


A mass spectrometry workflow for measuring protein turnover rates in vivo

Mihai Alevra^{1,5}, Sunit Mandad^{1,2,3,5}, Till Ischebeck⁴, Henning Urlaub^{2,3}, Silvio O. Rizzoli^{1*} and Eugenio F. Fornasiero ^{1*}

Proteins are continually produced and degraded, to avoid the accumulation of old or damaged molecules and to maintain the efficiency of physiological processes. Despite its importance, protein turnover has been difficult to measure in vivo. Previous approaches to evaluating turnover in vivo have required custom labeling approaches, involved complex mass spectrometry (MS) analyses, or used comparative strategies that do not allow direct quantitative measurements. Here, we describe a robust protocol for quantitative proteome turnover analysis in mice that is based on a commercially available diet for stable isotope labeling of amino acids in mammals (SILAM). We start by discussing fundamental concepts of protein turnover, including different methodological approaches. We then cover in detail the practical aspects of metabolic labeling and explain both the experimental and computational steps that must be taken to obtain accurate in vivo results. Finally, we present a simple experimental workflow that enables measurement of precise turnover rates in a time frame of ~4–5 weeks, including the labeling time. We also provide all the scripts needed for the interpretation of the MS results and for comparing turnover across different conditions. Overall, the workflow presented here comprises several improvements in the determination of protein lifetimes with respect to other available methods, including a minimally invasive labeling strategy and a robust interpretation of MS results, thus enhancing reproducibility across laboratories.

Introduction

The stability of the proteome is an essential aspect of living organisms. The mechanisms that regulate the maintenance of a functional proteome are important in several aspects of biology, especially in connection with pathologies such as neurodegeneration, protein accumulation, prion diseases and aging¹. Measuring protein turnover in vitro (i.e., in cell cultures) using isotope-labeled amino acids is an accessible technique that can be applied by virtually any laboratory with moderate experience in cell culture and MS². The situation in vivo is markedly complicated by the sizeable reuse of amino acids within the animal organism during physiological protein metabolism^{3,4}.

Traditionally, this problem has been approached by using custom diets and advanced analysis tools, which are not readily accessible to most laboratories⁵. For these reasons, although approaches to measurement of proteome-wide turnover have existed for more than a decade^{6–8}, measurement of protein lifetimes has not yet become common practice in MS laboratories. At the same time, previous approaches have been difficult to reproduce between laboratories⁴. Nevertheless, protein stability in vivo remains of great importance and keeps attracting interest in the scientific community^{5,9–13}. We have, therefore, developed tools to render the measurement of protein lifetimes in mice in vivo not only precise but, importantly, easily reproducible in virtually any laboratory with access to shotgun proteomic technologies⁴. The strategy described here is based on a pulsing workflow that utilizes the isotopically stable ¹³C₆-lysine during metabolic labeling. This essential amino acid is absorbed through the diet and is incorporated into proteins depending on their relative turnover. The analysis of protein labeling is complicated by the reuse of unlabeled amino acids, as we describe in detail in this protocol.

Methods for estimating protein stability in mice in vivo

Historically, the main strategy for estimating protein turnover in living organisms has been based on pulsing isotopically labeled amino acids (or amino acid precursors) and monitoring their appearance

¹Department of Neuro- and Sensory Physiology & Center for Biostructural Imaging of Neurodegeneration (BIN), University Medical Center Göttingen, Göttingen, Germany. ²Department of Clinical Chemistry, University Medical Center Göttingen, Göttingen, Germany. ³Bioanalytical Mass Spectrometry Group, Max Planck Institute of Biophysical Chemistry, Göttingen, Germany. ⁴Department of Plant Biochemistry, Albrecht-von-Haller-Institute for Plant Sciences and Göttingen Center for Molecular Biosciences (GZMB), University of Göttingen, Göttingen, Germany. ⁵These authors contributed equally: Mihai Alevra, Sunit Mandad. *e-mail: srizzol@gwdg.de; efornas@gwdg.de

in the proteins that are synthesized in animals^{7,9,12,14–18}. For simplicity, protein turnover can be considered as a kinetic process in which an old protein population is replaced with a new population by neosynthesis and degradation of the older proteins. In a system in which all the new proteins are synthesized from a new pool of fully labeled amino acids, this process can be approximated to a simple first-order kinetic. The situation in vivo is complicated by the fact that the pool of old (unlabeled) amino acids cannot be quickly replaced by labeled amino acids. This is because in vivo amino acids can be reused and the old (unlabeled) amino acids can become incorporated into new proteins. If the exact amino acid reuse is neglected, one can observe only relative differences in turnover between proteins by metabolically labeling animals for different amounts of time, followed up by measurements in which one assesses the speed at which each protein species becomes labeled.

In a seminal work, Price et al. used a pulsing approach and estimated the half-life of ~1,000 proteins in the mouse brain⁷. To achieve protein metabolic labeling, they supplemented the mouse diet with *Spirulina platensis* (cyanobacteria) that was previously labeled with ¹⁵N. Mice were metabolically labeled for different time periods and then killed; proteins were extracted and the relative incorporation of ¹⁵N was measured. Although Price et al. were pioneers in the estimation of protein lifetimes, the use of ¹⁵N-labeled peptides complicates the analysis of the MS data, requiring a sophisticated processing algorithm¹⁶. With this approach, ¹⁵N can enter at any position of each peptide, which causes a general mass spectral shift toward higher masses upon labeling. To be correctly analyzed, a peptide needs to be in a relatively ‘uncrowded’ position of the mass spectrum, which restricts the number of peptides that can be efficiently analyzed for each protein⁷, thus biasing the precision of the measurements⁴. Moreover, owing to the largely unknown metabolism rates of different amino acids and other ¹⁵N-labeled molecules present in the diet, it is virtually impossible with this approach to devise a formal description of the turnover of the entire proteome, although some efforts have been made in this direction^{16,19}. As a result, although comparative experiments between different experimental conditions are in principle possible, the lack of precision in the determination of lifetimes and the absence of reliable confidence intervals in these measurements do not allow a simple, practical implementation.

Other groups have applied metabolic labeling of proteins to mice or rats, mostly aiming at characterization of the longer-lived proteins, through the use of long feeding pulses. Examples include the work of Savas et al.⁹, Toyama et al.¹⁰, Toyama et al.²⁰ and the more recent work of Heo et al.¹¹. As an example, Toyama and collaborators pulse-fed a ¹⁵N diet to pregnant rats and continued the pulse in the progeny for up to 6 weeks after birth¹⁰. The progeny were successively chased with ¹⁴N food for different time periods and the relative incorporation of ¹⁵N in proteins was measured. In this case, the lifetimes were not estimated, but the overall stability of the proteins was studied, revealing the extremely long-lived proteins (ELLPs). These efforts, although important for the study of extremely stable proteins, tend to neglect or underestimate the shorter-lived fraction of proteins, which corresponds to ~95% of the proteome⁴. Importantly, none of these approaches take into consideration the amino acid reuse during the labeling process that takes place in vivo, precluding the possibility of measuring the exact lifetimes of proteins.

In addition to isotope-labeled amino acids, deuterium labeling has been used successfully as an alternative approach to labeling proteins⁶. In a typical deuterium workflow, animals receive intraperitoneal injections of deuterated water (²H₂O) and have ad libitum access to 8% ²H₂O in the drinking water supply for different time intervals²¹. During the synthesis of nonessential amino acids, the animals incorporate the deuterium into the C–H bonds, thus labeling the newly synthesized proteins⁶, along with all other biomolecules in the organism. Following protein preparation, the metabolic labeling of proteins can be measured over time by analyzing the shift in peptide isotope weights toward higher-end masses²¹. Freeware software is available for analyzing the shift in peptide isotope composition^{22,23}, although it is important to clarify that while ²H₂O labeling is in theory simple and cost-efficient, the exact time course of amino acid production degradation and the reuse of ²H cannot be formally modeled in an organism. This is because the quantitative aspects of the biotransformations linked to ²H are largely unknown, as ²H₂O can be incorporated into virtually all biomolecules²⁴. The current models work by approximating the problem of protein labeling with ²H to a mono-exponential saturation toward an equilibrium level. This assumption neglects the fact that the body of a mammal works as a reservoir for precursor molecules that are constantly reused for the synthesis of new proteins, which makes it difficult to accurately define protein lifetimes with ²H₂O labeling.

A method for precise determination of protein lifetimes in mice in vivo

To overcome these limitations, we have recently introduced and thoroughly validated a technique that enables the measurement of protein lifetimes⁴ while taking advantage of a commercially available

diet for SILAM²⁵. The approach is an extension of the stable-isotope labeling of amino acids in cell culture (SILAC), which has been largely adopted for protein quantification in dissociated cells²⁶. Approaches using a SILAM scheme for absolute protein quantification have been around for more than a decade, including those of several important *in vivo* works^{25,27,28}. Our *in vivo* pulsing strategy workflow utilizes an isotopically stable ¹³C₆-lysine, which is an essential amino acid that needs to be absorbed through the diet. In addition, lysine is not turned directly into other amino acids but is catabolized to specific metabolites such as pipercolic acid²⁹, reducing the isotopic labeling of other amino acids to virtually zero. Following metabolic labeling, the incorporation of ¹³C₆-lysine is quantified through shotgun MS. This is coupled with a proteome-level description of the lysine pool reuse based on both actual measures and mathematical modeling. Overall, our strategy comes with several improvements for the determination of lifetimes, including a simple and robust interpretation of MS results, which allows the obtainment of data for many peptides for each protein. This is a major advantage with respect to approaches based on ¹⁵N diets that limit the analysis to a handful of arbitrarily selected peptides^{4,7}. Importantly, even if the users modify this strategy by altering the pulsing and chasing schemes, our reliable analysis framework will still enable them to extract reliable protein lifetime parameters. For this reason, future results will be easily shared and replicated by other laboratories. This is an important aspect for the development of the field, because lifetime evaluations *in vivo* have been largely qualitative or comparative^{9–11}. Moreover, this refined strategy includes several labeling pulsing times and allows the accurate monitoring of protein labeling over different time scales. One additional improvement of this strategy is the precise evaluation of the reuse of labeled lysine, which enables a study of the stability of the entire proteome. As a result, this experimental design, coupled with the description of lysine reuse, enables investigators to determine the exact half-lives of proteins and can easily be extended to different mouse genetic lines and possibly to other animal models. In principle, one could also use it for other species such as bacteria or plants, if lysine is made into an essential amino acid by targeted mutations.

Overview of the procedure

The procedure is summarized in Fig. 1 and consists of five modules: mouse metabolic labeling and tissue collection (Steps 1–10); sample processing (Steps 11–51); MS analysis (Steps 52–70); data interpretation, lifetime determination and statistics (Steps 71–92); validation and controls (Step 93; several options available). The aspects from Steps 1–70 are based on existing procedures that we have specifically tailored for the purpose of measuring protein turnover in mice. In these three initial modules of the protocol, we cover in detail the timing, the quantity of isotopic food and other aspects while offering practical tips to render the measurements reliable and robust. The data interpretation and the final validations (Steps 71–93) are the most complex steps. For this reason, we provide in the protocol the extensive biochemical methods, computational tools and scripts that enable the data interpretation and validation, along with practical examples that might help with troubleshooting the most common problems.

Applications and future directions

Protein stability measurements are important to several domains of biology, encompassing a number of pathological alterations such as cancer and neurodegeneration. Mice are extensively utilized as genetic models, and protein turnover measurements have the potential to become as widely used as protein abundance measurements for general screening purposes. In general, the assessment of protein turnover parameters should be considered as an additional technical option in the palette of tools available for molecular biologists and could be used both as a validation and a discovery tool.

A direct application of the protein stability measurements is to determine the turnover of a protein of interest in specific organs or in subcellular structures^{4,30}, and eventually under genetic or pharmacological modulations of the pathway of interest. This would reveal the proteins that have compromised stability in a set of relevant modulations, in the same manner in which such proteins are currently identified in protein abundance studies. Importantly, in lifetime measurements, each hit contains an internal control because, for each protein, the ratio between the ‘heavy’ pulsed peptides and their ‘light’ counterparts is measured. As a result, the precision of these measurements is generally higher than that of protein abundance measurements⁴. This implies that small differences that might be overlooked in protein abundance studies would be readily detected with a turnover approach. Therefore, protein turnover measurements would be useful as a complement to, and in

