Global Protein Oxidation Profiling Suggests Efficient Mitochondrial Proteome Homeostasis During Aging*§

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The free radical theory of aging is based on the idea that reactive oxygen species (ROS) may lead to the accumulation of age-related protein oxidation. Because the majority of cellular ROS is generated at the respiratory electron transport chain, this study focuses on the mitochondrial proteome of the aging model Podospora anserina as target for ROS-induced damage. To ensure the detection of even low abundant modified peptides, separation by long gradient nLC-ESI-MS/MS and an appropriate statistical workflow for iTRAQ quantification was developed. Artificial protein oxidation was minimized by establishing gel-free sample preparation in the presence of reducing and iron-chelating agents. This first large scale, oxidative modification-centric study for P. anserina allowed the comprehensive quantification of 22 different oxidative amino acid modifications, and notably the quantitative comparison of oxidized and nonoxidized protein species. In total 2341 proteins were quantified. For 746 both protein species (unmodified and oxidatively modified) were detected and the modification sites determined. The data revealed that methionine residues are preferably oxidized. Further prominent identified modifications in decreasing order of occurrence were carbonylation as well as formation of N-formylkynurenine and pyrrolidinone. Interestingly, for the majority of proteins a positive correlation of changes in protein amount and oxidative damage were noticed, and a general decrease in protein amounts at late age. However, it was discovered that few proteins changed in oxidative damage in accordance with former reports. Our data suggest that P. anserina is efficiently capable to counteract ROS-induced protein damage during aging as long as protein de novo synthesis is functioning, ultimately leading to an overall constant relationship between damaged and undamaged protein species. These findings contradict a massive increase in protein oxidation during aging and rather suggest a protein damage homeostasis mechanism even at late age. Molecular & Cellular Proteomics 15: 10.1074/mcp.M115.055616, 1692–1709, 2016.

Reactive oxygen species (ROS)¹ are highly reactive intermediates leading to oxidative damage of virtually all biomolecules (1, 2). Mitochondria are known as the main source of endogenous ROS, mainly generated as by-products of oxidative phosphorylation (OXPHOS) at complexes I and III of the respiratory electron transport chain (3, 4). Consequently, mitochondria are inevitably the most prominent target of ROS-induced damage (5). The accumulation of oxidatively damaged macromolecules, particularly proteins, has critical consequences, for example on mitochondrial structure and activity of the respiratory chain (6–8). Accordingly, ROS are involved in several diseases (9, 10), and the free radical theory of aging postulates that the cumulative ROS-induced damage plays a causative role in aging (11, 12). From the chemical and analytical point of view, oxidative damage of proteins is complex and leads to a variety of products, with the accumulation of irreversible oxidative protein modifications contributing to the development of disease (13, 14) and to aging (12, 15).

Although ROS inflicted damage has frequently been reported for higher organisms and humans (16, 17), their long lifespan and laborious molecular manipulation has drawn attention toward alternative model systems such as Podospora anserina. To ensure the mitochondrial proteome of the aging model Podospora anserina as target for ROS-induced damage, this study focuses on the mitochondrial proteome of the aging model Podospora anserina as target for ROS-induced damage. To ensure the detection of even low abundant modified peptides, separation by long gradient nLC-ESI-MS/MS and an appropriate statistical workflow for iTRAQ quantification was developed. Artificial protein oxidation was minimized by establishing gel-free sample preparation in the presence of reducing and iron-chelating agents. This first large scale, oxidative modification-centric study for P. anserina allowed the comprehensive quantification of 22 different oxidative amino acid modifications, and notably the quantitative comparison of oxidized and nonoxidized protein species. In total 2341 proteins were quantified. For 746 both protein species (unmodified and oxidatively modified) were detected and the modification sites determined. The data revealed that methionine residues are preferably oxidized. Further prominent identified modifications in decreasing order of occurrence were carbonylation as well as formation of N-formylkynurenine and pyrrolidinone. Interestingly, for the majority of proteins a positive correlation of changes in protein amount and oxidative damage were noticed, and a general decrease in protein amounts at late age. However, it was discovered that few proteins changed in oxidative damage in accordance with former reports. Our data suggest that P. anserina is efficiently capable to counteract ROS-induced protein damage during aging as long as protein de novo synthesis is functioning, ultimately leading to an overall constant relationship between damaged and undamaged protein species. These findings contradict a massive increase in protein oxidation during aging and rather suggest a protein damage homeostasis mechanism even at late age. Molecular & Cellular Proteomics 15: 10.1074/mcp.M115.055616, 1692–1709, 2016.

¹ The abbreviations used are: ROS, reactive oxygen species; FA, formic acid; FASP, Filter-aided sample preparation; FDR, false discovery rate; Fwhm, full width at half maximum; HCD, higher-energy collisional dissociation; IAA, iodoacetamide; iTRAQ, isobaric tags for relative and absolute quantification; P. anserina, Podospora anserina; PSM, peptide spectrum match; PTM, posttranslational modification; SRM, selected reaction monitoring; TEAB, triethylammonium bicarbonate.
This fungus is a well-established model system in aging research because of its senescence syndrome and a short lifespan of ~25 days (18–21). Importantly, for P. anserina, it has been shown that in mitochondria the generation of ROS at the electron transport chain increases with age: For instance, the age-related accumulation of endogenous hydrogen peroxide is indicated by increased secretion of this ROS from old individuals. Deletion of a gene encoding for a mitochondrial fission factor leads to a strong increase in the healthy lifespan and goes along with a delay in hydrogen peroxide release in comparison to the wild type (22). The mutation of a nuclear gene, Grisea, coding for a transcription factor that is involved in the control of high affinity copper uptake leads to a switch from a copper-dependent standard respiration to an iron-dependent alternative respiration. This switch, because of a loss of respiratory complex III, a major generator of superoxide anion, leads to a decreased generation of this ROS (23). Although carbonylation of proteins visualized by the commonly used Western blot technique did not identify a prominent age-related change of carbonylated proteins in P. anserina (24), a strong decrease of carbonylated proteins was found in a strain in which a gene was overexpressed that encodes a mitochondrial methyltransferase which protects against ROS generation. The healthy lifespan of the corresponding transgenic strain was increased by 115% compared with that of the wild type (25). So far, a proteomic view to understand the effects of ROS-induced protein damage, such as carbonylation, in molecular detail during aging is missing.

Because of its high resolution power of (modified) proteins, 2D-electrophoresis has successfully been applied for the identification and quantification of oxidative protein modifications on the proteome level (26). Carbonylated proteins were detected with fluorophore-labeling, differential ProteoTope radioactive quantification (17, 27), and the immunochemical detection technique (25, 28–30). Despite their popularity, antibody and 2D gel-based methods for identification and quantification of oxidative protein damage have well-known limitations, such as under-representation of certain protein categories, limited dynamic range and comigration of proteins (31–33). In particular, these detection techniques allow only the analysis of one specific protein modification at a time. Furthermore, observed oxidative protein modifications by gel electrophoresis have to be interpreted with caution, because proteins can undergo artificial oxidation in polyacrylamide gels (34, 35). Because oxidation results in a specific mass shift, it can be precisely pinpointed with tandem mass spectrometry of intact proteins or their proteolytic digest (36). Hence, gel-free mass spectrometry analyses with previous enrichment step of low-abundant oxidized peptides have been developed (37). However, they are limited to only a few selected protein oxidations (38, 39).

In order to perform a large-scale, unbiased temporal analysis of prominent ROS-induced protein oxidations, we developed a gel-free quantitative proteomic workflow using chemical labeling of peptides with iTRAQ reagents (40, 41). This enables parallel quantification of protein species from mitochondria at different age stages; also, a beforehand data analysis with a novel statistical framework allows an interpretation and comparison of temporal trends of both oxidized and nonoxidized protein species to verify proteome homeostasis during increasing ROS exposure with age.

EXPERIMENTAL DESIGN AND STATISTICAL RATIONALE—

Growth of P. anserina and Isolation of Crude Mitochondria—The cultivation of the wild-type strain s and the isolation of crude mitochondrial fractions from P. anserina were performed as previously described (42). Cultures of six individuals serving as biological replicates were harvested at four different age stages (6 days, 9 days, 13 days and 16 days) resulting in a total of 24 mitochondrial samples.

Experimental Workflow for In-filter Protein Digestion and iTRAQ Labeling—The mitochondrial samples were processed according to the in-filter protein digestion (FASP II) procedure described by (43) with minor modifications. FASP combines the advantages of in-gel and in-solution digestion for mass spectrometry-based proteomics and enables the solubilization of crude mitochondria with SDS and detergent removal prior to LC-MS analysis. Because we focus on the mitochondrial proteome a solubilization step is inevitable for high protein coverage. Fifty micrograms of crude mitochondria were denatured and solubilized in SdT-lysis buffer by sonication for 3 min and subsequent shaking at RT for 30 min. Next, samples were mixed with 8 μM urea and prepared using the FASP II protocol. All solutions for FASP II included additionally 1 mM DTT and 1 mM EDTA to avoid artificial oxidation during sample processing. In addition, triethylammonium bicarbonate (TEAB) was used as a tertiary amine buffer instead of ammonium bicarbonate. After centrifugation the resulting peptides were acidified with 50% (v/v) ACN in 0.5% (v/v) TFA and dried using a SpeedVac. Digested and carbamidomethylated samples were labeled with iTRAQ 4plex tags according to the manufacturer’s protocol. Deviant from manufacturer’s protocol, peptide samples were labeled with half the amount of iTRAQ reagent after tryptic digestion for multiplexing all age stages from one of each biological replicate. Briefly, each iTRAQ reagent was dissolved in 70 μl ethanol and the aliquots of iTRAQ 114, 115, 116, and 117 were combined with the peptide mixtures of mitochondria from four different age stages (6, 9, 13 and 16 days), respectively. Consequently, for each experiment 35 μl of reagent solution was used for 50 μg of sample, which was resuspended in 25 μl iTRAQ dissolution buffer (0.5 mM TEAB). After 1.5 h of incubation at RT, labeling reaction was stopped with 10 mM glycine. The differently-labeled samples of all 4 age stages from one individual were pooled and dried again in a SpeedVac. Prior to nLC-MS analysis all samples were desalted by solid-phase extraction using Spec C18AR pipette tips according to the manufacturer’s protocol. To address any potential label bias, labels for all age stages of one biological replicate were swapped, too. The assignment of label tags to samples is summarized in Supplemental Table S1.

