protein expression changes between the two age groups. Unexpectedly, the vast majority of proteins were found in equal quantities in old and young animals (Fig. 3A). An average expression change of more than twofold was detected in less than 1% of all quantified proteins and less than 3% changed by more than one third in both brain tissues (Supplemental Table 3). These data indicate that age-related changes on the protein level in mice are very minor in the two analyzed brain regions.

Statistical Analysis of Senescence-Related Proteome Changes in Frontal Cortex and Hippocampus—We asked whether aging causes specific changes in tissue proteomes. For this purpose we calculated the Pearson correlation coefficients between all pairs of biological samples using the abundance ratios of all quantified proteins. Specific age related proteome changes should be reflected by higher Pearson correlation coefficients between the animals of one age group as compared with the coefficients when comparing old and young animals. However, the correlation between pro-

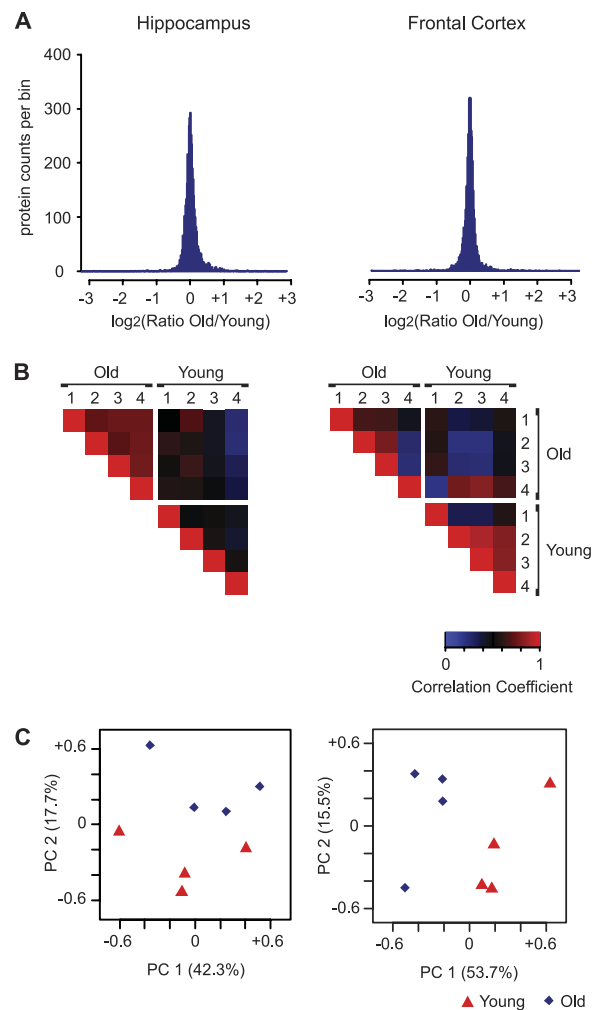


FIG. 3. A, Abundance of the vast majority of proteins is unchanged during aging in mice as indicated by a histogram representation of protein abundance ratios between old and young animals in hippocampus or frontal cortex. **B**, Pearson correlation between the proteomes of mice within the young or old age groups is not generally higher than between old and young individuals. Correlation coefficients in the matrix are color coded as shown. **C**, Principal component analysis of the individual proteomes.

teomes within young or aged animals was not generally higher than that between old and young mice (Fig. 3B). The differences between individual proteomes can be attributed to biological variability between the mice rather than to differences in sample preparation. This was apparent from the high degree of reproducibility obtained when analyzing each of three technical replicates of two selected hippocampal tissue lysates that were prepared and analyzed using the same protocol (Supplemental Fig. 1). To investigate the potential proteome differences between young and old mice by a different method we performed principal component analysis. The distances between the samples of the same age group was not generally smaller than the distances to samples from the other age group, confirming the findings ob-

tained by correlation analysis (Fig. 3C). Finally, statistical analysis of hippocampus and frontal cortex datasets via a two-sided *t* test at a permutation-based false discovery rate of 1% yielded only five significantly regulated proteins each (Supplemental Tables 1 and 2). Collectively, these results indicate that on the protein level of the two tissues, biological variability between individuals is more pronounced than changes induced by the process of aging, even in inbred mice with a matching genetic background.

The Aging Tissue Proteomes of Cerebellum, Heart, and Kidney—To investigate whether the absence of major changes in protein abundance is restricted to specific regions of the mouse brain, we additionally measured the proteomes of cerebellum, heart and kidney. In contrast to the previously described experiments, protein lysates within age groups of young or old animals were pooled, resulting in only two individual proteomes for each tissue. Similar to the situation in hippocampus and frontal cortex, the abundance of the vast majority of proteins was unaffected (Fig. 4, Supplemental Tables 3 and 4), therefore generalizing our findings to additional tissues.