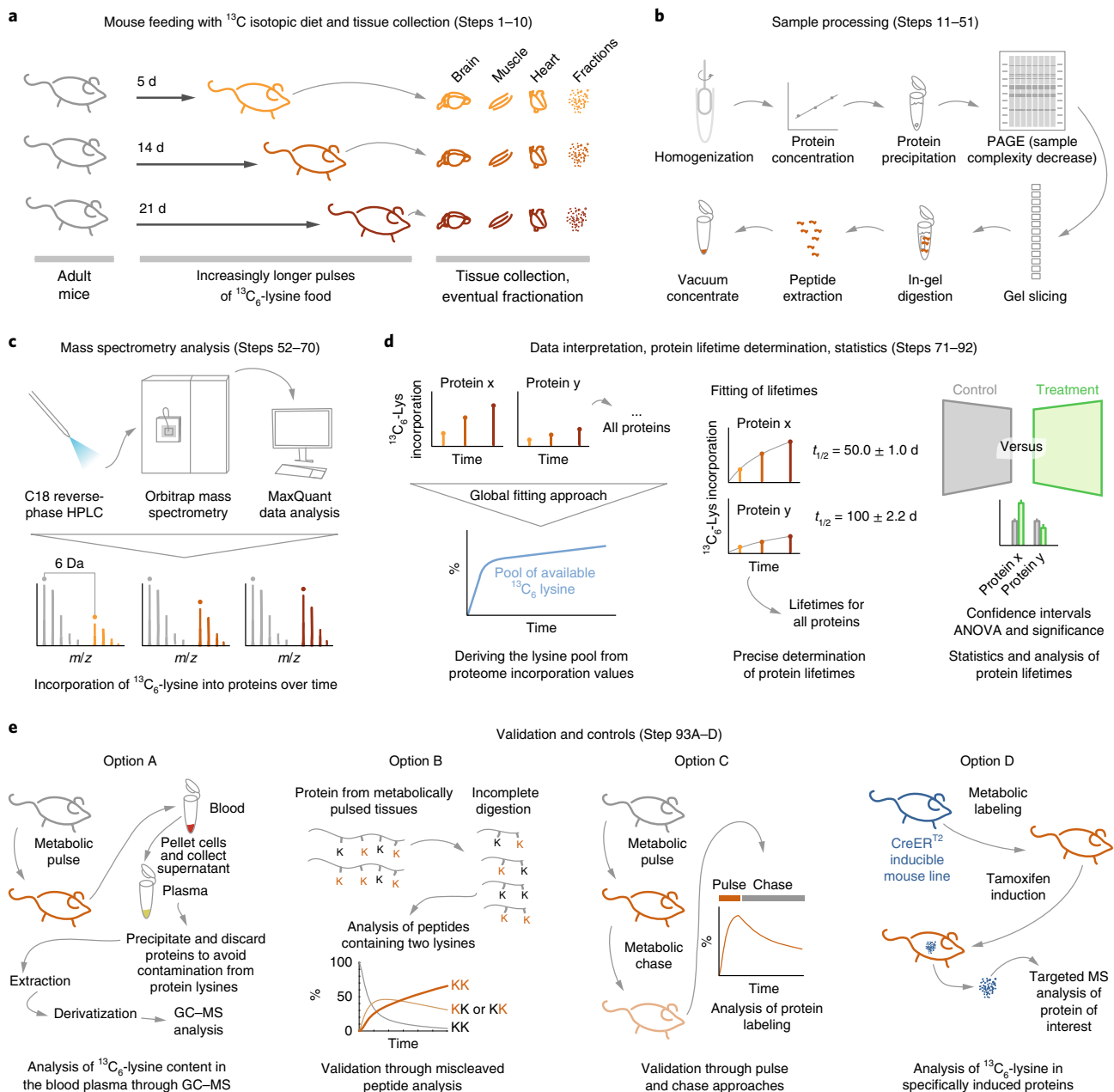


Fig. 1 | Flowchart of the protocol. **a**, Steps 1–10: pulse-feeding of mice for metabolic labeling, followed by tissue collection. The most appropriate feeding scheme for the protein of interest can be defined by following the guidelines in Supplementary Table 1. **b**, Steps 11–51: sample processing for MS. This stage can be adapted to specific applications. Importantly, because the metabolically labeled and the unlabeled populations of proteins coexist in the same sample, fractionation methods such as PAGE can be applied to simplify the sample and provide deeper proteome coverage. **c**, Steps 52–70: protein analysis by nano reverse-phase HPLC coupled with high-resolution mass spectrometry with an instrument such as an Orbitrap. This step allows the measurement of relative incorporation of $^{13}\text{C}_6$ -lysine at the proteome level. These values will be used for data interpretation and lifetime measurements. **d**, Steps 71–92: data interpretation, precise determination of lifetimes, statistics. This protocol includes all the scripts needed to derive protein lifetimes directly from MS results. **e**, Step 93A–D: validation and controls. We provide a gallery of four different controls and cross-validation steps that can either be used to check the quality of the measurements or might be useful in extending this protocol to other animal models or different amino acids. Black K, $^{12}\text{C}_6$ -lysine (Lys0); Brown K, $^{13}\text{C}_6$ -lysine (Lys6) (Option B). See main text for details.

some cases a replacement for, protein abundance studies. This can be particularly efficient because the same samples can be used for both purposes by combining turnover and label-free quantifications³¹.

Among other examples, a simple practical aim could be to downregulate or knock out a specific protein that is part of a putative complex and then to determine the effects on the formation and/or the stabilization of the complex by monitoring the lifetimes of the interaction partners. This is particularly relevant *in vivo*, where complex stability is influenced by molecular crowding and by the

exact stoichiometry of the individual components³². This would reveal differences in situations in which various compensatory processes might still maintain a constant protein level, even though the protein turnover, i.e., the speed at which a protein is produced and degraded, might be altered. Along the same lines, this approach can be used for the study of any of the proteins that are thought to be important for the regulation of degradation pathways, such as specific E3 ligases or regulators of the proteasome or the lysosome pathways. Several other practical applications could be envisaged, including the study of specific protein modifications on lifetimes, the effects of autoimmune disease on the stability of receptors and cellular structures, and the metabolism of cancerous tissues at different stages or under different pharmacological treatments.

Among the most promising future developments of this technique, we would like to mention the possibility of combining it with more advanced MS workflows, including data-independent acquisition methods³³ and similar approaches that have recently become more accessible and reproducible^{34,35}. Another obvious future development will be the use of multi-pulsing strategies adding other specific amino acids. These more complex pulsing schemes might be instrumental in shedding light on the *in vivo* behavior of proteins that might be replaced with unusual dynamics³⁶. This is especially relevant for proteins that have completely different turnover profiles at different developmental steps during animal development. In fact, some proteins, such as the crystallins in the eye lens, are synthesized only very early in development and are virtually never exchanged during the animal lifetime³⁷. Along these lines, we have previously applied a second metabolic pulse with Arginine10 (¹³C₆-¹⁵N₄ arginine)⁴. Even if arginine deprivation is linked to growth impairments³⁸ and can thus be considered an essential amino acid, *in vivo* it is efficiently metabolized to proline. This complicates the MS analysis and, in the absence of a precise description of arginine metabolism, restricts the analysis to peptides devoid of proline. A possible future circumvention of this problem could be based on neutron-encoded stable isotope labels³⁹, which are commercially available and might be adapted to pulse SILAC strategies to measure protein turnover.

Finally, all the conceptual and practical knowledge that is included in this detailed protocol can be used to extend these lifetime measurements to other frequently used animal models such as rat, fruit flies, nematodes and zebrafish, for which the SILAC food is already commercially available.

Advantages and limitations of the approach

The main general limitations of protein turnover measurements *in vivo* at the moment are the lack of a simple workflow for reproducible measurements and the absence of an experimental framework that can be followed by non-experts in the field. These aspects have rendered the protein turnover field a closed community predominantly concentrating either on technical aspects of protein labeling^{7,16,17} or on the proteins that are most easily addressed by simple labeling approaches, such as the ELLPs⁹⁻¹¹.

To circumvent these limitations, the approach we have developed takes into consideration the reuse of lysines in the organism and enables a precise determination of protein lifetimes. Compared to the approaches used in the past, the main advantages of this workflow are (i) rapid implementation for any laboratory using protein MS; (ii) robust analysis and reliable results that can be easily achieved with the scripts we include in this protocol; (iii) the flexibility of the method and the validation steps, which can be fine-tuned for each practical application; (iv) the modular applicability to specific biological questions, such as neurodegenerative diseases and genetic mouse models; and (v) the possibility of validating lifetimes with high precision.

Although SILAC diets are commercially available, a practical limitation for the application of the approach is the high cost of the isotopically labeled mouse food. Aware of this limitation, in this protocol, we have streamlined the design of experiments and we provide different experimental designs, based on the most common needs, that would minimize animal number, labeling time and costs (see Supplementary Table 1). As an example, a minimal design could restrict the use of labeled food to a few hundred grams, enabling reliable results with an affordable investment (on the order of €/1,500).

Another limitation of the method, which is shared with other shotgun MS approaches used to quantify proteins, is a relatively poorer measurement precision for proteins with low relative abundance. There are two possible solutions to this problem: the first is to simplify the complexity of the sample that is analyzed, for example, fractionating the sample based on its biophysical features so that the most abundant proteins do not obscure the detection of the less abundant ones. Here, we detail a thoroughly time-tested method based on PAGE for this purpose^{40,41} (Steps 15–51) to simplify the

application of this protocol for laboratories that do not have access to other more recent MS solutions to this issue, such as high-pH reverse-phase peptide fractionation⁴². The second solution is to enrich the protein or the structure of interest either by specific immunoprecipitation or by subcellular fractionation. We have applied both methods to samples that were metabolically pulsed, and we confirmed the compatibility of these approaches with this method⁴. Owing to space limitations, in this protocol we do not provide an extensive description of these steps, although we refer to the appropriate protocols for these purposes (Step 10). Having a reasonably high amount of protein in the sample is a very strict requirement for this method. We advise using an initial amount of 100 μ g of total protein for each sample. If this amount is exceedingly high, for example, because the biological sample under scrutiny is too small (e.g., a small brain area), one could collect similar samples from several mice and measure the pooled samples, although this increases the number of analyzed mice and thus the price of the labeling procedure.

Specific needs in the determination of lifetimes might include the study of the lifetime differences for either extremely short-lived proteins (<1 d) or ELLPs (>60 d). With our protocol, such proteins are identified relatively easily (provided that they are sufficiently abundant) and we know in advance whether they are extremely short- or extremely long-lived. However, it might become challenging to define their exact lifetimes. Specifically, for extremely short-lived proteins, approaches that include short pulses of intravenous injections of isotopically labeled metabolic precursors should be considered. Our scripts have been designed to be usable for calculating the lifetimes for such short pulses. One need only input the correct parameters of the available amino acid pool. It should be noted that, whenever possible, intravenous delivery should be avoided to prevent excessive delivery of precursor molecules, which would affect the physiology of the system. For ELLPs, the most obvious adaptation of our protocol is to include longer pulses, as we suggest in Supplementary Table 1.

Another challenge in the field is the study of protein lifetimes in tissues composed of fast-dividing cells with extremely high mitotic activity, such as those observed in transformed cells in cancer. In this particular case, the determination of protein lifetimes is confounded by the fact that the cells composing the tissue have faster turnover rates than those of the proteins that compose them. In simple terms, the protein turnover system is not at equilibrium. In this case, other computational approaches for determining lifetimes might be required, including, for example, the implementation of a three-compartment model function fitting with four rate constants, as elegantly proposed in 2012 by Guan et al.¹⁶.

An additional potential limitation of the methods that use SILAM labeling is the fact that, upon trypsinization, some peptides do not contain lysines (because they can be cleaved on arginine residues). In theory, this problem can be circumvented by using the endoproteinase LysC, which cleaves peptide bonds only at the carboxyl side of lysine. In reality, this solution is suboptimal because the proteome coverage may be compromised as a consequence of the decreased number of peptides generated. In our workflow, trypsin digestion provides a median of six peptides containing lysine per protein identified and allows us to determine the turnover of ~80% of identified proteins. This is superior to techniques based on ¹⁵N diets or ²H₂O labeling, in which peptide analysis is more cumbersome and allows determination of turnover rates for only ~50–60% of the proteins identified^{21,43}.

Experimental design

The final design of the experiments will depend on the exact purpose of the study, the budget that can be dedicated to the project and the constraints on the amount of the protein(s) of interest in the analyzed samples. In this protocol, we describe a minimal workflow, which includes the feeding of one mouse for each of three different time periods for a single experimental condition (for a total of three animals). If the samples analyzed meet the optimal protein amounts necessary for MS analysis (100 μ g), this workflow will allow the precise fitting of protein lifetimes for large proteomic datasets. Thus, it can be used for comparing the effect of specific modulations on protein lifetimes by measuring a set of three mice for any additional genetic or pharmacological treatment of interest.

If differences in lifetimes are minimal, or if the precision of the measurements is particularly important to the study, we advise using three animals for each pulsing time, for a total of nine mice for each condition. This solution reduces both the biological and the technical variability and renders the measurements more reliable. As for the pulsing periods, a large fraction of the proteome is relatively shortlived^{4,7}; thus long pulses are not recommended for most cases, because they substantially increase the cost of the experiments without substantially improving the precision of the

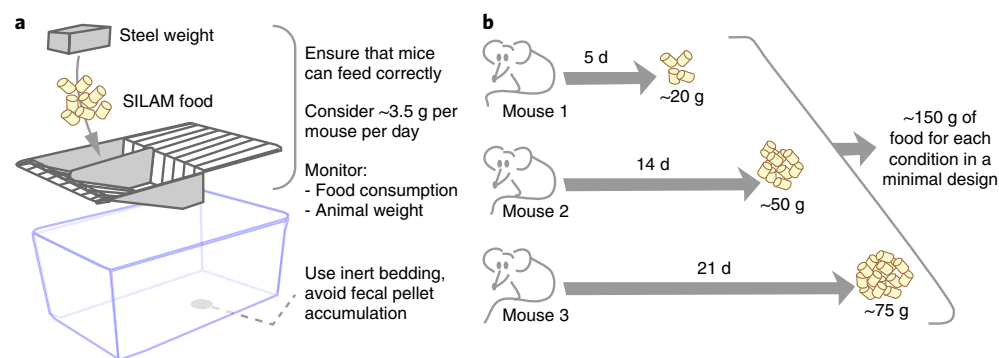


Fig. 2 | Mouse metabolic labeling setup and SILAC food estimates for a minimal experimental design. **a**, Schematic representation of the most important aspects to consider during metabolic labeling. A metal weight can be used to allow mice to grasp and eat the food correctly. Mice should be placed in single cages in the case that competition for access to food is feared. Monitor food consumption and animal weight to ensure correct food consumption. Because mice have a tendency to eat fecal pellets, which can interfere with labeling, it is useful to change the bedding or remove them. **b**, Estimated food consumption for a minimal experimental design. Refer to Supplementary Table 1 for more complex experimental designs. The total amount of food for a mouse over 40 d will correspond to ~150 g of SILAC food. With this minimal experimental design, the cost of the food can be maintained under ~\$/€2,000. It is safe to always have an extra amount of labeled food in case the mice eat slightly larger amounts.

results. Specifically for short-lived proteins, the addition of short pulsing times will improve the precision of the measurements without a marked increase in the experimental costs. By contrast, if the main interest is to determine differences in the stability of longer-lived proteins and ELLPs, it might be necessary to design longer pulse schemes. For more specific considerations on the exact workflow, refer to Supplementary Table 1.