Nano-LC-MS/MS Analysis—All desalted samples were resuspended in 2% ACN in 0.1% FA (1 μg/μl) by sonication for 10 min prior to one-dimensional nLC-ESI-MS/MS analysis. Measurements were performed on a LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) coupled to a nanoACQUITY gradient UPLC pump system combined with an autosampler (all Waters, Milford, MA). The nanoACQUITY UPLC system was equipped with a reversed phase UPLC HSS T3 column (1.8 μm, 75 μm x 250 mm, Waters) and a PicoTip Emitter (Silica TipTM, 10 μm i.d, New Objective, Woburn, MA) as a nanospray source. Four microfilters of the sample were loaded directly onto the analytical column using the
TABLE I

| Amino acid | Product                        | Molecular formula | Monoisotopic mass shift |
|-----------|--------------------------------|-------------------|------------------------|
| Arg       | Glutamic semialdehyde          | −5H +1C −3N +1O   | −43.05343              |
| Arg       | +14 Daa                        | −2H +1O           | +13.97927              |
| Asp       | 3-hydroxyaspartic acid         | +1O               | +15.99492              |
| Cys       | Sulfenic acid                  | +2O               | +31.98983              |
| Cys       | S-nitrosylation              a  | H(−1) N O         | +28.990164             |
| His       | 2-oxohistidine                | +1O               | +15.99492              |
| His       | 4-hydroxyynonenal              | H(18) C(9) O(2)   | +156.115030            |
| Leu       | +14 Da a                       | −2H +1O           | +13.97927              |
| Leu       | 3-hydroxyecine                 | +1O               | +15.99492              |
| Met       | Methionine sulfoxide           | +1O               | +15.99492              |
| Met       | Methionine sulfone             | +2O               | +31.98983              |
| Phe       | Hydroxyphenylalanine           | +1O               | +15.99492              |
| Pro       | Pyrrolidinone                  | −2H −1C −1O       | −30.01057              |
| Pro       | Pyroglutamic acid              | −2H +1O           | +13.97927              |
| Pro       | Hydroxyproline                 | +1O               | +15.99492              |
| Thr       | 2-amino-3-ketobutyric acid     | −2H               | −2.01560               |
| Trp       | Kynurenine                     | −1C +1O           | +3.99490               |
| Trp       | Hydroxytryptophan              | +1O               | +15.99492              |
| Trp       | Hydroxykynurenine              | −1C +2O           | +19.98983              |
| Trp       | N-formylkynurenine             | +2O               | +31.98983              |
| Trp       | 6-nitrotryptophan              | −1H 1N +2O        | +44.98508              |
| Phe       | 2-nitrophenylalanine           | −1H 1N +2O        | +44.98508              |

* a Precise structure of these products is unknown.

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All examined single amino acid modifications induced by oxidative damage. Molecular formula and monoisotopic mass shift of the difference between the native amino acid and the oxidized product are given. Based on protein modifications for mass spectrometry (www.unimod.org).

Protein Identification and Quantification—MS data search was performed against a P. anserina protein database (version 6.32) from http://podospora.igmors.u-psud.fr/, containing 10612 sequences (44), using the SEQUEST algorithm embedded in Proteome-Discoverer 1.3.0.339 (Thermo Electron © 2008–2011). All acquired raw-files from three technical replicates of one biological replicate were combined in one data search, for which following search parameters were applied: (1) fully tryptic as enzyme specificity, (2) a maximum of two missed cleavages, (3) precursor ion mass tolerance of 5 ppm, (4) fragment ions mass tolerance of 1 Da, (5) Carbamidomethylation of Cys and (6) iTRAQ4plex(N-term) as fixed as well as (7) iTRAQ4plex(K,Y) as variable modifications. The S/N threshold of Peak Filters in the Orbitrap was set to 3. Of note, database searches for suspected protein modifications are limited in the Proteome-Discoverer 1.3.0.339 to four. Therefore, database searches had to be sequentially conducted considering different oxidative amino acid modifications as fixed modification to allow detection of all possible posttranslational modifications (PTMs). All searched oxidative modifications are specified in Table I. Protein quantification is performed with the Reporter Ions Quantifier tool embedded in the Proteome-Discoverer software. Settings were kept at the default values: (1) integration window tolerance of 20 ppm, (2) integration method of most confident centroid, (3) mass analyzer is the Orbitrap with (4) MS² order, and (5) HCD as activation type. Reporter based quantification is normalized by the protein ratio median. The factor normalizes all peptide ratios by the median protein ratio. Additionally, to minimize unwanted quantification of co-isolated peptides the allowed relative isolation interference was set to < 20% of precursor signal intensity. For the determination of the false discovery rate (FDR) a decoy database search was performed with the percolator validation in Proteome Discoverer. The q-value is the minimal FDR at which the identification is considered correct and was set to 1%. The q-values are estimated using the distribution of scores from the decoy database search (45). For data analysis the mass spec format-(msf)-files were filtered with peptide confidence “high” and one peptide per
protein with peptide rank 1. Protein or peptide grouping were disabled to achieve the highest number of protein identifications. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org) via the PRIDE partner repository (46) with the dataset identifier PXD001023.

Calculation of Age Dependent iTRAQ Protein Ratios Between the Age Stages—To compare all mitochondrial proteomes from different ages to each other we calculated the following iTRAQ protein ratios for each biological replicate: (1) 114/115 (6/9 days), (2) 114/116 (6/13 days), (3) 114/117 (6/16 days). The iTRAQ protein ratios were calculated as the median over all distinct peptide ratios belonging to a protein. For protein quantification in six biological replicates we performed a multiconsensus report in which all calculated protein ratios were displayed separately for each biological replicate, in order to survey a possibly variance between biological replicates. Consequently, we obtained three different iTRAQ ratios per protein (i, ii, iii) for each biological replicate, resulting in (3 × 6) iTRAQ protein ratios in total, also here referred to as replicate ratios. In this study we carried out several database searches depending on different oxidatively amino acid modifications as well as nonoxidized peptides leading to a total of 23 multiconsensus reports. For detailed information about each protein quantified in multiconsensus reports from six biological replicates and for each of examined modifications all tables are deposited in the ProteomeXchange repository with the dataset identifier PXD001023 as well as all MS/MS spectra of identified and quantified peptides.

Protein Ratios and Computation of a Quality Score for Each Age Stage—To calculate a final protein ratio for each of the three age stages, all individual biological replicate ratios were combined into one iTRAQ protein ratio per age ratio (i, ii, iii) using again the median. Unfortunately, not in all experiments a value for every of the six possible replicate ratios could be measured for each available protein and each age stage. Even if a ratio is available for all of the six biological replicates they often exhibit an experimental variation in strength and direction of their signs (up- and down-regulated).

Hence, because of the need to estimate the actual quality of each single measurement, we computed the quality score \( q \) for each protein ratio, enabling us to evaluate the reliability of the computed final protein ratio. The quality score is a value within the interval 0;1 where 0 indicates a highly reliable experimental measurement and 1 an unreliable protein ratio because of strong experimental variation. It is important to highlight that we do not use this quality score in order to test for significance of the expression at one time point in contrast to another. We needed this scoring function only to decide whether the measurement of a single protein ratio under a specific condition is reliable; because our data exhibit the drawbacks described in results sections (see also supplemental Fig. S2).

The quality score bases upon three assumptions and was computed as described in the following. Per protein P and experiment E, we obtain a set \( N_{P,E} = \{x_1, x_2, \ldots, x_m\} \) of 6 results. A result \( x_i \) is either a real number for the ratio of abundances of the protein P or \( x_i \) set to “not a number” (NA) if no peptide of the protein has been detected in the \( i \)th replicate of experiment E. Obviously, a result \( x_i = NA \) or \( x_i = 1 \) produces no valuable information on the up- or down-regulation of the protein. We eliminate these results and get a subset \( M_{P,E} = N_{P,E} \) of \( m \) positive real numbers that are either larger than one (up-regulated) or smaller than one (down regulated). The assignment of a protein P to be up/down-regulated in experiment E relies on the set of replicates \( M_{P,E} \). An unambiguous assignment is impossible if half of the results in \( M_{P,E} \) indicate up-regulation and the other half down-regulation. The number of values that identifies a unique type of regulation is relevant for reliability assignment. The same is true for the variance of real values in \( M_{P,E} \). To account for the reliability and unambiguity of our assignment of a protein P to be up or down-regulated we compute a score based on three features of \( M_{P,E} \):

(1) Number of replicates in which a specific protein is detected. In the best case we obtain one ratio for each of the six possible replicates per protein that would be rated with 1 and in the worst case no detected replicate ratio - with 0:

\[
q_v = \frac{m}{M} \quad (\text{Eq. 1})
\]

For example: Of the six possible replicate ratios we detect four:

\[
\frac{4}{6} = 0.67
\]

(2) Quotient of up- to down-regulated replicates. In the best case each detected replicate exhibits the same tendency. That is all replicates are either up- or down-regulated which should lead to a value of 1. In the worst case there are as many up-regulated as down-regulated replicates, which should be rated with the worst value of 0:

\[
r_{\text{ratio}} = \frac{\max(d, u)}{m} \geq 0.5 \quad (\text{Eq. 2})
\]

\(d\) is the number of down-regulated and \(u\) the number of up-regulated replicates in M. Hence, in the worst case we now get a value of 0.5 (instead of 0), because there are as many up- as down-regulated replicates. Next, we “stretched” the interval 0.5;1 to the required interval 0;1:

\[
q_r = \frac{r_{\text{ratio}} - 0.5}{0.5} \quad (\text{Eq. 3})
\]

(3) The variance of the replicates ratios. Next, we have to evaluate the variances of the replicates. In the best case there is no variance in M and all ratios exhibit exactly the same values which should be rated with 1. For this purpose we use the coefficient of variance \( q_v \) which results in 0 if there is no variance among the ratios and it does not have an upper limit. To map this one-sided open range to the interval (0, 1) we computed the quality of variance with

\[
q_v = e^{-q_v} \quad (\text{Eq. 4})
\]

That is if the ratios show no variance we calculate 1 and the greater the variance the more \( q_v \) approaches asymptotically 0:

\[
\lim_{q_v \to 0} q_v = 0 \quad (\text{Eq. 5})
\]

Finally, we combined all values to one quality score using the geometric mean—

\[
q = \sqrt[6]{q_v \times q_r \times q_t} \quad (\text{Eq. 6})
\]

which ensures that \( q \) is 0 if one of the three values is 0.