Comparison with Other Large-Scale Studies—Age-related changes in transcript abundance in mammalian tissues have previously been investigated by microarrays. Although earlier investigations only reported a very small number of genes whose transcription changed during aging (6, 7), more recent publications did find a larger number of significantly regulated genes in mouse cortex (8, 9). However, in these latter studies, as in the proteome studies, the vast majority of transcripts did not change significantly. Furthermore, the transcripts that did appear to change during aging (8) generally did not exhibit corresponding changes in our quantitative proteomics dataset of frontal cortex (Supplemental Fig. 2). During the preparation of this manuscript a report appeared that addressed proteomic changes in the aging mouse brain by two-dimensional gel electrophoresis (27). The authors suggested significant changes in the abundance of several proteins such as proteasomal subunits, chaperones and mitochondrial proteins. Almost all these proteins were quantified in our data set in both the frontal cortex and the hippocampal datasets (111 out of 114 unique SwissProt identifiers). However, we did not detect any significant age-related changes of any of these proteins and their mean ratio changes were less than 5% (Supplemental Table 5).

As an orthogonal method, we substantiated the SILAC quantifications of selected candidate proteins by performing Western blots (Supplemental Fig. 3). We chose candidate proteins that appeared to change in aging mice in the above study (27), such as the mitochondrial protein VDAC1, the proteasomal subunit Psma5 and chaperones (calreticulin and Hsc71). As expected, the results verify our SILAC based finding that levels of these proteins remain virtually unchanged. We also obtained evidence that the few proteins that we did find to change are actually differentially expressed during aging.

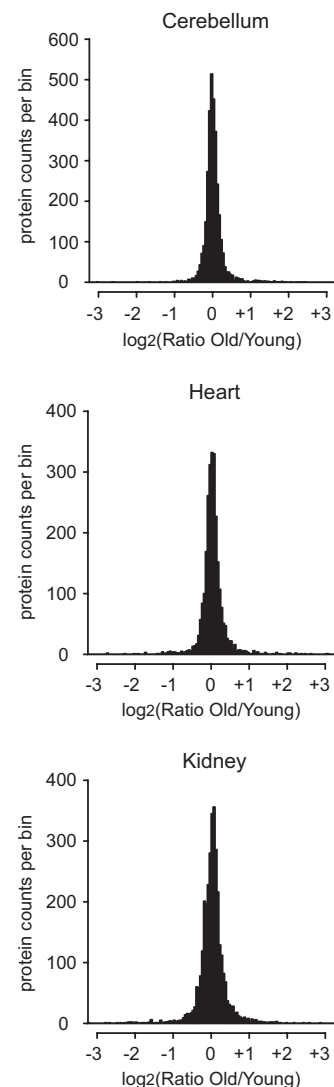


FIG. 4. Histograms of the protein abundance ratios between old and young animals in cerebellum, heart and kidney (representation analogous to Fig. 2A).

Specifically, the Western blot against complement C1q subcomponent subunit B shows an about twofold accumulation with age in hippocampus, as expected from the SILAC ratio of 2.25.

DISCUSSION

Using high resolution mass spectrometry combined with the SILAC mouse technology, we performed the most comprehensive study of mammalian tissue aging at the proteome level to date. Reaching a depth of more than 4000 quantified proteins, our study covers the vast majority of tissue protein mass. Strikingly, our results indicate that abundance changes are low at least up to 26 months, an age at which less than half of the initial population survives (37). It should be noted that many low abundant proteins are not covered by our dataset. Nevertheless, if regulatory pathways in control of synthesis or

degradation of specific proteins subsets were affected in aging tissues, global changes at a larger scale would have been observed. Technological limitations are not likely to be the reason for not detecting profound proteome changes. Previous studies from our and other laboratories have demonstrated that SILAC-based quantitative proteomics is a sensitive method to measure effects on protein expression in knockout mice (23) or in the study of global differences in protein abundance between primary cells and cell lines from mice (12, 13, 38). Likewise, knockdowns of single genes can result in easily detectable abundance changes of hundreds of proteins (39). Finally, the recently described super-SILAC strategy, which also uses a complex SILAC-labeled proteome as an internal standard for tissue proteome quantification, readily detected the expected differences between tumor types (40). These studies clearly demonstrate that our experimental approach was well suited for the discovery of global proteomic changes in mice with aging.

We specifically focused on two brain regions, frontal cortex and hippocampus, for which acquisition of the proteomes of individual animals allowed us to perform statistical analysis of protein abundance between age groups. Multiple studies have addressed the effects of aging on the transcriptional level in mouse brain by microarrays (6–9). The overlap of significantly regulated candidate genes between individual studies was often low and the vast majority of mRNAs clearly did not change in abundance with age. Therefore, our observation of the absence of large scale gene expression changes at the proteome level generally reflects previous results at the level of the transcriptome.

In conclusion, this study demonstrates that expression levels of the vast majority of proteins remain virtually unchanged during aging in multiple mouse tissues. This suggests that the proteome is efficiently maintained to a relatively high age. As our data are averages obtained from a large number of cells, they do not exclude strong age-related variability between individual (5) or specific sub-populations of cells, such as tissue stem cells. As proteomic technology advances, it would be interesting to directly investigate these potential cell specific effects of aging.

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☒ This article contains [supplemental Figs 1–3 and Tables 1–5](#).

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