Mouse metabolic labeling (Steps 1–9)

The metabolic labeling of mice with SILAC food was introduced more than a decade ago^{25,27,28}. For our purposes, the pulsed metabolic labeling of mice is performed with a rather simple setup (Fig. 2a), but there are a number of important aspects that should be considered in order to perform it correctly.

As previously mentioned, SILAC food costs need to be considered when planning metabolic pulsing experiments. The price of 1 kg of ¹³C₆-lysine food is on the order of ~\$/€10,000; thus large experiments require very careful planning. A minimal labeling design can be used to limit the costs of the experiments (Fig. 2b). An important aspect to consider is that mice will tend to be skeptical of new diet formulations for some days. To prevent the pulsing procedure from being biased by this effect, it is useful to first habituate mice with a less expensive ¹²C₆-lysine formulation that is identically produced and packaged, which allows the mice to get used to the new food in a few days. We recommend monitoring of food consumption and mouse weight across the entire labeling procedure to avoid any chance of labeling biases that could compromise the entire outcome of the workflow. Usually, mouse cage setups are designed to hold relatively large quantities of food. For this reason, food pellets are provided in abundance and the weight of the food itself is sufficient for the animal to grasp and eat the food. When small amounts of food are used, the weight of the food itself might not be sufficient for the animals to correctly grasp the pellets and eat them. To overcome this problem, a simple solution is to add a steel weight that presses the food down and allows its correct consumption (Fig. 2a). Owing to the importance of the metabolic pulsing to the overall outcome of the entire workflow, several other details are thoroughly discussed in the protocol and presented in Fig. 2.

Tissue and subcellular fractionation (Step 10)

A virtually unlimited number of tissue and protein fractionation steps can be combined with this metabolic labeling approach. Owing to space limitations, in this protocol we avoid engaging in a detailed description of all possible fractionation methods. For subcellular fractionation and, in particular, for brain organelles such as synaptosomes, synaptic vesicles and mitochondria, we refer to previously published protocols^{44–47}. In principle, the study of cell-type-specific proteomes could also be combined with this technique^{4,48}, allowing the evaluation of the differential turnover of the same protein species in different cells. Importantly, for all preparations, we advise collection of 100 µg of

total protein for optimal results. If a pooling strategy cannot be used, it is possible to apply an MS method that does not require fractionation (see below), although this results in a limited amount of detected proteins and scarce proteome coverage.

MS analysis (Steps 52–70)

The MS analysis we describe here in detail is based on the initial separation of the samples through PAGE⁴⁰, followed by in-gel digestion and reverse-phase HPLC-MS. This allows the measurement of relative incorporation of ¹³C₆-lysine at the proteome level and the heavy-versus-light values for each protein, corresponding to the amount of labeled ¹³C₆-lysines versus the amount of unlabeled ¹²C₆-lysines. These are the required values for the interpretation of the results and the lifetime measurements. Several other possible shotgun MS approaches could in principle be used to obtain similar values. Aware of this, we have provided scripts for computational analysis (see below) that are compatible with the results from other approaches that provide the heavy-versus-light fraction for each protein as an output.

Computational analysis (Steps 71–93)

The main complexity in the application of this method concerns the computational aspects and the correct calculation of the lifetimes. To circumvent this issue, we provide in this protocol several tools that simplify all computational steps (Supplementary Data 1). These include (i) a graphical user interface script (turnoverGUI.m) used to create a simple file template for inputting the data, import data, estimate the lysine pool dynamics from proteomic data, calculate protein lifetimes and confidence intervals based on the pool of lysines, and compare lifetimes from different conditions and obtain statistically relevant differences; (ii) a script to predict the labeling efficiency for proteins containing two lysines (predictDoubleLabel.m); (iii) a script to predict the relative labeling of proteins in a chase experiment for more advanced applications (predictPulseChase.m); (iv) a script to compare the goodness of fit for multiple datasets using different lysine pool dynamics (comparePools.m); (v) a script to estimate the lysine pool dynamics from independent measurements, such as those that can be obtained from the blood serum (fitDirectPool.m); (vi) a script to generate simulated datasets that can be used for testing purposes (simDataset.m); and (vii) a series of supporting scripts that are used by previous scripts (readDataXLS.m, writeDataXLS.m, fitSetWriteXLS.m, fitSingle.m, timeMsg.m, genTemplateXLS.m, fitGlobalPar.m and fitSet.m). All these scripts are based on MATLAB, fully annotated and entirely inspectable and modifiable based on the needs of specific applications. We compiled all the scripts, and we explain them stepwise in this protocol in a way that allows their utilization by users with no previous experience with MATLAB.

Data interpretation and amino acid pool fitting (Steps 79–92)

To obtain precise lifetimes, it is necessary to describe the change from the incorporation of normal ¹²C₆-lysines to the labeled ¹³C₆-lysines over the duration of the experiment. The mathematical modeling and the fitting of the data are summarized in Fig. 3. Briefly, we have shown that lysines exist in free solution ('s') or are incorporated into proteins ('p'), in either a non-labeled (light (L)) or a labeled form (heavy (H))⁴. The average interactions between these four pools can be approximated by a fast process (dominated by food absorption and excretion, rate parameter b) and a slower process (corresponding to protein synthesis and degradation, rate parameter a). Together with the pool size ratio (r ; which indicates bound versus free lysines), four coupled differential equations are solved analytically, using the information gathered with a pulsing experiment⁴. The change of the lysine pool can thus be described as a bi-exponential process (Fig. 3c) with three unknown pool parameters $[a, b, r]$. The labeling of an individual protein of interest (H_{poi}) is influenced by the pool and has a specific turnover time constant, which is analytically solved. For each $[a, b, r]$ pool parameter triplet, all data obtained by MS can be fitted, producing residuals for each protein that can be summed for all proteins (Fig. 3c, lower panels). The residuals are then minimized during a global pool fit, repeating the individual H_{poi} fits for different $[a, b, r]$ triplets until optimal pool parameters are found. The best parameters can then be used for obtaining all individual lifetime measures (half-lives).

We have previously published the average behavior of the lysine pool in mice⁴. The values can be immediately used for initial lifetime estimations and are included as pre-sets in our script. At the same time, it is possible that specific pharmacological or genetic manipulations will affect the lysine pool. For this reason, in this protocol we include a specific script that can be used to estimate the pool of available ¹³C₆-lysines from a global fit of the labeling results. Once the most accurate lysine pool parameters are chosen, the heavy-versus-light ratios can be used to calculate protein lifetimes.

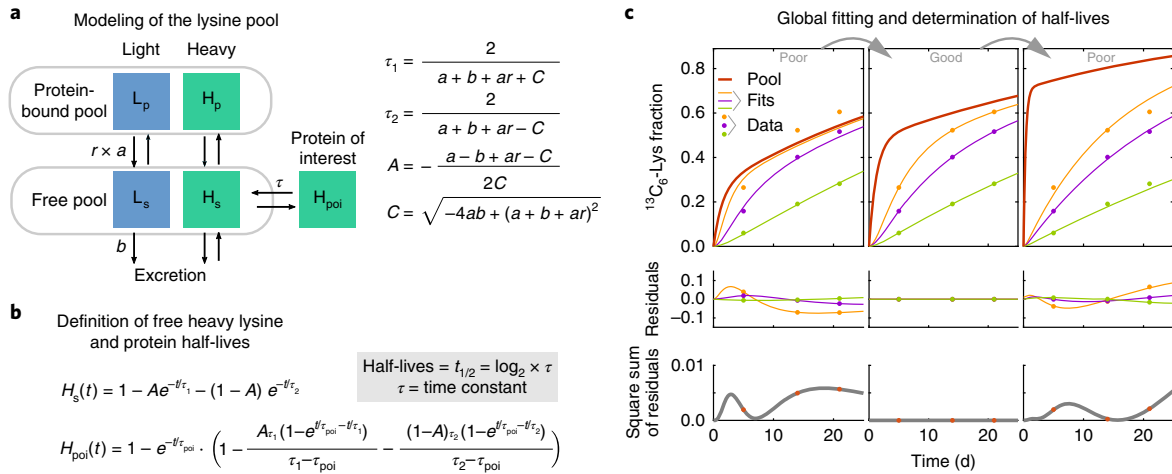


Fig. 3 | Mathematical modeling and fitting of the data. **a**, Schematic representation of the relationships between the lysine pool components. Lysines exist in free solution (s) or are incorporated into proteins (p), in either non-labeled (light, 'L') or labeled (heavy, 'H') form. The average interactions between these four pools are described by a fast process (food intake and excretion, rate b , with amplitude A and time constant τ_1) and a slower process (protein synthesis and degradation processes, rate a with time constant τ_2). Together with the pool size ratio (r), four coupled equations can be defined and analytically solved⁴. **b**, The function for free heavy lysine $H_s(t)$ can thus be described as a bi-exponential process with the three unknown pool parameters a , b and r , using the terms C , A , τ_1 and τ_2 . The labeling of an individual protein of interest (H_{poi}) follows H_s using a simple, protein-specific turnover time constant τ , which is analytically solved. **c**, During global fitting, used to find the correct parameters of the lysine pool, for each pool parameter triplet, theoretical H_{poi} curves can be fitted to the experimental (H/total) ratios (upper three graphs). For each protein, these fits produce residuals and sum of squared residuals (the middle and lower three graphs, respectively). The sum of squared residuals is then minimized during the global pool fit procedure, repeating the individual H_{poi} fits for small steps of the different pool parameter triplets until the optimal pool parameters are found. The best parameters, exemplified in the 'Good' situation in the center of this panel, are finally used for the full experimental dataset to obtain all individual time constants τ (or half-lives: $\tau_{1/2} = \log(2) \times \tau$).

The best measures for the lifetimes obtained through fittings are means with confidence intervals⁴⁹. Comparing means when the errors are represented by confidence intervals is not standard practice in statistical workflows. Thus, to obtain statistical differences between two conditions, we provide a statistical script that performs the initial comparisons of protein turnover results.

Final validations (Step 93)

Owing to the wide range of applications of protein turnover measurements, each specific experiment might require a set of tailored validation steps. We include in this protocol a gallery of four controls that can be useful for these validation steps (summarized in Fig. 1e and detailed in the protocol). These include (i) a gas chromatography–MS (GC–MS) method for the direct measure of free $^{13}C_6$ -lysines from biological fluids (such as blood plasma) or tissue samples (option A), which is useful for monitoring the availability of labeled lysines during the labeling procedure; (ii) a protocol and a script that allow definition of the labeling profile of double-labeled peptides ('miscleaved' peptide analysis) (option B; this enables the experimenter to monitor and validate the overall labeling of the proteome while also eventually including the results of double-labeled peptides in the computation of the lifetimes); (iii) a script to predict, on the basis of the lifetime results, the relative labeling of specific proteins in a chase experiment (option C); (iv) a method that allows the measurement of the labeling of an exogenous protein after genetic induction (option D). This method can be used to confirm that the available $^{13}C_6$ -lysine concentration in a particular organ corresponds to the specific value calculated from a global fit of the labeling results (as discussed in the previous paragraph). These final validations may be required only by laboratories with special experimental needs. The paragraphs below provide a more detailed description of each of the validation approaches.

Step 93A: evaluation of lysine labeling in the plasma through GC–MS. Although the global fitting approach is a successful method for estimating the change in lysine labeling, at the experimental level the pool of available lysines can be measured directly from the amount of free lysines in the plasma (or in other biological fluids). It is therefore critical to remove any lysines that have been incorporated into proteins before performing the analysis. Depending on the volume of the sample, several approaches can be used for this purpose, all of which are based on protein precipitation followed by high-speed centrifugation to remove the precipitate.

Step 93B: validation through miscleaved peptide analysis. Validation of the data can be performed by analyzing the labeling profile of peptides that contain two (or more) lysines. Following metabolic labeling in animals, unlabeled lysines from the tissues ($^{12}\text{C}_6$ -lysines) mix with the heavy lysines from the food ($^{13}\text{C}_6$ -lysines). As a result, peptides containing two lysines can be found in one of three different states: (i) both lysines are unlabeled or 'light' (L; two $^{12}\text{C}_6$ -lysines); (ii) both lysines are labeled or 'heavy' (H; two $^{13}\text{C}_6$ -lysines); and (iii) one lysine is labeled and one is unlabeled; thus the peptide is considered 'medium' (M; one $^{12}\text{C}_6$ -lysine and one $^{13}\text{C}_6$ -lysine). We provide a script for predicting the labeling status of a peptide based on its estimated lifetime. The predicted result can be compared to the experimentally measured labeling status as described in the protocol.

Step 93C: validation through pulse-and-chase approaches. The data can be further validated through pulse-and-chase approaches. Because there is an infinite number of possible designs, we included a small script that can be used to predict the relative labeling of a protein for which the pulse-and-chase parameters have been defined. This is particularly useful for estimating the relative labeling of a protein following a known chase time in an animal that was previously pulsed for a determined number of days.