We assume a protein ratio to be reliable if \( q \geq 0.6 \) holds. This semi-arbitrary threshold bases upon the fact that two of the three subvalues \( q_v \) and \( q_r \) relied on discrete values (\( n \) and \( m \)). A threshold of 0.6 for both subvalues ensures that more than half of the possible replicates are available, and more than twice as many replicates for a protein ratio are up- than down-regulated and vice versa. Furthermore, because of the fact that the coefficient of variation is expressed as percentage and is subsequently stretched to the interval [0, 1], a threshold of 0.6 ensures that the protein ratios do not vary more than 50%. A subsequent manual checkup of the q-values confirmed that 0.6 indicates a reliable protein ratio (see examples in supplemental Table S2). For example, if there are 3 replicates available out of the 6
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possible replicates this results in \( q_{n^*} = \frac{3}{6} = 0.5 \) whereas four possible (more than half as many) replicates lead to \( q_{n^*} = \frac{4}{6} = 0.666\ldots \).

Criteria for Significant Up- and Down-regulation—The Reporter Ions Quantifier tool exported the replicate ratios always compared with the lowest mass iTRAQ reagent for each biological replicate, which was in our case the youngest sample: (i), (ii), (iii). Conversely, for a more intuitive interpretation and especially to compare the protein ratios easier with previous studies, we determined the inverses for all ratios; that is subsequently the following protein ratios are considered as abundance changes of oxidized or nonoxidized species from an older in comparison to a younger individual (iv) 115/114 (9/6 days), (v) 116/114 (13/6 days), (vi) 117/114 (16/6 days).

To decide whether a protein (modified- or unmodified) was up- or down-regulated on a certain day in respect to the reference day 6 we had to choose a reliable threshold. Unfortunately, on average the fold changes were very close to 1, that is we had to take into account that changes of protein abundances in our experiments normally are small.

Furthermore, regarding the drawbacks of our measurements described in results sections (see statistical approach: quality score) it was not reasonable to use a conventional parametric significance test to check whether the abundance of a protein (modified or unmodified) is high or low. For example, a two sided \( t \) test presumes a normal distribution and an appropriate sample size. It also just quantitatively takes into account the variances or the means, respectively, of the single (actually available) values and of each local distribution but not the qualitative properties like same regulative direction. We take into account the latter in combination with the quality score by using an appropriate approach which considers the complete data and all distributions as one.

We computed specific thresholds based on the distribution of our data. For this purpose, we first computed for all ratios the respective logarithmic ratios (to the base of 2) to obtain symmetric behavior. Subsequently, we calculated the quartiles for all of the logarithmic ratios and defined the 25% and the 75% quartile as our lower- and upper-threshold, respectively. That is 50% of all ratios lay within this interquartile range and all others beyond these borders (see supplemental Table S3 and are stronger down- or up-regulated than 50% of all other ratios. The supplemental Table S3 indicates that these 50% of all ratios are within the interval [0.9, 1.54]. Hence, a decrease of more than 10% and an increase of more than 54% in protein abundance were assumed to be significant.

Comparison of Modified and Unmodified Protein Profiles—With our experimental approach it is possible to detect and quantify both oxidized and nonoxidized species for each protein via iTRAQ in a single analysis, which largely eliminates technical variance. Therefore, comparing the abundance changes of modified and unmodified species at different age stages is possible, and we recorded the trend of changes on the peptide levels in protein oxidation and protein amount over the age stages in order to determine age-related protein damage. By this means, the actual relative change in oxidation degree for a certain protein during aging based on the determined ratio of oxidized to nonoxidized species could be calculated.

To test whether abundance changes of oxidized and nonoxidized protein species is similar, we compared these profiles and decided whether they are equal by means of the two-sided two-sample Kolmogorov-Smirnov-Test, which tests for differences between two one-dimensional distributions (47). The \( n \) replicate-ratios for profile 1 and the \( m \) replicate-ratios for profile 2 were used to compute the empirical distribution functions \( F_{1,n} \) and \( F_{2,m} \). Hence, the two-sided hypothesis can be formulated as

\[
H_0: F_1(x) = F_2(x) \quad \text{(Eq. 7)}
\]

(similar or indifferent distributions or profiles, respectively) and

\[
H_1: F_1(x) \neq F_2(x) \quad \text{(Eq. 8)}
\]

(different distributions or profiles, respectively).

Subsequently, based on the \( D \)-statistic

\[
D_{n,m} = \sup_x |F_{1,n}(x) - F_{2,m}(x)| \quad \text{(Eq. 9)}
\]

a \( p \) value has been computed as described in (48) and a significance threshold of 0.05 was applied, meaning that if the \( p \) value was less than or equal to 0.05 the null-hypothesis was rejected and we assumed that two profiles were significantly different. To control for multiple hypothesis testing we applied the false discovery rate method introduced by Benjamini and Hochberg (49).

Confirmation of Both Oxidized and Nonoxidized Species for Selected Proteins Via SRM MS—For selected proteins, additional MS experiments were performed to confirm the data obtained by iTRAQ quantification.

Relative quantification of the protein abundance ratios using crude AQUA peptides was performed using a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific) in SRM mode. Twenty-four suitable peptides, 3 per protein, were selected from the iTRAQ data set based on sequence uniqueness, length between 5–20 amino acids, missed tryptic cleavage sites, and amino acid oxidation predisposition. Corresponding crude heavy peptides (nontagged SpikeTides L) were synthesized from JPT Peptide Technologies containing C-terminal lysine or arginine stable isotopes to induce mass shifts of 8 or 10 Da per peptide (see on Passel PASS00738). Four transitions per peptide were selected based on maximum signal intensities observed during nLC-iTRAQ MS/MS and collision energy for each peptide was generated in-silico using Skyline software (version 2.6.0.7176; MacCoss Lab, University of Washington; (50)) on PASS00738. The four age stages of the six independent biological replicates were prepared in the same manner as described in experimental section above (Experimental workflow for in-filter protein digestion and iTRAQ labeling), however without iTRAQ labeling step. One hundred femtomole or one picomole of each heavy peptide was spiked into the tryptic digested samples prior to the desalting procedure (see on PASS00738). SRM measurements were performed as described previously by (51) with slight modifications. Samples (5-μl injection) were loaded onto the column with 2% buffer B. Peptides were eluted from the column with a multistep gradient of buffer A and buffer B which was established as follows: for 0–5 min: 2% buffer B; for 5–10 min: 2–5% buffer B; for 10–71 min: 5–35% buffer B; for 71–77 min: 35–85% buffer B; and for 77–105 min: 2% buffer B. The SRM instrument method consisted of one SRM scan event over a 105 min run-time whereby 206 transitions were measured with a 10 s cycle time (0.05 ms scan time per transition). Fixed parameters were 0.2 fwhm Q1 for precursor ions and 0.7 fwhm Q3 resolution for product ions.

For targeted quantification of the corresponding proteins with their oxidative modification sites, the six independent iTRAQ-labeled samples (see supplemental Table S1) were remeasured on the TSQ Vantage in SRM-mode. The selected oxidized peptides, precursor ions, SRM transitions and collision energies used for this analysis are deposited to the PeptideAtlas (PASS00738). Collision energy for each peptide was generated again by the skyline software and further experimentally refined for the iTRAQ-labeled peptides according to signal-to-noise measurements during SRM trials. The SRM instrument method consisted of one SRM scan event over 105 min run-time, whereby for all SRMs a scan width of 0.01 m/z and a scan time of 0.02 s was set, and fixed parameters were 0.2 fwhm Q1 and 0.7 fwhm Q3 resolution for peptide fragments and 0.3 fwhm Q3 resolution
for iTRAQ reporter ions. Each biological replicate was analyzed three times.

All SRM-data have been deposited to the PeptideAtlas SRM Experiment Library (PASSEL) and are accessible via the website http://www.peptideatlas.org/PASS/PASS00738 (52).

**SRM Data Analysis**—For SRM data normalization of nonoxidized protein species the total protein amount of each sample was determined by precursor ion quantification (53). For this normalization, all 24 samples from SRM experiment were measured on a LTQ Orbitrap XL mass spectrometer, and for each sample the total protein area from all identified protein areas was summed to generate a normalization factor. More details are available in supplemental methods. Data analysis for age-related protein abundance changes was carried out by using Skyline 3.1.0.7382 software (50). All SRM-data were manually inspected to ensure correct peak identification whereby not accurately identified peptides based on selected transitions were excluded from the data set. Further, two samples were excluded from the further data analysis, based on peculiarities of the samples exhibiting shifting of retention times for all peptides and a lower peptide and protein identification rate compared across all samples. The ratios between the peak areas of each light and heavy peptide were calculated using Skyline and exported to Excel for further statistical analysis. To account for differences in protein amount across the samples, the peptide peak ratios of the different samples were normalized based on the total protein area of each corresponding sample. As described above, the equal age ratios for each peptide of one of each biological replicate were calculated: (i), (ii), (iii). Subsequently we determined the inverses for all age ratios: (vi), (v), (vi) and logarithmised to base 2. The final protein age ratios were calculated as the median over all logarithmic iTRAQ age ratios from technical and biological replicates for final protein age ratios. Furthermore, we determined the inverses for all age ratios: (vi), (v), (vi) and logarithmised to base 2. The final protein age ratios were calculated as the median over all logarithmic distinct peptide ratios belonging to a protein. Additionally, the mean and the standard deviation were calculated in the same manner (supplemental Table S8).

Data analysis for age-related changes in protein oxidation of the corresponding proteins was performed by using Skyline 2.6.0.7176 software (50). Again all SRM-data were manually inspected to ensure correct peak identification and not accurately identified peptides by transition peaks were excluded from data set. Calculated areas of the iTRAQ reporter ions from each biological and technical replicate were summarized for each protein in an excel worksheet, and the same iTRAQ ratios per age ratio were computed: (i), (ii), (iii). All iTRAQ age ratios were normalized on the factors from respective biological replicate which were obtained during data analysis for unmodified protein species via Proteome Discoverer 1.3, and were therefore most reliable for normalization. The inverses for all iTRAQ age ratios were determined: (vi), (v), (vi) followed by the logarithm to base 2. The median was computed over logarithmic iTRAQ age ratios from technical and biological replicates for final protein age ratios. Furthermore the mean and the standard deviation over logarithmic iTRAQ age ratios from technical and biological replicates were determined (supplemental Table S9).

**Estimation of Modification Site Occupancy**—Peak areas of proteins of and of modified and unmodified peptides were obtained from Proteome Discoverer 1.4.1.14. Fractional intensity of each of the four iTRAQ labeled samples was calculated from respective peptide peak area and iTRAQ reporter ratios so that all 4 labeled samples sum up to the peptide peak area. For this purpose, the normalized (setting protein median ratio between each sample to one) relative intensity of iTRAQ reporter ratios was used. For each peptide of each age state and biological replicate the median fractional peptide intensity was calculated from first all quantified peptide spectrum matches and second from all three technical replicates. Then fractional peptide peak areas were normalized between biological replicates by division with the corresponding protein peak area in each biological replicate. Site occupancies in the range between 0 (no modification) and 1 (completely modified) were calculated under the simplified assumption that a) total peak area of a peptide is the sum of the peak areas of the one modified and the one unmodified peak area, and b) that the “flyability” (i.e. ionization efficiency) of modified and unmodified peptide is identical based on the formulas given in (54). Occupancy calculations were realized by using an in-house Perl script.

**RESULTS**

**Optimal Experimental Workflow for Comprehensive Data Acquisition of Oxidative Protein Modifications and Global Proteome Analysis**—The technical goal of this study was to develop an in-solution sample processing workflow (Fig. 1A) using iTRAQ labeling to enable the simultaneous, unbiased identification and quantification of oxidized and nonoxidized proteins from mitochondria of *P. anserina* to comprehensively analyze aging-related modified proteins. To avoid artificial protein modifications from SDS-PAGE, we adapted the filter-aided protein digestion (FASP) prior to iTRAQ labeling. Labeling efficiency was determined by comparison of database searches conducted with or without iTRAQ modification as parameter for each biological replicate (see supplemental Table S4). Based on these results, we can conclude that an average labeling efficiency of 92% was achieved in our experiments. Furthermore, one experiment was repeated with a label swap in order to verify any potential labeling bias, and we found that this switch did not significantly affect iTRAQ protein ratios; an eventual label bias can be neglected for regulated proteins (see supplemental Table S5).

iTRAQ labeling enables pooling the four age stages of one biological replicate and allows for simultaneous relative quantification of oxidized and nonoxidized protein species within a single LC-MS/MS run. This reduces experimental variance; however, it also leads to higher sample complexity. Moreover, a critical factor of reporter ion quantification is precursor mixing (55), leading to compression of observed iTRAQ protein ratios. This analytical challenge could be properly addressed by achieving high separation power with a 25 cm column and an optimized four-hour linear solvent gradient, resulting in over 1000 quantified proteins. Additionally, each experiment was analyzed on a LTQ Orbitrap Velos with CID-HCD coupled method in three technical replicates (Top10, Bottom10, Exclusion list-Top10) to account for the expected low amount of modified (oxidized) protein species. Combining all acquired raw-files from three technical replicates of one experiment in a single database search increased ∼3-fold the number of quantified iTRAQ-labeled peptides. Mainly, the Bottom10 method enlarged the detection of unique peptides of proteins by an average of 655 (Fig. 1B). To verify the robustness of our experimental setup, technical variance and biological variance were estimated from three biological replicates. Therefore, we calculated the mean standard deviation of measured protein ratios related to their age stage for technical and biological replicates and confirmed that the mean standard deviation from technical replicates is smaller than the mean standard deviation from biological replicates (sup-
In detail, we detected a mean standard deviation of 0.08 for technical variance and 0.10 for biological variance in day 9, 0.07 and 0.11 in day 13 and finally 0.09 and 0.15 in the oldest age stage (day 16). A complete overview of measured iTRAQ protein ratios from representative experiments, as well as mean values and standard deviations between technical and biological replicates can be found in supplemental Table S6.

General Survey of Global iTRAQ-based Proteome Analysis—Samples of six independent *P. anserina* individuals were analyzed according to the defined experimental design (Fig. 1A). The optimized proteomic strategy allowed an identification and quantification of in total 2341 proteins, whereof for a number of 746 both protein species (unmodified and oxidatively modified) were detected (Fig. 2A). No unmodified peptides could be quantified for 333 proteins. In these cases, only the oxidized protein form is listed. Overall, the iTRAQ proteomic analysis revealed remarkably moderate changes in protein oxidation and total protein abundance. Fig. 2B provides a compilation of quantified protein oxidation sites before statistical analysis of temporal changes and reveals a high level of mono-oxidation in amino acid residues. The data show that methionine is the main oxidation site within the detected proteins, which is reasonable because of the easy oxidation of sulfur-containing amino acid side chains. Other prominent identified modifications in decreasing order of occurrence are mono-oxidation of several amino acid residues, then carbonylation as well as formation of N-formylkynurenine and pyrrolidinone.

Statistical Approach and Time Course of Oxidative Protein Damage—In order to determine age-related protein damage, the ratios of iTRAQ-labeled peptides from four age stages were used to calculate the trend in protein oxidation and amount. Unfortunately, advanced bioinformatics tools for a comprehensive, comparative analysis of oxidized protein species and their nonoxidized counterparts in complex samples are currently missing, which made it necessary to develop a new, customized statistical approach. First, computational analysis and evaluation of the measured iTRAQ data had to be established: a quality score q for each protein ratio from six experiments was calculated that accounted for the deviation one biological replicate in one single 380 min nLC MS/MS run. All samples were acquired on a LTQ Orbitrap Velos instrument with CID/HCD method in three technical replicates (Top10, Bottom10, Exclusion list-Top10). Peptides were identified and quantified with Proteome Discoverer 1.3. B. Number of unique peptide sequences as well as total number of identified peptide spectral matches (PSMs) quantified with each of the three MS-methods, shown for three biological replicates (n = 3). Higher numbers of quantified iTRAQ-labeled peptides were gained by merging of technical replicates in one database search. Notably, the Bottom10 method enlarged the detection of unique peptides of proteins by an average of 655. Detailed list of iTRAQ protein ratios, their averages and standard deviation between technical replicates can be found in supplemental Table S4.
of our data from normal distribution and missing data values, which enabled to evaluate the reliability of the computed final protein ratio for each age stage. To clarify the need for such a quality score we illustrate the relative frequencies of measurements with missing replicate values and different regulatory direction using two barplots (see supplemental Fig. S2). Overall, only a third of all protein ratios are computed using six single values deduced from six available replicates (supplemental Fig. S2a - rightmost bar). In addition, only half of all protein ratios exhibit a distinct regulatory direction when going to the level of the corresponding single replicate ratios. That is all available replicates for one protein ratio are indicating an up- or down-regulation (supplemental Fig. S2b - rightmost bar). Secondly, a specific threshold based on the box plot distribution of our data was computed to decide whether a protein (modified or unmodified) was significantly up- or down-regulated on a certain day in respect to the reference day 6 (supplemental Table S3). After application of these defined statistical criteria on our proteome data, still 20% of oxidized proteins remained suitable for further analysis of their regulation.

Fig. 3A illustrates the distribution of modified proteins before and after the statistical analysis and exposes the total number of proteins with significantly altering abundance during aging, sorted by the oxidative modification. In the end, 18 types of protein oxidation remained after applying statistical criteria with methionine oxidation still being the most prominent, and were subjected to further data analysis. As depicted in Fig. 3B, these significant, differentially oxidized proteins were plotted against their calculated abundance ratio for each age stage to gain a complete overview of oxidation trends during the course of aging: concomitantly with increasing age, there is an increase in the number of proteins with significant abundance and oxidation changes. However, there exists no age-related uniform trend of changes in protein oxidation.

**Fig. 2. General survey of global iTRAQ-based proteome analysis (n = 6).** A, Total number of all quantified protein species in nonoxidized and oxidized form. In total, 2341 proteins were quantified of which 32% of proteins carried an oxidative amino acid modification (B). Relative amount of all analyzed amino acid modifications. Database searches using Proteome Discoverer 1.3 were sequentially conducted to identify 22 different oxidative amino acid modifications.
Fig. 3. Statistical analysis of significant changes in oxidative protein damage (n = 6). A, Protein modifications between different age stages passing defined statistical criteria are shown in red. Criteria are: 1. Number of quantified biological replicates ≥3, 2. Consistent up- or down-regulation of protein damage, 3. Variance of protein damage between biological replicates. In total 20% of protein modifications passed statistical criteria and were subsequently used for further data-analysis. B, Regulation trend of significant changes in protein oxidation at different age stages. Most proteins show a decrease in oxidative damage during the course of aging. Few proteins exhibit small increases in abundance of protein oxidation of which most were quantified in the oldest age stages.
because individual proteins display an increase as well as a decrease in protein damage with higher age. 