Step 93D: validation by expression of an exogenous inducible protein. The actual labeling of the lysine pool in the organ (or subcellular fraction) can be monitored by expressing an exogenous protein at a defined timepoint during the procedure. Ideally, the protein will be induced transiently and will 'sample' the pool of lysines for a short time interval (~1 d). Owing to the limited sampling time, the protein will act as a sensor of the available lysine pool during its lifetime without influencing the implementation of this validation step. Time-resolved protein expression can be achieved by genetic induction using a Cre-ERT system⁵⁰, which is activated by tamoxifen injection. This strategy can be combined with specific promoters to achieve tissue or cell specificity (see, as an example, the work from Erdmann et al.⁵¹). Other expression methods, such as targeted viral inductions, can also be used successfully for this purpose.

Materials

Biological materials

- Mice. Six 20-week-old male C57BL/6J, Black 6 mice (Jackson Laboratory, stock no. 000664). For final validation in Step 93D, specific tamoxifen-inducible mouse driver lines could be used **!CAUTION** All procedures involving animals must conform to governmental and institutional animal care guidelines. All the mouse data presented in this protocol were obtained through experiments that were approved by the Lower Saxony State Office for Consumer Protection and Food Safety (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit).

Reagents

!CAUTION The Material Safety Data Sheets for the reagents listed below should be read before use. Each reagent should be used and disposed of in accordance with the respective guidelines. Appropriate personal safety protective equipment should be worn at all times **▲CRITICAL** Unless otherwise noted, the reagents listed here can be obtained from any major chemical manufacturer.

Metabolic labeling of animals

▲CRITICAL These diets are stable for ~12 months if stored in a cool, dry place away from light (4 °C). Owing to their high price, it is advisable to estimate the amount of food required for the experiments and pay particular care to their conservation. For estimates of food consumption, refer to Supplementary Table 1 **▲CRITICAL** For final validation, other labeling diets can be used.

- Lys(0)-SILAC-Mouse mock diet ($^{12}\text{C}_6$ -lysine; Silantes Proteomics, cat. no. 230004600)
- Lys(6)-SILAC-Mouse labeling diet ($^{13}\text{C}_6$ -lysine; Silantes Proteomics, cat. no. 230924630)

(Optional) Stimulation of tamoxifen-induced protein expression Step 93D)

- Tamoxifen-free base (Sigma-Aldrich, cat. no. T5648)
- Corn oil (delivery vehicle for fat-soluble compounds; Sigma-Aldrich, cat. no. C8267)

Tissue dissection and subcellular fractionation

- (Optional) Anesthetizing agents (for euthanasia and tissue-collection steps; please act in conformity with governmental and institutional animal care guidelines)

- Hanks' Balanced Salt Solution for dissection (HBSS; Sigma-Aldrich, cat. no. 55021C)
- (Optional) Reagents required for subcellular fractionation in Step 10. Several subcellular fractionations can be coupled with this method; the required reagents depend on the method of choice (Experimental design)

Sample preparation for GC-MS

- Methanol (HPLC grade)
- Chloroform (HPLC grade)
- Methoxyamine hydrochloride (Sigma-Aldrich, cat. no. 226904)
- Water-free pyridine (Sigma-Aldrich, cat. no. 270970)
- *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA; Sigma-Aldrich, cat. no. 69479) ▲ **CRITICAL**
MSTFA is stable for 1 month at 25 °C after opening.

Sample preparation for liquid chromatography-MS

- Phenylmethylsulfonyl fluoride (PMSF; Roth, cat. no. 6367.3)
- C18 matrix (Empore; Fisher Scientific, cat. no. 15334911)
- Formic acid (FA; Fluka, cat. no. 94318)
- RapiGest (Waters, cat. no. 186001861)
- Dithiothreitol (DTT; Merck, cat. no. 43815)
- Iodoacetamide (Sigma-Aldrich, cat. no. I1149)
- LiChrosolv-grade water (Merck, cat. no. 1153332500)
- LiChrosolv-grade methanol (Merck, cat. no. 1060072500)
- LiChrosolv-grade acetonitrile (ACN; Merck, cat. no. 1000302500)
- Ammonium bicarbonate (ABC; Sigma Aldrich, cat. no. A6141)
- Sequencing-grade modified trypsin (Promega, cat. no. V5111)
- Trifluoroacetic acid (TFA; Fluka, cat. no. T6508)
- Acetone (Merck, cat. no. 1000141011)
- NuPAGE sample loading buffer (Invitrogen, cat. no. NP0007)
- NuPAGE antioxidant (Invitrogen, cat. no. NP0005)
- NuPAGE MOPS running buffer (20×; Invitrogen, cat. no. NP000102)
- NuPAGE Novex 4–12% Bis-Tris gels (1 mm; Invitrogen, cat. no. NP0321BOX)
- Benzonase nuclease (Sigma-Aldrich, cat. no. E1014-5KU)
- Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, cat. no. 23225)
- Sucrose (320 mM)
- HEPES-NaOH (5 nM)
- Coomassie Brilliant BlueG-250 dye (0.08% (wt/vol); Thermo Fisher Scientific, cat. no. 20279)
- Orthophosphoric acid (1.6% (vol/vol); Merck, cat. no. 10005731000)
- Ammonium sulfate (8% (wt/vol); Sigma Aldrich, cat. no. A4418-500G)
- Methanol (20% (vol/vol); Merck, cat. no. 1060091011)
- Calcium chloride (CaCl₂; 100 mM; Sigma Aldrich, cat. no. 449709-10G)
- ddH₂O
- Kasil liquid potassium silicate (PQ)
- Formamide

Equipment

Metabolic labeling of animals and tissue collection

- Cages with compartments for suitable delivery of small quantities of food (Fig. 2a)
- Weighing scale for small animals (a 100-g-range scale and a plastic box can be used for this purpose)
- Dissection tools (forceps, tweezers and scissors)

Tissue sample preparation

- Microcentrifuge tubes (1.5 and 2 ml)
- Conical tubes (15 ml)
- Benchtop microcentrifuge, preferably refrigerated (Eppendorf, model no. 5415 R) ▲ **CRITICAL** For subcellular fractionation in Step 10, ultracentrifuges may be required. For detailed descriptions, see the most appropriate protocols^{44–47} as well as the 'Experimental design' section.
- Parafilm M (Sigma-Aldrich, cat. no. P7793-1EA)

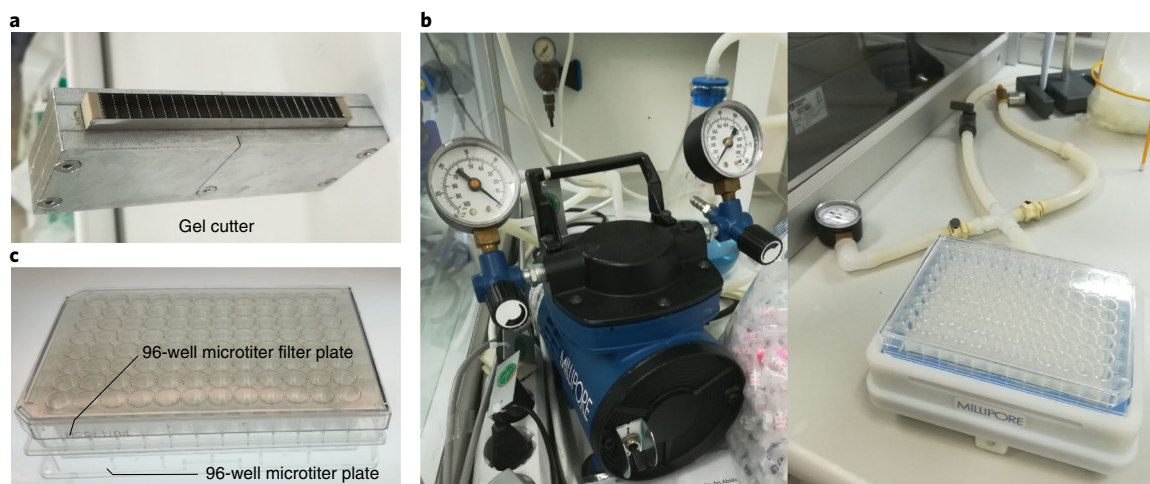


Fig. 4 | Custom-made gel cutter and 96-well washing setup. **a**, Picture of custom-made gel cutter. **b**, Picture of vacuum manifold pump (left) connected to 96-well plate kit (right) for the removal of solvents and extraction of peptides from 96-well microtiter filter plates. **c**, Arrangement of 96-well microtiter filter plate containing gel slices (top) and 96-well microtiter plate (bottom) for extraction of peptides during and after trypsin digestion.

Protein analysis by reverse-phase liquid chromatography–MS

- HPLC system (Thermo Fisher Scientific, model no. UltiMate 3000 RSLCnano)
- Mass spectrometer compatible with MaxQuant (Q-Exactive or latest Bruker instruments, where MaxQuant analysis can be applied)
- UV spectrophotometer for protein estimation
- Laminar flow hood
- Vacuum concentrator (SpeedVac; Thermo Scientific)
- Pestle tissue grinder (Thomas)
- Water bath sonicator (Sonorex)
- Fused silica capillary (i.d. 75 μm , o.d. 363 μm ; Polymicro Technologies, cat. no. TSP-0753375)
- Self-packed fused silica capillary columns (75- μm i.d., 15-cm length; Polymicro Technologies; columns should be filled with Reprosil-Pur Basic C18-AQ 1.9- μm -pore-size beads (Dr. Maisch). Alternatively, use commercially available Acclaim PepMap 100 C18 liquid chromatography (LC) columns (Thermo Fisher Scientific), following the instrument setup provided by the supplier
- Trapping C18 column (Acclaim PepMap 100 C18; 300- μm i.d. \times 5 mm, 5 μm , 100 \AA ; Dionex)
- ThermoMixer (Eppendorf)
- MultiScreenHTS vacuum manifold (Merck Millipore, cat. no. MSVMHTS00)
- Magnetic stirrer
- Vial with small stirrer
- Large stirrer
- Three-way valve channel pipeline (Swagelok 316)
- Vortex
- Microtiter filter plates (96-well; Merck, cat. no. MSRPN0450)
- Microtiter plate (96-well; Merck, cat. no. MDCPN2M50)
- Microtiter plate polyolefin foil (96-well; HJ-Bioanalytik, article no. 900320)
- (Optional) Gel cutter (custom made; see Fig. 4a)

GC–MS

- Autosampler (PAL3 RTC 120)
- Gas chromatograph (Agilent, model no. 7890B) connected to a mass selective detector (Agilent, model no. 5977N)
- Column (30 m \times 0.25 mm; 0.25- μm coating thickness; Agilent, cat. no. HP5-MS)

Software

- MaxQuant (http://www.coxdocs.org/doku.php?id=maxquant:common:download_and_installation#download_and_installation_guide)

- MATLAB (v.2014b or more recent) with ‘Optimization Toolbox’ and ‘Statistics and Machine Learning Toolbox’ (MathWorks). A free 30-d trial version of MATLAB can be downloaded from <http://www.mathworks.com>.
- Updated Microsoft Office (<https://products.office.com/en-us/home>) or Open Office (<https://www.openoffice.org/>)
- We used MSD ChemStation software D.01.02.16 (Agilent). The MSD ChemStation is no longer available, but Agilent software capable of performing the same functions is available (<https://www.agilent.com/en/products/software-informatics/openlab-software-suite>)

Reagent setup

Homogenization buffer

This solution is 320 mM sucrose and 5 mM HEPES–NaOH, pH 7.4. It can be stored at 4 °C for up to 1 week.

Colloidal Coomassie staining solution

This solution is 0.08% (wt/vol) Coomassie Brilliant Blue G-250 dye, 1.6% (vol/vol) orthophosphoric acid, 8% (wt/vol) ammonium sulfate and 20% (vol/vol) methanol. Coomassie staining solution can be stored at 25 °C for up to 1 month.

HPLC buffer A

This solution is 0.1% (vol/vol) FA in LiChrosolv-grade water. This buffer should be prepared fresh, but it can be stored at 25 °C for up to 6 months.

HPLC buffer B

This solution is 0.1% (vol/vol) FA and 95% (vol/vol) ACN in LiChrosolv-grade water. This buffer should be prepared fresh, but it can be stored at 25 °C for up to 6 months.

ABC buffer

This solution is 100 mM ABC (pH 8) ▲ **CRITICAL** Prepare freshly and discard within a day. Check the pH of the buffer with a pH paper indicator.

Reducing solution

This solution is 10 mM DTT in 100 mM ABC buffer ▲ **CRITICAL** Prepare shortly before use.

IAM

Iodoacetamide solution (IAM) is 55 mM iodoacetamide in 100 mM ABC ▲ **CRITICAL** Prepare shortly before use.

100 mM CaCl₂

Once sterile filtered, this buffer can be stored at 25 °C for up to 1 year. This buffer can be divided into aliquots and stored at –20 °C for up to 1 year.

Digestion buffer

Mix 6.5 ml of LiChrosolv-grade water, 6.5 ml of 100 mM ABC and 650 µl of 100 mM CaCl₂ ▲ **CRITICAL** Prepare shortly before use.

Trypsin buffer

Resuspend 20 µg of trypsin in 1.4 ml of digestion buffer ▲ **CRITICAL** Prepare shortly before use.

Sample loading buffer

This solution is 5% (vol/vol) LiChrosolv-grade ACN and 0.1% (vol/vol) FA in LiChrosolv-grade water ▲ **CRITICAL** Prepare shortly before use.

Extraction solution for lysine measurements

This buffer is methanol/chloroform/ddH₂O (129:60:25 (vol/vol/vol)); it can be stored at 25 °C for up to 1 month.

Methoxyamine hydrochloride solution

This solution is 3% (wt/vol) methoxyamine hydrochloride in water-free pyridine. This buffer can be stored at 25 °C for up to 3 d.