**Age-related Changes in Protein Damage Based on Only Oxidized Protein Species**—Usually proteomic studies target only the modified (here oxidized) proteins in complex biological samples for subsequent identification and quantification. Even though our data affords quantification of both the oxidized and cognate nonoxidized peptide species (see the following sections), we investigated first only the oxidized protein species. By this means, we are able to compare our results with previous works on oxidative protein modification in *P. anserina*, and to analyze those proteins, where only quantification of the oxidized peptides was possible. supplementary Fig. S3 shows the proteins with the highest significant age-dependent changes in oxidation level, ignoring their unmodified counterparts (if detected). In accordance with the statistical analysis mentioned in the previous chapter, only oxidized protein species with at least 0.66 (log2[-0.6])-fold decrease or 1.5 (log2[0.6])-fold increase in amounts normalized to day 6 had to be considered and belonged to 8 diverse biological processes based on the clusters of orthologous groups of proteins (COGs from [www.jgi.doe.gov](https://www.jgi.doe.gov)) (56, 57).

Below, we describe some proteins in more detail because of their protective role against aging.

Three chaperones (Pa_1_6520, Pa_3_9400, and Pa_6_2570) were found with increased protein damage of about 1.5 (log2[0.6]) to 2 (log2[1])-fold during aging. One of them, the mitochondrial protein SSC1 (Pa_6_2570) belongs to the 70-kDa heat shock protein family, functioning as molecular chaperone to protect cells against the adverse effects of stress. For five proteins only oxidized peptides could be detected, suggesting they are prone to ROS-induced oxidation. These were a putative oxygenase (Pa_4_520), the putative cytochrome P450 (Pa_1_9520) and a putative transporter protein (Pa_4_6490), which all showed an overall decrease in protein damage. Increase of oxidation during aging was observed for the putative methyltransferase (Pa_5_11950). Additionally, the putative polyketide synthase (Pa_1_11870) showed a sudden 1.5 (log2[0.6])-fold increase in protein damage from day 13 to 16.

**Changes in Abundance and Oxidation Positively Correlate for the Majority of Proteins During Aging in *P. anserina***—Comparison of trends between modified and unmodified protein species were statistically evaluated by correlation analysis. Thus, we compared the trends of all oxidized protein species passing the statistical criteria with their unmodified counterpart and verified with the two-sided two sample Kolmogorow-Smirnoff test and are listed in supplementary Fig. S4, and after additional Benjamini and Hochberg test still 14 of them remained. Those with p value ≤ 0.05 for both tests are highlighted in supplementary Fig. S4 (red asterisk) and marked with the symbol [*] throughout the text. Notably, half of these proteins are participating in energy metabolism, as depicted in supplementary Fig. S4. Among these proteins, most exhibited an overall decreasing oxidation degree with aging. However, for some proteins first an increase and then a decline at older age stages and conversely was observed, indicating a more complex process during aging (supplementary Fig. S4). Frequently several probable amino acid oxidations were discovered for a certain protein, often with similar trends, exemplified by the putative electron transfer flavoprotein beta-subunit 6 (Pa_6_1650). Still, one must bear in mind that determination of the exact localization of the oxidized amino acid residue within a peptide sequence was very challenging and often not possible, because often a complete series of fragment ions is required for unequivocal assignment. Therefore, we presented and quantified all possible (according to database search results) amino acid modifications for a given peptide to monitor the degree of protein oxidation in supplementary Fig. S4.

Of particular interest is the putative mitochondrial ATP synthase gamma chain (Pa_1_9450) [*] that revealed a continuous increase in oxidative damage up to a 2.4 (log2[1.2])-fold change, whereas abundance of the nonoxidized protein species was unaltered during aging. This protein subunit belongs
to the complex V of respiratory chain and is the only protein implicated in energy metabolism that exhibited a trend of increasing oxidation. In contrast, an overall decrease of protein oxidation is confirmed for the mitochondrial OXPHOS system, because protein subunits of complex I, II, IV and V showed a decline of about 20% at different time points. According to our results complex I and V could be oxidative hotspots, because several subunits of one complex are damaged by oxidation. However, the abundance change in oxidation degree decreases for almost all subunits of each complex with age or correlates with their respective protein amount. Also, the plasma membrane ATPase type III (Pa_3_6820 [*]) showed a significant decline in protein oxidation. Moreover, three proteins (Pa_1_4920, Pa_6_5750 and Pa_6_2570 [*]) that are involved in cellular stress response, such as chaperones, revealed a slight decrease in oxidized protein species. RNA helicase mss116 (Pa_6_3510 [*]) contributes to the translation mechanism and exhibited a remarkable increase of about 2 (log2[1])-fold in oxidized protein species from day 6 to day 9. Moreover, two proteins (Pa_2_1640 and Pa_4_9350) related to lipid metabolism showed a marked increase in oxidation degree during aging. We also detected several cytoplasmic proteins - probably because of isolation of crude mitochondria- which evidently changed their oxidation degree during aging. Representative proteins with a possible link to aging are the putative woronin body major protein HEX1 (Pa_1_17000 [*]) and the putative eukaryotic translation initiation factor 5A-2 (Pa_3_4460) with an overall decreasing oxidation degree and further a putative GTP-binding protein (Pa_1_1540) with a remarkable increase in oxidation of aspartic acid residues during aging.

Confirmation of Age-related Changes in Abundance and Oxidation for Selected Proteins by SRM Analysis—To validate the most notable age-related changes in protein oxidation and total protein abundance, a targeted proteomic approach for relative quantification was used. Consequently, age-related protein abundance changes were verified by the use of selected AQUA peptides, whereby for verification of age-related changes in protein oxidation the iTRAQ-labeled samples were re-measured in SRM modus on a TSQ mass spectrometer. SRM data analysis for nonoxidized and oxidized species of selected proteins are presented in supplemental Table S8 and S9, respectively. To facilitate data interpretation the temporal trends of changes in protein oxidation or protein abundance from analyzed peptides obtained either on an Orbitrap Velos or a TSQ instrument are shown in direct comparison (Fig. 5). Fig. 5 illustrates that the application of targeted quantification by SRM MS provided a good means of confirmation since for all analyzed proteins the trend profiles for changes in abundance were highly similar between iTRAQ-based and AQUA-based relative quantification, even though the absolute log2 ratios are not always equal during aging. For changes in protein oxidation, five proteins exposed similar results for iTRAQ ratios obtained on Orbitrap Velos versus TSQ, whereas just for four protein results did not agree: the putative laccase (Pa_6_7880), a copper-containing oxidase enzyme, the cytochrome c oxidase (Pa_6_5480), the putative SAM-dependent o-methyltransferase PaMTH1 (Pa_2_7880) and the putative ATP-dependent RNA helicase mss116 (Pa_6_3510). Thus these proteins weren’t any more credible for a statement about the processes during biological aging. Nevertheless, it was possible to confirm an increasing oxidation...
### FIG. 5.

Confirmation of iTRAQ data results by SRM quantification of both oxidized and nonoxidized species for selected proteins. Additional SRM experiments were performed on a TSQ mass spectrometer and directly compared against the iTRAQ data obtained on an.

| Accession | Description | Peptide sequence | change oxidative damage | change protein abundance |
|-----------|-------------|------------------|-------------------------|--------------------------|
| Pa_1_0450 | Putative mitochondrial ATP synthase gamma chain precursor | VYQSAETKPL(+10)EGEGKK; NIVLNFSVGK; YQILFR | log2 ratio | 6/6 | 9/6 | 13/6 | 16/6 |
| Pa_mito_nad5 | NADH-ubiquinone oxidoreductase chain 5 | YTITNL10LGGMQ(+10)TK | log2 ratio | 6/6 | 9/6 | 13/6 | 16/6 |
| Pa_6_5480 | Cytochrome c oxidase polypeptide 5 encoded by the cox5 gene | GNWAELET(+O)AEK; EWGEAT(+O)NEYLK; EWQEADEYLK; AGNS6DLTG996EGYK | log2 ratio | 6/6 | 9/6 | 13/6 | 16/6 |
| Pa_3_0400 | Putative mitochondrial GpE protein homolog precursor | AL(+O)TMVPEEK; DLVDVVDFNDR, EHNTVFHTQOK | log2 ratio | 6/6 | 9/6 | 13/6 | 16/6 |
| Pa_6_2570 | Putative mitochondrial precursor of heat shock protein SSC1 | DIOEVL(+0)YGGMTR; TTPSVVAFADGER, DAGQAOGLVLR, ADSVLDTDEK | log2 ratio | 6/6 | 9/6 | 13/6 | 16/6 |
| Pa_6_3510 | Putative ATP-dependent RNA helicase mss110 | YIDP(-2H-1C-10)TFQATV; TLAFWPLQDK, AIWSIR | log2 ratio | 6/6 | 9/6 | 13/6 | 16/6 |
| Pa_5_11950 | Putative methyltransferase | L(+O)WVW(+O)NGL(+O)DHL(+O)JK | log2 ratio | 6/6 | 9/6 | 13/6 | 16/6 |
| Pa_6_7880 | Putative tscase precursor | GW(+O)QQPLSEPSLYDTMCADFPDK; W(+O)MMHEAPR, EAEESCSW(+O)R, NWYDDSIEGEGGR, WFTPOK | log2 ratio | 6/6 | 9/6 | 13/6 | 16/6 |
| Pa_2_7880 | Putative SAM-dependent O-methyltransferase encoded by the PaMTH1 gene | LOGSIA(+0)MIFAR; AEIVTLIESPK, TLEGFDLIEVANK, DGYAGYV | log2 ratio | 6/6 | 9/6 | 13/6 | 16/6 |
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...tion level for the chaperone SSC1 with high age (day 16) although log2 ratios differed between the Orbitrap Velos and the TSQ. Concerning this matter the same applies for the putative methyltransferase (Pa_5_11950).

Of particular interest is the confirmation of the changes in protein abundance and oxidation for the ATP synthase gamma chain (Pa_1_9450), further substantiating the age-related protein damage. Furthermore the NADH-ubiquinone oxidoreductase (Pa_mito_nad5, complex I) and the chaperone protein GrpE were confirmed in their age-related changes in protein abundance and oxidation.

Modification Site Occupancy Indicates Low Protein Oxidation Level—Fractional modification site occupancies were estimated for the mono-oxidation of methionine and leucine, and carbonylation of proline which leads to the product pyroglutamic acid (supplemental Table S10). For most sites, occupancies only varied slightly between the different age stages. The distribution of leucine and methionine occupancies peaked around 0.2 (supplemental Fig. S5), whereas for the few estimated proline carbonylations, the occupancy was mostly less than 0.1. Hence, for the majority of sites, the nonoxidized form was in excess. Nevertheless, for some peptides and proteins only the oxidized form could be identified, which may be because of high occupancy, yet its missing unmodified counterpart precluded occupancy calculation. In this respect, it was unfortunately not possible to calculate the occupancy for the leucine oxidation site on the gamma subunit, because the unmodified peptide was not identified. However, data shows that some sites of the gamma and other ATP synthase subunits exhibit relatively high occupancy; in more detail occupancy for another site on the gamma subunit was 0.58, for two sites on the alpha subunit it was 0.48 and 0.63, respectively, and for two sites on the beta subunit it was 0.48 and 0.38. Altogether, these data suggest that some regions of the holoenzyme are hotspots for ROS. For the two other proteins with changes unequivocally confirmed by SRM, i.e. the NADH-ubiquinone oxidoreductase (Pa_mito_nad5, complex I) and the chaperone protein GrpE, mean site occupancies were 0.41 and 0.14, respectively.

DISCUSSION

A prominent theory of aging research explains aging as the result of ROS-induced accumulation of random molecular damage to biomolecules, where the majority of ROS are generated in mitochondria. Therefore oxidative modifications of mitochondrial proteins should contribute to their functional decay with age (12, 58). Accordingly, protein damage has been assumed to be a causative factor or at least a key contributor to degeneration and death, also in humans (59). In P. anserina different studies demonstrate that oxidative stress increases during aging and that interfering with the generation or scavenging of ROS has a strong impact on aging and lifespan control (22, 23, 25).

Despite this and other evidence, the detailed role of oxidative stress in aging is only insufficiently explained at the molecular level, such as the proteome, because of difficulties in tracking in vivo oxidants and oxidized biomolecules (60). As discussed in the following sections, these technical challenges were overcome by a newly developed workflow consisting of large-scale, untargeted protein modification analysis and statistical evaluation of results to scrutinize the role of ROS-induced protein damage for aging.

Assessment of the Untargeted iTRAQ-based Proteomic Approach to Monitor ROS-induced Protein Oxidation in P. anserina—In the present work, a large dataset for age-dependent protein abundance changes of oxidatively modified and unmodified protein species was obtained. A clear advantage of the used untargeted proteomic approach is no a priori limitation to a particular protein modification. However, previous untargeted proteome analyses have concentrated almost exclusively on the identification of post-translational modifications, rather than on their quantification (61, 62). Based on these previous qualitative studies it is well-known that an extremely diverse mixture of protein oxidation products occurs during aging, which we aimed to analyze quantitatively, too.

The applied workflow enabled identification and quantification of in total 2341 proteins, whereof both species (unmodified and oxidatively modified) were detected in 746 proteins, and no oxidized counterpart peptides could be quantified for 1273 unmodified proteins. The underlying reasons for the latter are probably both biological and technical. For instance, a previous determination of the oxidation degree in ATP synthase from P. anserina (42) suggests a low amount of oxidized species for many proteins. Moreover, the oxidized peptide is more hydrophilic than its corresponding nonmodified form, leading to differences in elution and consequently different interferences from co-eluting background ions during RP chromatography. Despite these challenges, our study stands well against previous quantitative analyses, even when pre-enrichment of modified peptides was done. Compared with (37), who identified and quantified over 200 carbonylated proteins of crudely enriched rat skeletal muscle mitochondria by virtue of pre-enrichment, we were able to quantify almost 100 carbonylated proteins without using any enrichment pro-
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procedure for low-abundant oxidized peptides. Admittedly, targeted quantitative enrichment of thiols groups with OxiCAT achieved better coverage of cysteine oxidation status, for example 400 different protein thiols in Saccharomyces cerevisiae (63) and thiol redox status for 140 proteins in Caenorhabditis elegans lifespan (64, 65) were obtained. Even though powerful technologies to target large-scale methionine oxidation sites exist (66), they have not been applied to mitochondrial aging. Unsurprisingly, our untargeted modification approach does not achieve the same qualitative and quantitative proteome coverage for a single oxidative modification as recent targeted studies and therefore we might have missed information pertaining to oxidized peptides which are critical for the proper function of a protein (e.g., active sites); but instead it has enabled tracing a much wider spectrum of modifications, amounting to more than 2000 identified protein species which allows the investigation of a global ROS-induced protein damage to verify the ROS theory of aging. The suitability of our iTRAQ-based shotgun proteomics workflow was validated by confirming selected peptides values with SRM analysis. The sometimes observed result differences between the two methods could probably be best explained by missing data points in the shotgun approach.

Can Previous Studies on Oxidative Damage and Protein Abundance Changes in Mitochondria During Aging be Extended?—Obviously the central biological question we wanted to address was whether our results are in line and extend former studies on the role of ROS in aging. Although many studies focused on the relationship between ROS-induced protein damage and aging, an age-related increase of bulk oxidized mitochondrial proteins could not be observed. Instead only individual proteins seem to be modified, such as proteins of the OXPHOS or aconitase (17, 24, 37). Indeed, an immunochemical analysis of carbonylated proteins failed to detect significant age-dependent difference in bulk carbonylation of mitochondrial proteins; nevertheless, a remarkable increase of proteins containing carboxymethylated lysine residues during aging in P. anserina could be shown (24). In the present study we were able to quantify 18 different protein oxidations concurrently over the age stages in P. anserina. Unfortunately, the used software (Proteome Discoverer 1.3) did not allow searches for oxidative modifications on lysine residues since already modified by the iTRAQ reagent. Overall, the iTRAQ proteomic analysis revealed remarkably moderate changes in protein oxidation and total protein abundance. It is known that this quantitative approach suffers to some extent from compression of iTRAQ protein ratios in complex samples but the direction of change (differential up-, down-, or unregulated) is itself unaffected (67). In agreement with our work other comparative approaches on P. anserina, such as transcriptome analysis, 2D-DIGE analysis or label free protein quantification, revealed the same moderate effects on the mitochondrial proteome during aging (68–70), suggesting a compensatory response to ROS-induced protein damage during aging of P. anserina. Further, a recent SRM analysis on the mitochondrial proteome of P. anserina PaSod3 over-expression mutant revealed that the protein changes in the mutant are much more remarkable than in the aging wild type (70). Our work extends previous studies which showed only minor changes in the protein abundance involved in oxidative phosphorylation, energy metabolism, stress response and protein quality control (68–70). Even though the made assumptions for occupancy calculation demand cautious result interpretation, it seems fair to say that overall a low site oxidation level could be maintained at all investigated age stages of P. anserina. This result again indicates a proteome homeostasis mechanism. It might further indicate that sites with a low or "decorative" level of oxidation do not impair protein function. On the contrary, the relatively high occupancy of several modification sites in the ATP synthase together with the observed significant increase of oxidation for its gamma subunit, hints toward functional impairment at late age. Furthermore, it can be concluded from the wealth of obtained data that protein abundance and oxidation during aging do not follow a simple trend, but instead show different and varying kinetics, although the overall changes are quite subtle, and the composition of the mitochondrial proteome of P. anserina can be considered as generally stable even when reaching late age.

Besides the important global assessment of mitochondrial protein damage, novel findings for individual proteins related to aging were obtained: (17) summarized examples of age-related proteins (ATP-synthase F1 complex, reticulocalbin) with redundant isoforms in mitochondria from three different species, P. anserina, rat and human. The study revealed that in the ATP synthase F1 complex the underlying posttranslational modifications are associated with distinct domains of ATP synthase subunits; whereas other parts of the protein remain unaffected. Furthermore, (37) approve the susceptibility of the OXPHOS machinery to carbonylation. In agreement with these observations our iTRAQ-quantification showed that the OXPHOS complexes are certainly targets of ROS, including several oxidized protein forms. In addition, our data revealed a remarkable increase of leucine oxidation within the ATP synthase gamma chain with age that could be also confirmed by the SRM validation (Fig. 5) and may therefore be a hotspot to ROS-induced damage during aging. However, regarding the complete proteome level, we end up with a different biological interpretation, namely protein damage homeostasis, because correlations between the amount of modified and unmodified peptide species were found. Indeed, no pronounced increase of global protein damage at late age could be observed; instead our data rather indicate a general reduction in protein oxidation and abundance mostly from day 13 to day 16. This does not mean that an absolute increase of oxidized protein species could not negatively affect cellular and organismal physiology even though the fractional amount does not change, but we hypothesize that as long as the
organismal scavenger systems and the protein de novo synthesis are properly functioning, the occurring protein damage can be largely, if not completely, compensated, which will be discussed in following sections.