Equipment setup

UltiMate 3000 RSLCnano HPLC gradient (Step 59)

Time interval (min)	Gradient: %B (vol/vol)
0	5
0	5
3	8
76	46
76	90
82	90
82.1	5
88	5

Agilent MS settings (Step 60)

Scan event	Method parameter	Value
Scan event 1 (MS1)	Analyzer	FTMS
	Mass range	350-1,600 <i>m/z</i>
	AGC target value	1 × 10 ⁶ ions
	Maximum ion time (IT)	50 ms
	Resolution at 200 <i>m/z</i>	60,000 FWHM
	Polarity	Positive
	Top N	30%
	Data type	Profile
Scan event 2 (MS2)	Analyzer	Orbitrap
	Resolution at 200 <i>m/z</i>	15,000 FWHM
	Spectrum data	Centroid
	Normalized collision energy (NCE)	30%
	AGC target value	1 × 10 ⁵ ions
	Isolation window	1.6 <i>m/z</i>

AGC, automatic gain control; FTMS, Fourier transform mass spectrometer, FWHM, full width at half maximum.

GC-MS settings using an HP5-MS column (Step 93A(ix))

Parameter	Setting
Helium flow	1 ml/min
Inlet temperature	250 °C
Temperature gradient	180-250 °C at 5 °C/min 250-320 °C at 15 °C/min 250-320 °C at 15 °C/min 320 °C for 3 min
Electron energy	70 eV
Transfer line temperature	280 °C
Ion source temperature	230 °C
Mass range	70-600 Da/e

Metabolic mouse labeling and tissue collection ● Timing variable, from a few days to months

! CAUTION All procedures involving animals must conform to governmental and institutional animal care guidelines.

▲ CRITICAL Because the entire procedure relies on the correct pulse feeding of mice, all experiments critically depend on this process. To avoid affecting mouse behavior, the entire procedure is designed for minimal interference with mouse physiology. To this aim, mice are fed ad libitum. Because metabolic diets are expensive, it is important to design the feeding scheme and calculate the required amount of food. As a reference value, an adult male mouse will eat ~3–4 g of food per day, whereas a female under normal conditions will eat slightly less. As a rule of thumb, each mouse eats 1/10th of its weight in dry pellet food daily.

▲ CRITICAL See Supplementary Table 1 for timing details.

1 If you fear food competition among the animals, before the beginning of the labeling procedure, divide the animals by placing them in separate cages.

2 Track animal weight and possibly food consumption daily during the labeling procedure (at the same hour of the day). Sudden changes in weight or eating behavior should be carefully monitored.

▲ CRITICAL STEP For ensuring the correct labeling of mice, it is necessary to evaluate weight differences across the labeling procedure and exclude from the study any animals with abnormal feeding behavior.

▲ CRITICAL STEP Mice become accustomed to specific diet formulations, and a change in the administered diet usually results in weight loss. For this reason, it is necessary to habituate the animals to the Lys(0)-SILAC-Mouse mock diet for some days before the start of the experiment. Usually 3–5 d are sufficient for the habituation process. Habituation can be considered complete if after 2–3 d mice return to their initial weight.

? TROUBLESHOOTING

3 Feed the mice with the Lys(0)-SILAC-Mouse mock diet for 3–5 d. Animals should become accustomed to the new diet in this time period. To speed up the habituation step, some pellets can be inserted directly into the mouse cage.

▲ CRITICAL STEP It is not advisable to leave too much food in the cage because it will become spoiled and the mice will end up playing with rather than eating the pellets. At the same time, most cages are not compatible with the small quantities of food pellets that are used for metabolic labeling. Mice should be able to grasp food from the bottom of the pellet rack, and if the food weight is not sufficient, they will not be able to eat correctly. To this purpose, a stainless-steel cube can be used to weight the food pellets (see cage setup scheme in Fig. 2a).

4 Following habituation with the Lys(0)-SILAC-Mouse mock diet, proceed to metabolically labeling the mice with the Lys(6)-SILAC-Mouse labeling diet.

▲ CRITICAL STEP The time and day of the beginning of the metabolic labeling should be planned carefully because the tissue collection and possibly the cell fractionation should be performed at precise time intervals following this step. For a standard protein turnover evaluation, we advise collecting tissues from three cohorts of animals after short (5 d), medium (14 d) and long (21 d) pulsing times (see also Supplementary Table 1). For more precise evaluations of long-lived proteins and ELLPs^{4,9,10}, longer time intervals might be required.

5 During the first days of the labeling procedure, remove the fecal pellets or change the bedding daily.

▲ CRITICAL STEP Rodents have the tendency to eat fecal pellets. To avoid the influence of the unlabeled amino acids from the feces on the labeling, it is important to make sure that the unlabeled feces are not eaten, because this might interfere with the experiment outcome. Once the feces are mostly labeled (after ~4–5 d), it is not so critical to eliminate fecal pellets. An alternative is to use metabolic cages, which collect the fecal pellets in a compartment inaccessible to mice, although this might affect mouse behavior.

6 After the desired pulsing time, prepare the dissecting tools, the materials and the solutions that will be used for the chosen tissue preparation and subcellular fractionation procedure.

7 Kill mice using the appropriate method, in conformity with local guidelines.

8 Collect mouse tissues of interest rapidly in 15-ml conical tubes filled with ice-cold HBSS buffer.

9 Wash the blood away from the organs thoroughly, changing the ice-cold HBSS buffer 2–3 times.

▲ CRITICAL STEP Blood is a ‘liquid organ’ and as such will contaminate protein measurements from other organs. To further decrease the contamination of blood, it is possible to cut the organs

into 1-mm cubes before these washing steps. A more laborious alternative is to perfuse the animal with HBSS buffer before dissection⁵².

- 10 (Optional) Decrease the complexity of the sample by performing further tissue or subcellular fractionation steps^{44–46} (Experimental design). Note that several tissue or subcellular fractionation approaches can be coupled with this method. A limiting factor can be the tissue size and the total protein content of the collected samples. For reliable MS measurements, we advise collection of at least 100 µg of total protein for each sample to be analyzed. The protein amount should be measured by a reliable assay before addition of the sample buffer. If one animal is not sufficient, the subcellular fractions of several animals can be pooled.
 - For synaptic vesicle isolation and the required reagents, refer to Ahmed et al.⁴⁴.
 - For isolation of the brain synaptosomal fraction, use either the above-mentioned protocol until Step 4 or, alternatively, refer to Dunkley et al.⁴⁵.
 - For the isolation of mitochondria, refer to Sims et al.⁴⁶.

▲ **CRITICAL STEP** For virtually all subcellular fractionation methods, the osmolarity of the solutions used is critical. Check the osmolarity whenever required.

■ **PAUSE POINT** Samples can be stored at –80 °C at this point for several months.

Sample processing ● Timing ~1 week

- 11 *Protein concentration determination (Step 11)*. Determine the protein concentration using a standard BCA (or Bradford) protocol. Reliable results can be obtained with the BCA Protein Assay Kit by following the guidelines provided by the supplier.

▲ **CRITICAL STEP** For the calibration curve, use the same buffer in which the tissue or subcellular fractions have been resuspended.

? **TROUBLESHOOTING**
- 12 *Protein precipitation (Steps 12–14)*. Precipitate ~200 µg of protein by adding a 10× volume of pre-chilled (–20 °C) acetone and incubating the solution at –20 °C overnight.

▲ **CRITICAL STEP** Avoid keratin. We recommend wearing gloves while handling the proteomic samples. Contamination with keratin will most likely suppress the proteomic result. We also recommend performing all the digestion steps (handling gel, gel cutting, addition of solvents and reagents) under a laminar flow hood.
- 13 Spin the precipitated protein by centrifuging in table-top centrifuge at 10,000g for 30 min at 25 °C.
- 14 Discard the supernatant carefully and dry the pellet for 2–3 min in a SpeedVac concentrator. Evaporate all the remaining acetone from the precipitated protein by leaving the vials open under a chemical hood at room temperature (RT; 20–25 °C).
- 15 *Gel electrophoresis (Steps 15–20)*. Add 10 µl of NuPAGE sample loading buffer, 4 µl of reducing agent (included in the NuPAGE kit) and 40 µl of water to each 200 µg of acetone-precipitated protein sample from Step 14 and vortex for 30 s.
- 16 Heat the samples at 95 °C for 5 min.
- 17 Shortly spin the tubes (1,000g, 25 °C, 30 s) to collect the entire sample at the bottom of the tubes.
- 18 Load 25 µl of protein sample (corresponding to 100 µg of total protein) onto a NuPAGE gradient gel (the 4–12% gradient NuPAGE gel has performed superiorly in our experience).
- 19 Run the gel at a constant voltage (i.e., 200 V for ~50 min).
- 20 Incubate the gel with ~100 ml of colloidal Coomassie G250 overnight to verify the correct protein separation.

? **TROUBLESHOOTING**
- 21 *In-gel digestion (Steps 21–41)*. Cut each gel lane into a defined number of small pieces. For this purpose, we use a custom-made gel cutter (Fig. 4), which allows easy cutting of each lane precisely into 23 pieces.
- 22 Further cut each gel slice into smaller pieces (usually eight pieces of ~1 mm × 1 mm) and place them into a 96-well microtiter filter plate.
- 23 Wash the gel slices to remove water-soluble contaminants by adding 150 µl of water to the top of each gel piece. Incubate for 15 min in a ThermoMixer at 300 r.p.m. at 37 °C.
- 24 Remove the solvent using the vacuum manifold pump kit (as shown in Fig. 4b).

▲ **CRITICAL STEP** Remove the solvents from the gel pieces by using the vacuum manifold pump and kit for 96-well microtiter filter plates. The regular pressure should be <–35 Pa. Alternatively, use pipette tips to remove the solvent manually from the microtiter plate or vials. Avoid pipetting

out gel slices. Preferably, use long gel-loading pipette tips to pipette out solvents because the fine tip avoids aspiration of the gel pieces.

? TROUBLESHOOTING

- 25 Wash the gel slices by adding 150 μ l of 100% (vol/vol) LiChrosolv-grade ACN and incubate them for 15 min at 37 $^{\circ}$ C, with shaking at 300 r.p.m.
▲ CRITICAL STEP Addition of organic solvents such as ACN will shrink the size of the gel pieces. This is due to removal of water from the gel pieces. Take extra care while handling small gel pieces. The small gel pieces tend to exit the well or be pipetted out because of their small size.
- 26 Remove the solvent. Evaporate the excess amount of LiChrosolv-grade ACN under a chemical hood for a few minutes.
▲ CRITICAL STEP Traces of ACN hinder the reduction, alkylation and trypsinization reactions of proteins. For this reason, it is critical to evaporate the ACN from the gel pieces. Incubation of gel pieces with ACN not only shrinks gel pieces but has been suggested to also expose proteins to the outer part of the gel, thus enabling efficient reduction, alkylation and final trypsinization of proteins.
- 27 Reduce the proteins by adding 100 μ l of 10 mM DTT to the gel pieces and incubating them at 56 $^{\circ}$ C for 1 h in a ThermoMixer at 300 r.p.m. This will reduce and thereby cleave the disulfide bridges from the proteins in the gel slices. Eliminate the solvent with the pump as illustrated in Fig. 4.
- 28 Wash the gel slices by adding 150 μ l of 100% (vol/vol) LiChrosolv-grade ACN and incubate them for 15 min at 37 $^{\circ}$ C, with shaking at 300 r.p.m. Remove the solvent with the pump.
- 29 Alkylate the cysteine side chains of the proteins by adding 100 μ l of 50 mM IAM to the gel pieces and incubate them for 20 min in the dark at 37 $^{\circ}$ C, with shaking at 300 r.p.m.
- 30 Remove the solvent with the pump.
- 31 Add 150 μ l of ABC buffer to the gel pieces and incubate them for 15 min at 37 $^{\circ}$ C, with shaking at 300 r.p.m.
- 32 Remove the solvent with the pump.
- 33 Add 150 μ l of LiChrosolv-grade ACN to the gel pieces and incubate them for 15 min at 37 $^{\circ}$ C, with shaking at 300 r.p.m.
- 34 Remove the solvent with the pump.
- 35 Once again, add 150 μ l of LiChrosolv-grade ACN to the gel pieces and incubate them for 15 min at 37 $^{\circ}$ C, with shaking at 300 r.p.m.
- 36 Remove the solvent with the pump.
- 37 Place a fresh 96-well microtiter plate underneath the 96-well filter plate (Fig. 4c).
▲ CRITICAL STEP From this step onward, all the liquids will be pooled and collected in the 96-well microtiter plate underneath. Do not discard the liquids.
- 38 Add 20 μ l of trypsin buffer to each gel piece.
▲ CRITICAL STEP Avoid shaking the plates when the trypsin is added because if the trypsin does not remain in the gel long enough, the proteolytic step will be inefficient. While extracting the peptides with the 96-well plate kit, make sure that the plate topology of the upper 96-well filter plate matches that of the 96-well microtiter plate underneath.
- 39 Incubate the gel pieces at 4 $^{\circ}$ C for \sim 30 min to allow the trypsin to diffuse into the gel.
- 40 After trypsin diffusion, cover the gel pieces with 40–80 μ l of digestion buffer.
- 41 To avoid evaporation, cover the 96-well filter plate tightly with Parafilm and incubate it at 37 $^{\circ}$ C overnight without shaking. In our experience, overnight trypsinization ensures complete digestion. The digestion effectiveness for the samples can be tested (see Troubleshooting advice for Step 66 for details).
- 42 *Extraction of peptides (Steps 42–51)*. Add 20 μ l of 5% (vol/vol) FA solution and incubate the gel pieces for 15 min at 25 $^{\circ}$ C to stop trypsinization.
- 43 Add 50 μ l of 50% (vol/vol) LiChrosolv-grade ACN in LiChrosolv water to each well and incubate the plate for 15 min at 37 $^{\circ}$ C, with shaking at 300 r.p.m., to extract the peptides.
- 44 Use the vacuum manifold pump to extract all the digested proteins from the 96-well filter plate to the 96-well microtiter plate underneath (as in Fig. 4).
▲ CRITICAL STEP Apply a pressure of 40–60 Pa. Higher or lower pressures are not compatible with the filter membrane of the 96-well microtiter filter plate.
- 45 Repeat Steps 43 and 44 once more to extract as many peptides as possible.
- 46 To make sure that the peptides have been completely extracted from the gel pieces, add 50 μ l of water to each well of the 96-well filter plate and incubate it for 15 min at 37 $^{\circ}$ C, with shaking at 300 r.p.m.

- 47 Extract the peptides into the respective wells of the 96-well microtiter plate underneath.
- 48 To each well of the 96-well filter plate, add 50 μ l of LiChrosolv-grade ACN; incubate the plate for 15 min at 37 °C, with shaking at 300 r.p.m.
- 49 Use the vacuum manifold pump to extract all the digested proteins from the 96-well filter plate to the 96-well microtiter plate underneath (all the extractions are collected together in the same 96-well microtiter plate underneath, for a total volume of ~250–300 μ l).
- 50 Dry the peptides with a SpeedVac concentrator.
- 51 Seal the 96-well microtiter plate containing the digested peptides with 96-well-plate polyolefin foil.
 - **PAUSE POINT** Once sealed, the dried samples can be stored at –20 °C for several months.

MS analysis ● **Timing variable, ~3 d per sample analyzed**

▲ **CRITICAL** Timing varies from several hours to several days, depending on the number of samples. As a reference, each lane analyzed requires ~24 h of machine time, and usually two machine replicates are run, so the analysis takes ~2 days per sample analyzed.

- 52 *Sample preparation for LC (Steps 52–55)*. Re-dissolve the dried peptides from Step 51 in 20 μ l of the sample loading buffer for MS containing 5% (vol/vol) LiChrosolv-grade ACN and 0.1% (vol/vol) FA in LiChrosolv-grade water.
- 53 Sonicate the vials in a water bath for 3 min (maximum cycle time).
- 54 Centrifuge the vials in a table-top centrifuge at 12,000g for 5 min at RT to pellet the debris.
 - ▲ **CRITICAL STEP** If there are visible aggregates floating in the resuspended peptide solution, spin the vials again for 10–15 min at 12,000g at RT.
- 55 Carefully pipette the supernatant into LC glass vials.
- 56 *Reverse-phase HPLC–MS (Steps 56–60)*. Couple the self-made or the commercially purchased column with the UltiMate 3000 RSLCnano HPLC system.
- 57 Inject 5 μ l of peptide solution on an online UltiMate 3000 RSLCnano HPLC system coupled to the Q Exactive HF.
- 58 Load peptides onto a reverse-phase C18 Acclaim PepMap100 5- μ m trapping-column for 3 min. After 3 min, switch the valve online on an analytical C18 column (30-cm length, 75- μ m i.d.). Although there are commercial alternatives (C18 Acclaim PepMap100 15-cm column), we prepare the C18 column in-house using a ReproSil-Pur C18 AQ 1.9- μ m reverse-phase resin.
- 59 Fractionate the peptides with the buffer B gradient at a flow rate of 300 nl/min over an 88-min gradient time (see Equipment setup). Set the pre-column and column temperatures to 50 °C during the chromatography.
- 60 Acquire the MS data by scanning the precursors in a mass range from 350 to 1,600 Da at a resolution of 60,000 at m/z 200. The 30 top most intense precursor ions are chosen from MS1 for MS2 fragmentation. For MS2, higher-energy collisional dissociation (HCD) fragmentation is usually performed with the AGC target fill value of 1×10^6 ions. The precursors are isolated with a window of 1.6 Da (Equipment setup). Note that, in our experience, two machine replicates are a good compromise between machine time usage and precision.

? **TROUBLESHOOTING**

- 61 *MS data analysis (Steps 61–70)*. Download the latest UniProt database (specific for the organism under investigation) from <https://www.uniprot.org/proteomes/>.
- 62 Open MaxQuant⁵³ v.1.0.3.5 based on the Andromeda search engine⁵⁴.
 - ▲ **CRITICAL STEP** Any version of MaxQuant software can be used to analyze the data. While using Bruker instruments, check if the version of MaxQuant is compatible with the instrument. Older versions of MaxQuant are not compatible with all Bruker output files. Additional practical information about the use of MaxQuant can be found in the protocol from Tyanova et al.⁵⁵.
- 63 In MaxQuant, under the ‘Andromeda’ tab, select your UniProt database and restart MaxQuant.
- 64 Load the RAW files into the MaxQuant software.
- 65 In MaxQuant, assign—for all the RAW files coming from the same lane (same experiment)—a unique identifier so that they are analyzed as a single sample.
 - ▲ **CRITICAL STEP** Use unique names for all the RAW files that represent different biological samples.
- 66 After efficient trypsinization, there are only two types of peptides in the samples, i.e., the light version and the heavy version. For this reason, once it is confirmed that the trypsinization is complete (Troubleshooting), one can specify a multiplicity of ‘2’ in MaxQuant (Fig. 5). For peptides containing more than two lysines, refer to the validation part of the protocol (Step 93B).

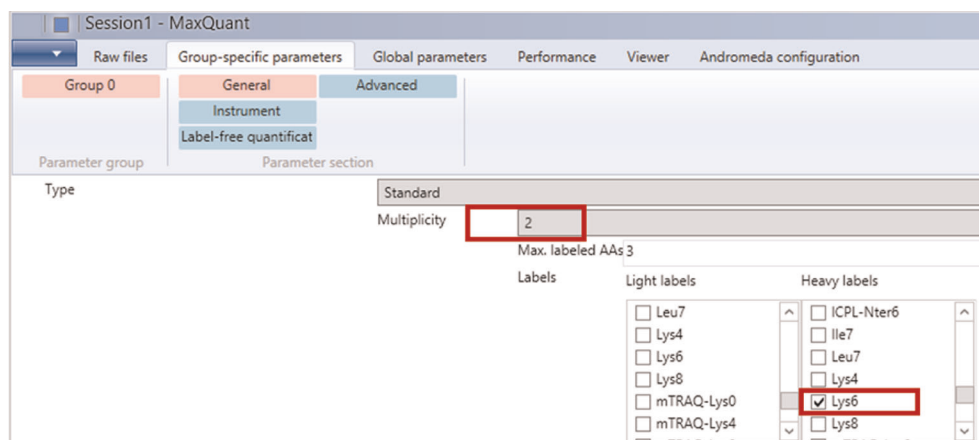


Fig. 5 | Sample MaxQuant screenshot showing the settings for SILAC quantification.

▲ CRITICAL STEP The multiplicity corresponds to the number of labels present in the sample. For a pulse with $^{13}\text{C}_6$ -lysine (Lys6), the multiplicity will be 2: Lys0 (light) and Lys6 (heavy).

? TROUBLESHOOTING

67 Select 'Lys6' from among the heavy labels (Fig. 5).

68 Select the database in 'Global parameters' (Fig. 5).

69 Untick or switch 'OFF' in the 'Requantify' option.

! CAUTION If the 'Requantify' option is switched 'ON', MaxQuant will consider background values (mainly corresponding to noise) as data for the missing SILAC pairs. This would result in an overall increase in proteome coverage (i.e., the number of proteins for which heavy-versus-light values will be reported), but the values will be incorrect and, as a consequence, the lifetimes will be incorrectly determined. Thus, to avoid reporting false protein turnover values, we recommend keeping the 'Requantify' option 'OFF'.

70 Set the peptides for quantification (protein quantification/peptides for quantification) as 'Unique + razor peptide' (Fig. 6). The search will provide the heavy-versus-light ratios that can be used to calculate protein lifetimes (see Data interpretation below).

▲ CRITICAL STEP This option could be set to 'Unique' in order to acquire accurate protein lifetimes by avoiding the possible homologous peptides. However, using this option might also lead to a decrease in the total protein coverage due to absence of peptides for protein quantification.

? TROUBLESHOOTING

Data interpretation ● Timing variable, ideally 1-2 d

▲ CRITICAL All MATLAB scripts (*.m) are available as Supplementary Data 1. The scripts must be copied into a common folder that is searchable by MATLAB (add the folder to the MATLAB search path). Make sure the 'Optimization' and 'Statistics and Machine Learning' toolboxes are installed.

The following steps are best run from the graphical user interface (turnoverGUI.m).

71 *Data import (Steps 71-78)*. First, make sure that the data are formatted as heavy-versus-light ratios. Use the data import script (included in Supplementary Data 1) to import the data obtained from the MS analysis. Start the graphical user interface by typing `turnoverGUI` into the MATLAB command window without parameters (and without the quotation marks). For evaluation purposes, an example is given in Supplementary Data 2.

? TROUBLESHOOTING

72 In the 'template' tab of the graphical user interface of the turnover script (Fig. 7), fill in the names of all unique conditions (treatments and/or tissues) in the 'Condition(s)' field (using a new line for each condition, with no empty lines).

73 Fill in all unique experimental pulse times (in days) in the 'pulse times' field. These are the same time pulses as those presented in Fig. 2b or in Supplementary Table 1.

74 Fill in the number of biological replicates. These would probably correspond to the number of single animals used, unless more than one mouse is pulled for subcellular fractionation.

75 Fill in the number of MS machine replicates. In our experience, two machine replicates are a good compromise between machine time usage and precision.

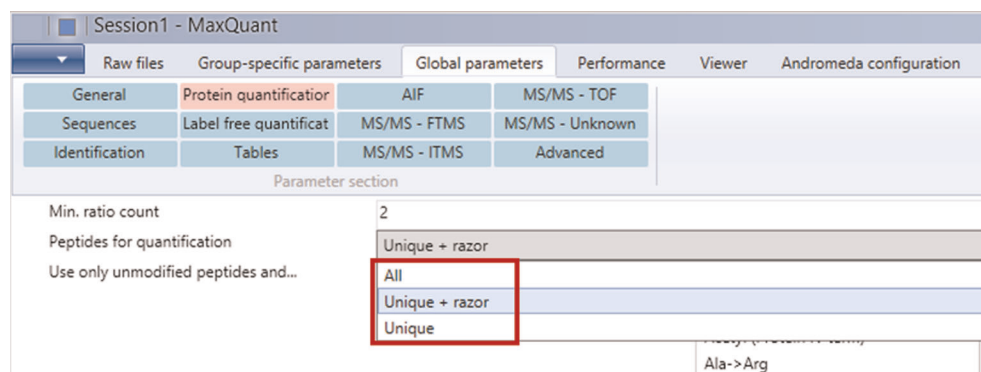


Fig. 6 | Sample MaxQuant screenshot showing the settings for quantification.

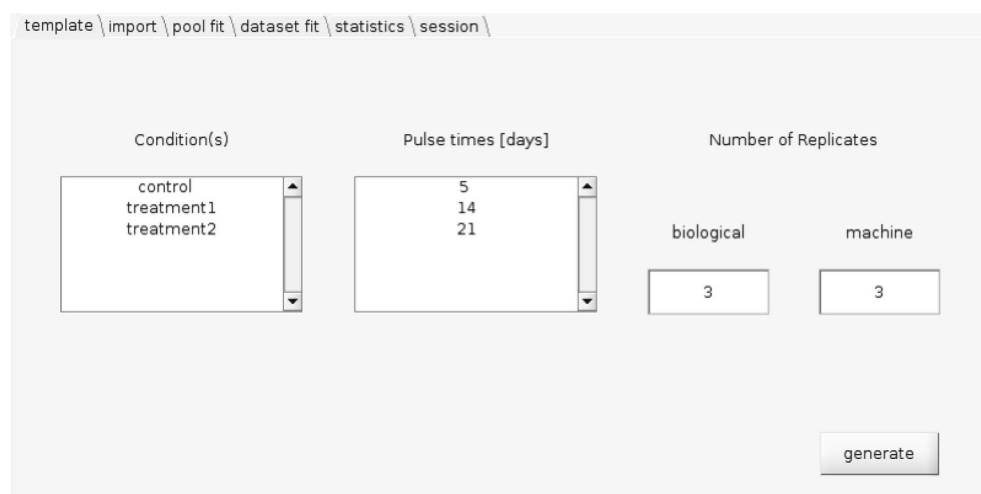


Fig. 7 | Exemplary screenshot of the graphical interface from the 'template' tab of the turnover script. See also Supplementary Data 1.