No Massive Increase in Protein Oxidation Degree During Aging in P. anserina—

How Could ROS-induced Protein Damage be Compensated?—Extensive correlative evidence has been collected over decades that corroborate the oxidative stress theory of aging including the discovery that aging and many age-related diseases are accompanied by considerable cellular oxidative damage (28, 39, 71–73) (74, 75). Despite these and many other reports supporting an increased ROS level, recently a series of conflicting results suggested a more complex interplay between endogenous oxidants, antioxidants, protein quality control and lifespan (76, 77). For instance, the comprehensive proteomic analysis of different mouse tissues revealed only minor age-related abundance changes and rather suggested a functional protein homeostasis up to a relatively late age (78). Notably, a redox proteomics study performed with the OxCat technique revealed a ROS burst as an early event in C. elegans life, but not during aging (65). Also, the previous SRM analysis of mitochondria from P. anserina showed that the examined oxidative modification in the ATP synthase subunit alpha remained rather unaffected by aging (42). These recent findings suggest that ROS level is well-controlled during aging and functionally regulates essential physiological processes in the cell (79, 80). For this purpose, pathways for special ROS signaling seem to exist which regulate also ROS homeostasis for alleviating the toxicity of ROS (2). For instance, our data reveal that methionine residues are the main oxidation site within the detected proteins, which could be removed, if repair mechanisms are sufficiently early activated. It is known that methionine residues constitute an important antioxidant defense mechanism because surface exposed methionine residues create an extremely high concentration of reactant, available as an efficient oxidant scavenger. Reduction back to methionine by methionine sulfoxide reductases allow the antioxidant system to be restored (81). Here, two different classes of methionine sulfoxide reductases, MsrA and MsrB, play a pivotal role (82); accordingly, msrA overexpression in Drosophila melanogaster and S. cerevisiae extended the lifespan and increased the resistance against oxidative stress, respectively. Methionine oxidation followed by reduction may act as a constant sink for reactive oxygen species, which primarily protects amino acids in close vicinity against ROS (83, 84). We identified unmodified peptid species of both putative methionine sulfoxide reductases (Pa_1_1390 & Pa_4_7040) and found that the protein amount of Pa_1_1390 remained stable during aging, whereas the putative methionine sulfoxide reductase Pa_4_7040 decreased slightly - interestingly in earlier age stages (day 9 and 13). This is supported by a recent transcriptome analysis revealing a decrease of the corresponding gene (Pa_4_7040) from day 6 to 14 with a factor of 0.67 (69) and indicating insufficient capacity of the Msr proteins to compensate oxidative damage alone. Indeed, concomitant increase of oxidized and nonoxidized forms of chaperons (GrpE, Hsp70 (SCC1) and Hsp60) suggests that P. anserina elicits a stress response for additional compensation of accumulated protein damage during aging.

Proteome Homeostasis Collapse Occurs at a Very Late Age—The here observed decrease of many mitochondrial proteins can be best explained by an age-dependent decrease in protein synthesis and an increase in protein removal by various proteolytic processes: Apparently the protein synthesis breaks down after day 13 and the organism cannot compensate the removal of ROS-damaged proteins anymore. Several studies revealed age-related increases in mtDNA mutations that contribute to physiological decline in aging and degenerative diseases (85–87) and are mirrored in senescent P. anserina (88, 89). These mtDNA mutations lead to deficiencies in remodelling of affected oxidized proteins that are encoded by the mtDNA, such as proteins of the respiratory chain that require replacement by de novo synthesis (21). This conclusion agrees with the observed general down-regulation of important nuclear as well as mtDNA-encoded mitochondrial proteins from day 13 on. Concerning the postulated increase of proteolytic processes with age, the recent genome-wide transcriptome analysis of P. anserina suggests that autophagy is a compensatory mechanism during aging when all other pathways failed to restore the proceeding accumulation of damaged biomolecules (69). A recent analysis showing that autophagy is up-regulated in old P. anserina, as monitored by the autophagy-dependent degradation of a PaSOD1::GFP reporter protein (90) underpins this assumption: autophagy evidently becomes induced during normal organismal aging when the proteasome and transcription/translation appear to be functionally impaired (69). Importantly, loss of autophagy significantly shortens lifespan (90). However, the exact role of autophagy in the degradation of oxidatively damaged mitochondrial proteins of P. anserina remains to be determined.

CONCLUSION

In summary, this study shows the potential of untargeted iTRAQ-shootgun proteomics and adequate statistics to monitor simultaneously many different oxidative modifications. Our approach allowed to conclude that: (1) no global increase in protein oxidation occurs in the mitochondria of P. anserina during aging, (2) not only protein amounts remain rather constant, but also the ratio of damaged to undamaged protein, and (3) ROS-induced protein damage is efficiently compensated by protein de novo synthesis until late age, when in P. anserina autophagy is activated. Hence, efficient proteome homeostasis is the key to maintain mitochondrial function during increasing ROS exposure with age.

Acknowledgments—
Mitochondrial Proteome Homeostasis During Aging

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| REFERENCES |
| --- |
| 1. Halliwell, B., and Gutteridge, J. (1990) The antioxidants of human extracellular fluids. Arch. Biochem. Biophys. 280, 1–8 |
| 2. D’Autreax, B., and Toledano, M. B. (2007) ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nat. Rev. Mol. Cell Biol. 8, 813–824 |
| 3. Lanciano, P., Khaltfau-Hassani, B., Selamoglu, N., Ghelli, A., Rugolo, M., and Daldal, F. (2013) Molecular mechanisms of superoxide production by complex III: a bacterial versus human mitochondrial comparative case study. Biochim. Biophys. Acta 1827, 1332–1339 |
| 4. Dröse, S., and Brandt, U. (2012) Molecular mechanisms of superoxide production by the mitochondrial respiratory chain. Adv. Exp. Med. Biol. 748, 145–169 |
| 5. Calabrèse, V., Scapagnini, G., Giuffrida Stella, A. M., Bates, T. E., and Clark, J. B. (2001) Mitochondrial involvement in brain function and dysfunction: relevance to aging, neurodegenerative disorders and longevity. Neurochem. Res. 26, 739–764 |
| 6. Lenaz, G., Bohinc, C., D’Autreax, M., Fatò, R., Formiggini, G., Genova, M. L., Giuliano, G., Pich, M. M., Paolucci, U. G. O., and Castelli, G. P. (2002) Role of mitochondria in oxidative stress and aging. Ann. N.Y. Acad. Sci. 959, 199–213 |
| 7. Cui, Z. J., Han, Z. Q., and Li, Z. Y. (2011) Modulating protein activity and cell function by methionine residue oxidation. Amino Acids 43, 505–517 |
| 8. Wang, C. H., Wu, S. B., Wu, Y. T., and Wei, Y. H. (2013) Oxidative stress response elicited by mitochondrial dysfunction: implication in the pathophysiology of aging. Exp. Biol. Med. (Maywood) 238, 450–460 |
| 9. Jenner, P. (2003) Oxidative stress in Parkinson’s disease. Ann. Neurol. 53 Suppl 3, S26–36: discussion S36–38 |
| 10. Lin, M. T., and Beal, M. F. (2006) Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 443, 787–795 |
| 11. Harman, D. (1956) Aging: a theory based on free radical and radiation chemistry. J. Gerontol. 11, 298–300 |
| 12. Harman, D. (1972) The biologic clock: the mitochondria? J. Am. Geriatr. Soc. 20, 145–147 |
| 13. Levine, R. L. (2002) Carbonyl modified proteins in cellular regulation, aging, and disease. Free Radic. Biol. Med. 32, 790–796 |
| 14. Cui, H., Kong, Y., and Zhang, H. (2012) Oxidative stress, mitochondrial dysfunction, and aging. J. Signal Transduct. 2012, 646354 |
| 15. Harman, D. (1992) Free radical theory of aging. Mutat. Res. 275, 257–266 |
| 16. Beal, M. F. (1995) Aging, energy, and oxidative stress in neurodegenerative diseases. Ann. Neurol. 38, 357–366 |
| 17. Groene, K., Krause, F., Kunstmann, B., Unterleggauer, H., Reifschneider, N. H., Scheckhuber, C. Q., Sastri, C., Stegmann, W., Wozny, W., Schwall, G. P., Poznanovic, S., Dencher, N. A., Jansen-Dürr, P., Osiewacz, H. D., and Schrattenholz, A. (2007) Differential proteome profiling of mitochondria from Podospora anserina, rat and human reveals distinct patterns of age-related oxidative changes. Exp. Gerontol. 42, 887–898 |
| 18. Osiewacz, H. D. (2002) Aging in fungi: role of mitochondria in Podospora anserina. Mech. Ageing Dev. 123, 755–764 |
| 19. Osiewacz, H. D. (2002) Mitochondrial functions and aging. Gene 286, 65–71 |
| 20. Lorin, S., Dufour, E., and Sainsard-Chanet, A. (2006) Mitochondrial metabolism and aging in the filamentous fungus Podospora anserina. Biochem. Biophys. Acta 1757, 604–610 |
| 21. Osiewacz, H. D., Hamann, A., and Zintel, S. (2013) Assessing organismal aging in the filamentous fungus Podospora anserina. Methods Mol. Biol. 965, 439–462 |
| 22. Scheckhuber, C. Q., Erjavec, N., Tinzl, A., Hamann, A., Nystrom, T., and Osiewacz, H. D. (2007) Reducing mitochondrial fission results in increased life span and fitness of two fungal aging models. Nat. Cell Biol. 9, 99–105 |
| 23. Cui, Z. J., Grief, J., and Osiewacz, H. D. (2006) Mitochondrial free radical generation and lifespan control in the fungal aging model Podospora anserina. Exp. Gerontol. 41, 439–447 |
| 24. Luč, K., and Osiewacz, H. D. (2009) Increasing organismal healthspan by enhancing mitochondrial protein quality control. Nat. Cell Biol. 11, 852–858 |
| 25. Kunstmann, B., and Osiewacz, H. D. (2008) Over-expression of an S-adenosylmethionine-dependent methyltransferase leads to an extended lifespan of Podospora anserina without impairments in vital functions. Aging Cell 7, 651–662 |
| 26. Bakala, H., Ladouce, R., Baraiba, M. A., and Friguet, B. (2013) Differential expression and glycative damage affect specific mitochondrial proteins with aging in rat liver. Biochim. Biophys. Acta 1832, 2057–2067 |
| 27. Chaudhuri, A. R., de Waal, E. M., Pierce, A., Van Remmen, H., Ward, W. F., and Richardson, A. (2006) Detection of protein carbonyls in aging liver tissue: a fluorescence-based proteomic approach. Mech. Ageing Dev. 127, 849–861 |
| 28. Aksenov, M. Y., Aksenova, M. V., Butterfield, D. A., Geddes, J. W., and Markesbery, W. R. (2001) Protein oxidation in the brain in Alzheimer’s disease. Neuroscience 103, 373–383 |
| 29. Kunstmann, B., and Osiewacz, H. D. (2009) The S-adenosylmethionine dependent O-methyltransferase PaMTH1: a longevity assurance factor protecting Podospora anserina against oxidative stress. Aging 1, 328–334 |
| 30. Surco-Laos, F., Cabello, J., Gómez-Orte, E., González-Manzano, S., González-Paramás, A. M., Santos-Buelga, C., and Dueñas, M. (2011) Effects of O-methylated metabolites of quercetin on oxidative stress, thermotolerance, lifespan and bioavailability on Caenorhabditis elegans. Food Funct. 2, 445–456 |
| 31. Sheehan, D., McDonagh, B., and Bárčena, J. A. (2010) Redox proteomics. Expert Rev. Proteomics 7, 1–4 |
| 32. Törnvall, U. (2010) Pinpointing oxidative modifications in proteins—recent advances in analytical methods. Anal. Methods 2, 1638–1650 |
| 33. Fedorova, M., Bollineni, R. C., and Hoffmann, R. (2014) Protein carboxylation as a major hallmark of oxidative damage: update of analytical strategies: protein carboxylation. Mass Spectrom. Rev. 33, 79–97 |
| 34. Frolich, J. M., and Reid, G. E. (2008) The origin and control of ex vivo oxidative peptide modifications prior to mass spectrometry analysis. Proteomics 8, 1334–1345 |
| 35. Perdivara, I., Deterding, L. J., Przybylski, M., and Tomer, K. B. (2010) Mass spectrometric identification of oxidative modifications of tryptophan residues in proteins: chemical artifact or post-translational modification? J. Am. Soc. Mass Spectrom. 21, 1114–1117 |
| 36. Meller, M., Rogowska-Wreszynska, A., and Rao, R. S. P. (2011) Protein carboxylation and metal-catalyzed protein oxidation in a cellular perspective. J. Proteomics 74, 2228–2242 |
| 37. Meany, D. L., Xie, H., Thompson, L. V., Arriaga, E. A., and Griffin, T. J. (2007) Identification of carboxylated proteins from enriched rat skeletal muscle mitochondria using affinity chromatography-stable isotope labeling and tandem mass spectrometry. Proteomics 7, 1150–1163 |
| 38. Mann, M., and Jensen, O. L. (2003) Protein analysis of post-translational modifications. Nat. Biotechnol. 21, 255–261 |
| 39. Baraiba, M. A., Ladouce, R., and Friguet, B. (2013) Proteomic quantification and identification of carboxylated proteins upon oxidative stress and during cellular aging. J. Proteomics 92, 63–70 |
| 40. Ross, P. L. (2004) Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol. Cell. 15.5 1707 |
Mitochondrial Proteome Homeostasis During Aging