- 76 Use the 'generate' button to create an Excel file containing the headers necessary for the correct import of the data and save it in the same directory where the scripts are.
- 77 Copy all experimental heavy/light ratios into the corresponding columns of the generated file, directly under the header rows. Copy the corresponding protein IDs into the left column. Save the modified file.
- ▲ CRITICAL STEP** Make sure all experimental data points in the Excel file are heavy-versus-light ratios. If correctly input, the ratios will be internally converted to heavy-versus-total during the import step. Missing conditions and replicates will be ignored.
- 78 In the 'import' tab of the graphical user interface (Fig. 8), load the Excel file created in Step 77. General information about the dataset is shown in a text window. A testing dataset for evaluation purposes is included as Supplementary Data 2.
- 79 *Fitting of pool parameters (Steps 79–84)*. In the 'pool fit' tab of the graphical user interface (Fig. 9), select the reference condition from which the pool parameters are to be calculated (usually control conditions). Set the minimum number of data points as the threshold for protein inclusion. The number of proteins thereby selected will be displayed on the top in the threshold drop-down menu.
- ▲ CRITICAL STEP** This part of the script will be used to calculate the pool of lysines available for protein synthesis with a global fitting approach from the labeling data as previously described⁴. The pool of available lysines has two components: a fast component deriving from the rapid incorporation of the ¹³C₆-lysines from the labeled food and a slower component that depends on the slow exchange of the labeled ¹³C₆-lysines with the unlabeled ¹²C₆-lysines deriving from the degradation of the proteome. For the determination of individual protein lifetimes, the exchange of the underlying pool of available heavy and light lysines must be estimated. The optimal pool

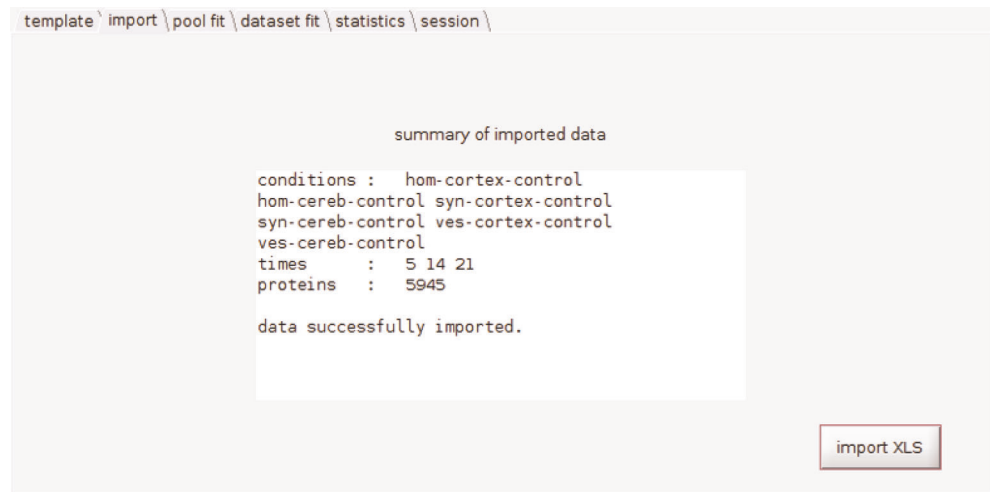


Fig. 8 | Sample screenshot of the graphical interface from the 'import' tab of the turnover script. See also Supplementary Data 1.

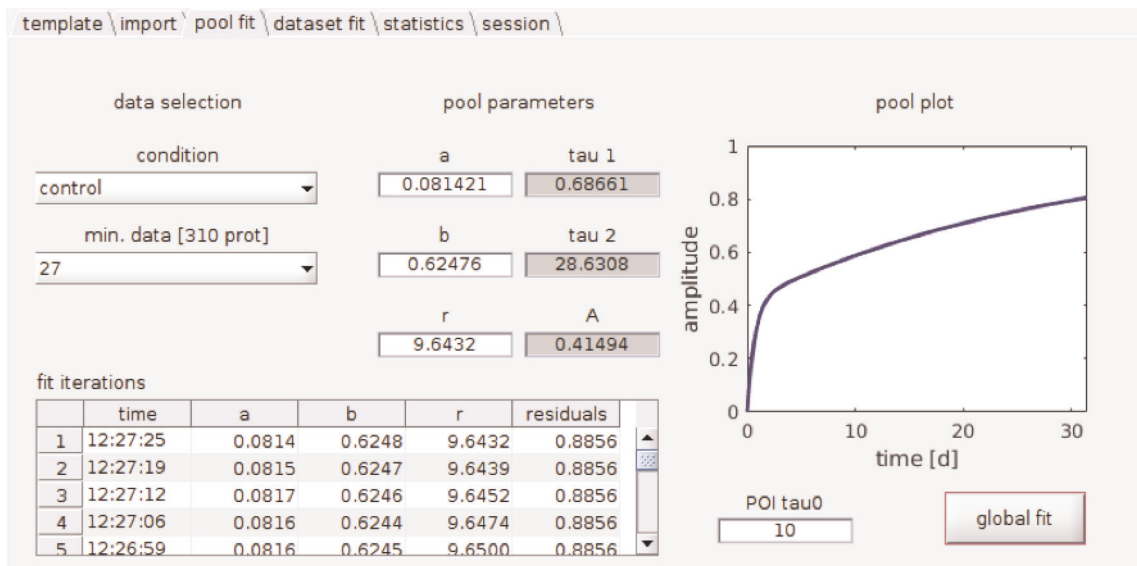


Fig. 9 | Sample screenshot of the graphical interface from the 'pool fit' tab of the turnover script. See also Supplementary Data 1.

parameters are found by minimizing the sum of residuals from a simultaneous fit of multiple proteins. It is computationally expensive to include the total set of proteins for this step, and, in our experience, including weak and noisy signals from proteins that are detected only in a small number of experiments does not substantially improve the quality of the pool fit. For this reason, a threshold can be set for the number of required data points for proteins to be included into the global fit.

80 Fill in the starting parameters (a, b, r) for the pool fit, or use the predefined values that were measured in the key reference work⁴.

▲ CRITICAL STEP The parameters describe global protein generation/degradation rates (a), feeding/excretion rates (b) and the ratio of protein-bound versus free lysine pools (r). In addition, derived parameters (τ_1, τ_2, A) are shown to the right for a better interpretation of the pool curve: τ_1 is the fast time constant of the exponential rise, τ_2 the slow time constant, and A is the amplitude of the fast component (and $1-A$ the amplitude of the slow component).

81 Fill in the starting time constant for the fit of individual proteins (POI τ_0). A value in the vicinity of the real protein time constant will improve the speed and robustness of the fit. A plot in this tab shows the temporal evolution of the pool using the current pool parameters and will be

updated upon manual parameter modification and after each step of the global fit procedure. The optimization should be run until a message window appears saying 'optimization of pool parameters', signaling completion.

▲ CRITICAL STEP Depending on the computer and the number of proteins, the following global fit can take from several minutes to several hours. It is interruptible by pressing Ctrl+C in the MATLAB command window but should be run until a message window signals completion. The progress to this point can be saved in the 'session' tab using the 'save session' button. A session variable will be created in the MATLAB workspace that can be saved to disk (by typing `save <filename> turnoverSession`). Loading the session (type `load <filename>`) and starting the `turnoverGUI.m` script with the session as parameter (`turnoverGUI(turnoverSession)`) will restore the saved session.

? TROUBLESHOOTING

- 82 Start the pool fit with the 'global fit' button. Statistics of the current iteration will be displayed in the adjacent log table, with the most recent entry at the top of the list.
- 83 After the completion message window appears, save the session to the MATLAB workspace (see above) to use the pool parameters at a later time point or with other datasets.
- 84 Different experimental conditions might induce a change in free lysine pool dynamics, resulting in different global pool fit results and possibly different lifetimes. To estimate how well different pools can model different datasets, an additional MATLAB script can be used in the following way: first, do a global fit as in the previous two steps and save the session using a unique name (e.g., 'ts1' for the first pool obtained from the first dataset) in the 'session' tab. Second, change the reference condition to the next relevant condition (Step 79, in the 'condition' drop-down menu) and repeat the process until all relevant datasets are fitted and saved as individual session variables (e.g., 'ts1', 'ts2'...). Third, type the following line into the MATLAB command window to call the comparison script using a cell array '{ }' of all sessions as the input parameter (in the example here with the three sessions):

```
comparePools ({ts1,ts2,ts3});
```

Wait until the script is finished. This gives, as a result, the matrix of normalized residuals. In the matrix, each column will correspond to a different dataset and each row to a pool parameter set $[a,b,r]$. The lowest relative residual (and therefore best result) should be 1, for the case that a dataset was fitted with the pool parameter obtained from the same dataset (and to be found in the diagonal of the matrix). For fits with foreign pools (non-diagonal elements), slightly increased residuals ($\sim <2$) indicate that these pools describe the dataset satisfactorily, while larger residuals (>2) suggest that the conditions should be analyzed using their own separate pool parameter sets. To provide an example of data that are not well fitted using the same pool, we include a supplementary dataset of simulated data (Supplementary Data 3). For more advanced users, we have also provided a script that can be edited and allows simulation of data and different noise levels (`simDataset.m`) and a script that can save the data generated in this way (`writeDataXLS.m`).

? TROUBLESHOOTING

- 85 *Fitting all proteins of interest (Steps 85 and 86)*. This part of the script allows the calculation of the lifetimes of the proteins, expressed as half-lives in days (the time required to replace half of a defined protein population). In the 'dataset fit' tab (Fig. 10), use the 'start fit' button to fit the time constants of all proteins of interest for all conditions of the dataset. During the fit, the button text changes to 'fitting...'. The fit process is uninterruptible and can take several minutes, so be patient. After completion, the button text changes back to 'start fit', and an overview of the fit result of one condition (selectable in the pop-up menu) will be shown in two plots.
- 86 Use the 'export fit' button to export the half-lives or time constants and their confidence intervals for all conditions to an Excel file. Save the Excel file in the MATLAB folder where the main script is also located.
- 87 *Statistics comparing different conditions (Steps 87–92)*. In the 'statistics' tab (Fig. 11), select two conditions for comparison and use the 'compare' button to test for significant differences between time constants for different conditions. A volcano plot will be shown on the left, from which a range of proteins can be selected based on their fold-changes (\log_2 , threshold shown as vertical blue lines) and P values ($-\log_{10}$, horizontal green line).

▲ CRITICAL STEP The uncertainty in the determination of protein lifetimes is given as a confidence interval. To compare the protein lifetimes and determine for each protein whether the lifetimes in

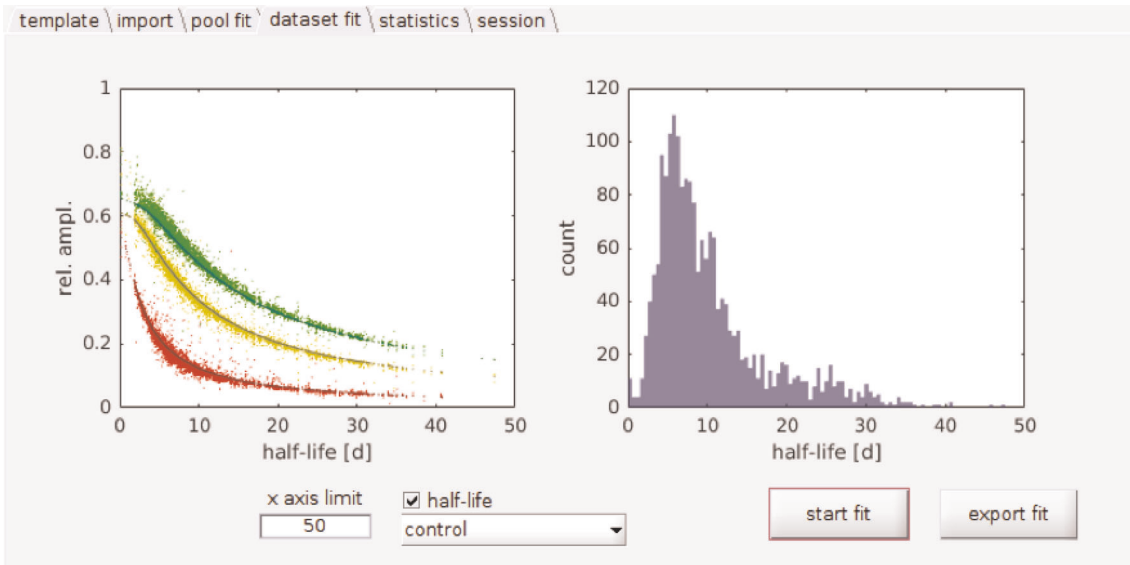


Fig. 10 | Sample screenshot of the graphical interface from the 'dataset fit' tab of the turnover script. See also Supplementary Data 1. Left, measured amplitudes (light colors) and theoretical amplitudes (dark colors) versus their half-lives or time constants. Right, histogram of all half-lives or time constants. The x axis limit can be modified in the respective edit field; the choice of half-life or time constant can be specified in the respective checkbox.

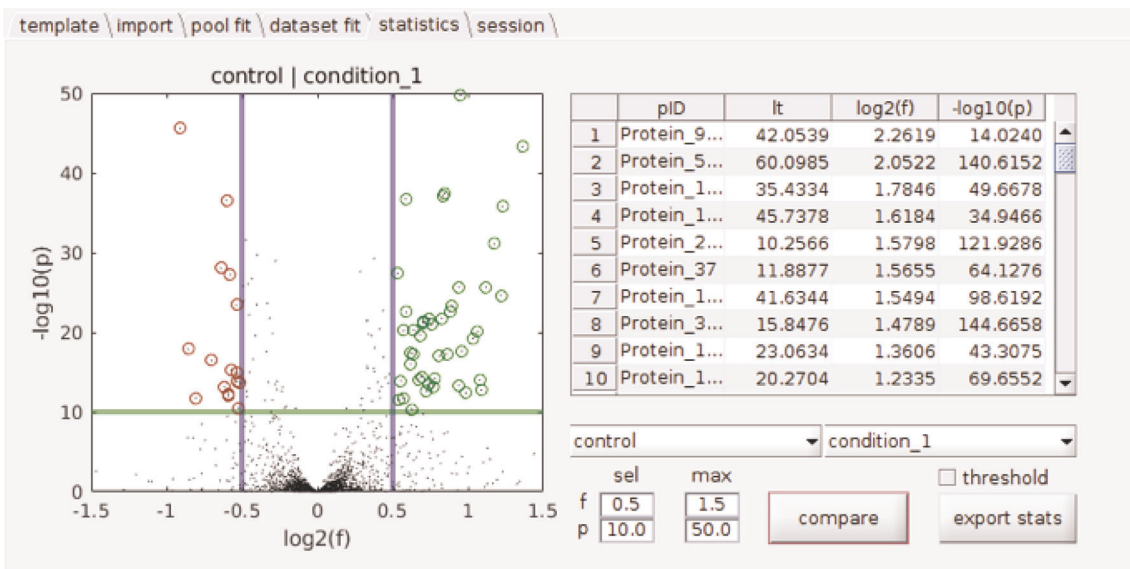


Fig. 11 | Sample screenshot of the graphical interface from the 'statistics' tab of the turnover script. See also Supplementary Data 1. On the volcano plot on the left, results are shown for the second condition versus the first condition tested. The fold-change (f) corresponds to the ratio of the lifetimes of the second condition ('condition_1' in the example) versus those of the first condition ('control' in the example). In the graph, the lifetime values that are relatively higher in the second condition ('condition_1' in the example) are shown in the rightmost part of the graph (in green), whereas the relatively lower values are shown on the leftmost part of the graph (in dark red). The table on the right contains the lifetime, fold-change and P values calculated for each protein.

two conditions are significantly different, our script calculates the fold-change (lifetime of condition 2 divided by lifetime of condition 1) and the respective P values and provides a volcano plot. The volcano plot is a specific type of scatter plot in which the significance (expressed as $-\log_{10}$ of the P values) is compared to the fold-change (expressed as \log_2 of the fold-change) on the y and x axes, respectively. With our script, the values can also be exported as an Excel file for further analysis. Also note that lifetimes <0.25 d indicate only that the proteins are extremely short lived. The precise study of proteins with a lifetime <1 might require additional experiments, including intravenous administration of isotopically labeled amino acids.