57. Tatusov, R. L., Koonin, E. V., and Lipman, D. J. (1997) A genomic perspective on protein families. Science 278, 631–637

58. Muller, F. (2000) The nature and mechanism of superoxide production by the electron transport chain: its relevance to aging. Age 23, 227–253

59. Devasagayam, T., Tilak, J. C., Boloor, K. K., Sane, K., Ghaskadbhi, S., and Lele, R. (2004) Free radicals and antioxidants in human health: current status and future prospects. Jap J 52, 794–804

60. Muller, F. L., Lustgarten, M. S., Jang, Y., Richardson, A., and Van Remmen, H. (2007) Trends in oxidative aging theories. Free Radic. Biol. Med. 43, 477–503

61. Tveen-Jensen, K., Reis, A., Moulis, L., Pitt, A. R., and Spickert, C. M. (2013) Reporter ion-based mass spectrometry approaches for the detection of non-enzymatic protein modifications in biological samples. J. Proteom. 92, 71–79

62. McClintock, C. S., Parks, J. M., Bern, M., GhattyVenkataKrishna, P. K., and Hettich, R. L. (2013) Comparative informatics analysis to evaluate site-specific protein oxidation in multidimensional LC-MS/MS data. J. Proteome Res. 12, 3307–3316

63. Brandes, N., Reichmann, D., Tienson, H., Leichter, L. I., and Jakob, U. (2011) Using quantitative redox proteomics to dissect the yeast redoxome. J. Biol. Chem. 286, 41893–41903

64. Kumsta, C., Thamsen, M., and Jakob, U. (2011) Effects of oxidative stress on behavior, physiology, and the redox thiol proteome of Caenorhabditis elegans. Antioxid Redox Signal 14, 1023–1037

65. Kulkarni, V., Thamsen, N., Nurm, Z., Niemuth, Nicholas J., Diederich, A.-K., and Jakob, U. (2012) Quantitative in vivo redox sensors uncover oxidative stress as an early event in life. Mol. Cell 47, 767–776

66. Ghesquiere, B., Jonckheere, V., Coleta, N., Van Durme, J., Timmerman, E., Goethals, M., Schymkowitz, J., Rousseau, F., Vanderklishov, J., and Gevaert, K. (2011) Redox proteomics of protein-bound metalloion oxidation. Mol. Cell. Proteomics 10, 1162–1166

67. Oh Y., Salim, M., New, J., Evans, C., Rehm, L., and Wright, P. C. (2009) iTRAQ underestimation in simple and complex mixtures: “The good, the bad and the ugly”. J. Proteome Res. 8, 5347–5355

68. Chimi, M. A., Dröse, S., Wittig, I., Heide, H., Steger, M., Werner, A., Hamann, A., Osiewacz, H. D., Schafer, E. R., Seelert, H., and Dencher, N. A. (2012) Reactive oxygen species target specific tryptophan site in the mitochondrial ATP synthase. Biochim. Biophys. Acta 1817, 381–387

69. Philipp, O., Hamann, A., Servos, J., Werner, A., Koch, I., and Osiewacz, H. D. (2012) A Genome-Wide Longitudinal Transcriptome Analysis of the Aging Model Proteome of Caenorhabditis elegans. PLOS ONE 8, e83109

70. Plohnke, N., Hamann, A., Poetsch, A., Osiewacz, H. D., Römer, M., and Rexroth, S. (2014) Proteomic analysis of mitochondria from senescent Podospora anserina casts new light on ROS dependent aging mechanisms. Exp. Gerontol. 55, 15–25

71. Finkel, T., and Holbrook, N. J. (2000) Oxidants, oxidative stress and the biology of aging. Nature 406, 239–247

72. Beal, M. F. (2002) Oxidatively modified proteins in aging and disease. Free Radic. Biol. Med. 32, 797–803

73. Fink, T., and Tatemichi, K. (2003) Oxidative stress and the biology of aging. Nature 423, 869–873

74. Vize, M. S., Teku, K. W., Tetherling, W. T., and Flannery, B. P. (1992) Numerical Recipes in C: the art of scientific computing, Second Edition, Cambridge Univ. Press, New York

75. Benjamini, Y., and Hochberg, Y. (1995) Controlling the False Discovery Rate: A practical and Powerful Approach to Multiple Testing. J. R. Statist. Soc. B 57, 289–300

76. MacLean, B., Tomazela, D. M., Shulman, N., Chambers, M., Finney, G. L., Frewen, B., Kern, R., Tabb, D. L., Liebler, D. C., and MacCoss, M. J. (2010) Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics 26, 966–968

77. Wolff, D., ter Veld, F., Kohler, T., and Poetsch, A. (2013) Combined application of targeted and untargeted proteomics identifies distinct metabolic alterations in the tetraacetylphytosphingosine (TAPS) producing yeast Wickerhamomyces anomalus. Mol. Cell. Proteomics 12, 144–156

78. Das, S., and Vandenbrande, A. (2012) Redox sensors uncover antioxidant defense and cellular regulation under oxidative stress in vivo. Physiol. Rev. 92, 811–826

79. Brandes, N., Reichmann, D., Tienson, H., Leichter, L. I., and Jakob, U. (2011) The oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but have little or no effect on life span in Caenorhabditis elegans. Genes Dev. 25, 3236–3241

80. Walther, D. M., and Mann, M. (2011) Accurate quantification of more than 4000 mouse tissue proteins reveals minimal proteome changes during aging. Mol. Cell. Proteomics 10, M110 004523

81. Rhee, S. G. (2008) Cell signaling. H2O2, a necessary evil for cell signaling. Science 319, 1882–1883

82. Held, J. M., and Gibson, B. W. (2012) Regulatory control or oxidative damage? Proteomic approaches to interrogate the role of cysteine oxidation status in biological processes. Mol. Cell. Proteomics 11, R111 013037

83. Levine, R. L., Berlett, B. S., Moskovitz, J., Mosoni, L., and Stadtman, E. R. (1999) Methionine residues may protect proteins from critical oxidative damage. Mech. Ageing Dev. 107, 223–332

84. Weissbach, H., Resnick, A. B., and Broit, N. (2005) Methionine sulfoxide reductases: history and cellular role in protecting against oxidative damage. Biochem. Biophys. Acta 1703, 203–212

85. Levine, R. L., Moskovitz, J., and Stadtman, E. R. (2000) Oxidation of methionine in proteins: roles in antioxidant defense and cellular regulation. J. IUBMB Life 50, 301–307
84. Friguet, B., Bulteau, A.-L., and Petropoulos, I. (2008) Mitochondrial protein quality control: implications in ageing. *Biotechnol J.* 3, 757–764
85. Chomyn, A., and Attardi, G. (2003) MtDNA mutations in aging and apoptosis. *Biochem. Biophys. Res. Commun.* 304, 519–529
86. Kujoth, G. C. (2005) Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. *Science* 309, 481–484
87. Wallace, D. C. (2010) Mitochondrial DNA mutations in disease and aging. *Environ. Mol. Mutagen.* 51, 440–450
88. Osiewacz, H. D., and Hermanns, J. (1992) The role of mitochondrial DNA rearrangements in aging and human diseases. *Aging* 4, 273–286
89. Osiewacz, H. D. (1997) Genetic regulation of aging. *J. Mol. Med.* 75, 715–727
90. Knuppertz, L., Hamann, A., Pampaloni, F., Stelzer, E., and Osiewacz, H. D. (2014) Identification of autophagy as a longevity-assurance mechanism in the aging model *Podospora anserina*. *Autophagy* 10, 822–834
91. Olsen, J. V., de Godoy, L. M., Li, G., Macek, B., Mortensen, P., Pesch, R., Makarov, A., Lange, O., Horning, S., and Mann, M. (2005) Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. *Mol Cell Proteomics* 4, 2010–2021