- 88 Modify the desired thresholds in their respective 'sel' edit fields, or left-click/left-drag the mouse pointer in the plot to change the selection thresholds directly.
- 89 To change the axis limits, edit the respective 'max' edit fields.
- 90 Use the 'export stats' button to export the current comparison as an Excel file. The comparison will contain all the proteins.
- 91 To export only the proteins that are selected by the threshold, activate the 'threshold' check box before the export; otherwise, all proteins will be exported.
- 92 At the end of the session, you can save all data under the 'session' tab with the 'save session' button. A session variable will be created in the MATLAB workspace that can be saved to disk (by typing `save <filename> turnoverSession`). Loading the session (type `load <filename>`) and starting `turnoverGUI.m` with the session as parameter (`turnoverGUI(turnoverSession)`) will restore the saved session.

Final validations

93 In this section, we describe four different procedures for validating the obtained results. Follow option A for evaluating the labeling of the lysine pool in the plasma of animals. This validation might be necessary if the labeling across different conditions is largely heterogeneous. Follow option B for an additional validation through miscleaved peptide analysis. This step is easy to implement in the case that miscleaved peptides are found in the database. Follow option C for an additional experimental validation through a pulse-and-chase approach. This option is useful for estimating complex labeling trajectories required by some specific experimental designs. Follow option D in the case that it is necessary to check for the labeling of an inducible protein, which might be relevant in the context of cell-specific proteomes (see the 'Experimental design' section for a more detailed description of these options).

(A) **Evaluation of lysine labeling in the plasma through GC-MS ● Timing 3 d, including the analysis, should be sufficient for 20 samples**

! CAUTION All steps during the extraction and derivatization procedure must be performed under a fume hood to avoid exposure to hazardous fumes.

▲ CRITICAL We encourage users to proceed with the GC-MS measurements of the free lysine pool if there is a large variation in optimal pool parameters obtained from different datasets (as seen in residual increases by a factor of >2 in Step 84). Alternatively, if there are specific issues related to the eating behaviors of the mice under different conditions, GC-MS measurement of the free lysine pool might be required.

▲ CRITICAL It is important that all fluids or tissue samples be quenched as soon as possible after killing the animal, because metabolite levels can rapidly change across tissues and fluids. Quenching can be performed by adding the extraction solution directly to fluids or by quickly freezing tissue samples in liquid nitrogen.

▲ CRITICAL Use only analytical-grade solvents. Plastic reagent caps can be used during all extraction and derivatization steps, because metabolites extracted from the plastic normally do not interfere with later analysis. As a control, a water sample should be carried through the whole procedure.

(i) Add two volumes of extraction solution for lysine measurements directly to the plasma (or, alternatively, to frozen plasma samples) to quench all enzymatic reactions. The starting amount of plasma should not be <10 µl. When using tissue samples, freeze-dry the material and grind it, for example, in a shaking mill. Extract 5–20 mg of freeze-dried material by shaking for 1 h in 500 µl of extraction solution.

(ii) Add 250 µl of water to the extracted samples.

(iii) Vortex the mixture and centrifuge for 5 min at 2,000g at RT.

(iv) Transfer the supernatant to a new tube.

■ PAUSE POINT Samples can be stored at –80 °C for several months.

(v) Evaporate 20 µl of the upper polar phase under nitrogen stream.

▲ CRITICAL STEP Depending on the sensitivity of the GC-MS system used, the amount of the upper phase that is evaporated can be varied to obtain sufficient signal while avoiding overloading of the column. Evaporation can also be performed in a SpeedVac concentrator. This concentrator must be equipped with a cooling trap to collect the evaporated organic solvents, which can be corrosive to the pump. Check the user manual of the machine in use beforehand.

- (vi) Derivatize the sample with 15 μ l of methoxyamine hydrochloride solution for 4–48 h at RT.
- (vii) For the next derivatization step, add 30 μ l of MSTFA, vortex and spin for 2 min at 20,000g at RT.
- (viii) Transfer the sample to GC–MS vials and incubate for 1–8 h.
▲ CRITICAL STEP Because MSTFA derivatization should not exceed 8 h, the GC–MS system should be ready to measure samples in this time frame. Therefore, it might be necessary to prepare samples in batches to ensure proper derivatization time.
- (ix) Run the GC–MS system using an HP5-MS column under the conditions specified in the ‘Equipment setup’ section.
▲ CRITICAL STEP In principle, other columns can be used, but care must be taken to avoid the elution of lysine from the column together with any major analyte, such as glucose. Changing the temperature gradient might help to overcome such problems.
- (x) Analyze the 4-trimethylsilyl derivative of lysine: determine the [^{13}C] to [^{12}C] ratio of lysine by quantifying the integrals of the mass-to-charge ratios of 317 Da/e and 322 Da/e. To account for the natural isotope distribution, the ^{12}C value must be multiplied by 1.499 and the ^{13}C value by 1.42.
- (xi) Import all measured heavy/light (H/L) ratios and measurement times into MATLAB using the graphical user interface script (turnoverGUI.m; included in Supplementary Data 1). For the following, the variable ‘HvsL’ is assumed to contain all H/L ratios from the previous step and the variable “t” is assumed to contain all corresponding times in days. As an example, input the data into MATLAB in the following format:

```
t = [5, 5, 14, 14, 21, 21]; HvsL = [1.3 1.4 1.8 2.0 2.3 2.5];
```

- (xii) Convert the H/L ratio to a heavy/total ratio by typing the following into the MATLAB command line: `y = HvsL ./ (HvsL+1);`
- (xiii) Fit a theoretical free lysine curve to the data, using the MATLAB script fitDirectPool.m (Supplementary Data 1) and the starting parameter `gPar0 = [a, b, r]`, obtained from the best global pool fit in Step 84:

```
gPar = fitDirectPool (t, y, gPar0);
```

- (xiv) Use the pool comparison tool as in Step 84, now with the obtained GC–MS pool parameter `gPar` (shown here for three saved sessions): `comparePool ({ts1, ts2, ts3}, gPar)`; the resulting matrix now includes the GC–MS pool parameter set in the last row of the matrix, allowing comparison to the other pools.

(B) Validation through miscleaved peptide analysis ● Timing variable, several hours to ≥ 1 week

▲ CRITICAL A few hours is sufficient for this analysis in the case that miscleaved peptides are found in the database; it takes ≥ 1 week in the case that trypsinization needs to be repeated and optimized.

- (i) For this validation use the usual sample processing workflow described in Steps 11–51, but modify Step 41, using a shorter trypsin incubation (1–2 h instead of the usual overnight incubation).

▲ CRITICAL STEP Trypsinization is usually very efficient, and the exact timing of this step needs to be calibrated ad hoc. For this validation experiment, it is necessary to ensure that the protein sample is trypsinized in a way that the majority of peptides contain at least two lysines. This can be achieved either by performing partial trypsinization (for 2–4 h) or by using other proteolytic enzymes (such as GluC or chymotrypsin, which do not cleave lysines).

? TROUBLESHOOTING

- (ii) To evaluate the relative labeling of the intermediate peptides, determine the three masses of the peptides L, H or M by MaxQuant, annotating the mass of one $^{13}\text{C}_6$ -lysine (M) and two $^{13}\text{C}_6$ -lysine (H) peptides as variable modifications to the usual $^{12}\text{C}_6$ -lysines. MaxQuant can be easily adapted for this modification by configuring the Andromeda search engine accordingly.

- (iii) To predict double labeling by incomplete digestion, use the MATLAB script `predictDoubleLabel.m` (Supplementary Data 1). The function is called from the MATLAB command window and needs the following three input parameters:
- The desired time points (t) for which the relative labeling should be predicted. For a comparison with experimental results, use the same time points (e.g., type $t = [5, 14, 21]$; for days 5, 14 and 21). For a more complete understanding of the labeling dynamics, use more frequent sampling (e.g., $t = 0 : .1 : 30$;
 - The protein lifetimes (τ) to use for the prediction. For a general understanding, only one or a few can be used (use $\tau = [1\ 10\ 100]$; to use time constants of 1, 10 and 100 d), or the time constants of an existing dataset fit (Step 84) can be used (type $\tau = \text{turnoverSession.dsFit}(1).\text{fit}.\tau$; to use the time constants of the first condition in the dataset of the saved session variable `turnoverSession`).
 - The pool parameters ($gPar$) containing parameters a, b, r (explained in Steps 79–84). Reference values can be used ($gPar = [0.0343, 0.4449, 11.837]$); or values obtained from the pool fit of a previously saved session ($gPar = \text{turnoverSession.pool.gPar}$;) can be used.
- (iv) Obtain the predicted labeling of ‘light’ (no label), ‘medium’ (one label) and ‘heavy’ (two labels) by typing: `[yL, yM, yH] = predictDoubleLabel(t, tau, gPar)`. Each of the resulting variables are then two-dimensional arrays ($T \times N$) containing relative labeling for N proteins at T time points, for example, `yM(1,2)` predicts the fraction of single labels at the first time point in t for the second given time constant in τ . These predictions can then be compared to measured values. Make sure to convert any measurements (e.g., absolute light, medium and heavy amplitudes) to relative values (e.g., $\text{light}/(\text{light}+\text{medium}+\text{heavy})$ for the light component).
- (C) **Validation through pulse-and-chase approaches** ● **Timing For the prediction: a few minutes, but several days if new data need to be gathered**
- (i) To predict the labeling ratio (heavy/total), use the MATLAB script `predictPulseChase.m` (Supplementary Data 1). Specify the following variables as arguments to the function:
- t , the time vector (in days) for which predictions need to be computed in days, for example, type $t = 0 : .1 : 30$; for a densely sampled exploratory calculation, or $t = [5\ 14\ 21]$; for a comparison with measured data points at days 5, 14 and 21.
 - $tStop$, the time (in days) of the pulse, for example, $tStop = 14$; for a pulse duration of 14 d followed by 7 d of chase in the above example.
 - τ , the time constant for one or multiple proteins. A previously saved session (Step 83) can be used ($\tau = \text{turnoverSession.dsFit}(1).\text{fit}.\tau$;' would use all protein time constants of the first condition of the dataset).
 - $gPar$, the pool parameters a, b and r given as a vector of 3, for example, $gPar = [0.0343\ 0.449\ 11.837]$ for default pool parameters, or from a previously saved session: $gPar = \text{turnoverSession.pool.gPar}$;
- (ii) Obtain the predicted labeling by typing: `[yP, yC] = predictPulseChase(t, tStop, tau, gPar)`; . The output arrays yP and yC are the labeling amplitudes for pulsed-only and pulse–chase experiments at given time points, for all time constants given.
- (D) **Validation by expression of an exogenous inducible protein** ● **Timing A few weeks to some months, including MS evaluation and analysis**
- (i) Feed the mice with the SILAC diet for the required number of days (usually >5 d; this validation is more reliable for longer pulsing times, because variable induction trajectories can influence the results).
- ▲ **CRITICAL STEP** For this validation step, short feeding periods (1–2 d) are not compatible with the expression times of most of the inducible systems available. Longer feeding schemes should be considered and analyzed during this validation.
- (ii) Induce the production of the exogenous protein with the most appropriate method. For tamoxifen induction, an induction scheme consisting of two intraperitoneal injections/day is usually sufficient. If the expression levels of the proteins of interest can be detected without problems with a conventional western blot strategy, there is a good chance they can be observed with MS.
- ▲ **CRITICAL STEP** For the incorporation test, induce the expression of a protein that is not endogenously synthesized in the tissue of the organism of interest. It is important to be able to distinguish the reporter protein from the endogenous proteins; otherwise, the labeling of

the endogenous protein will interfere with the results of this validation. If the induction scheme is particularly challenging for the animal, food consumption during the induction should be monitored.

- (iii) Kill the mice, following the appropriate guidelines.
- (iv) Prepare the tissue lysate as described in Steps 11–51 of this protocol.
- (v) Analyze the data according to Steps 71–92. Check that the H/L ratio of the overexpressed exogenous protein corresponds to the incorporation as predicted by the global fitting approach used (Step 79–84).

? TROUBLESHOOTING

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 Troubleshooting table			
Step	Problem	Possible reason	Solution
2	A mouse does not eat regularly (2–3 g of food/d)	Labeling-unrelated health problem of the mouse	Consider checking the health status of the animal, and eventually excluding the animal from the labeling procedure and replacing it
11	Protein concentrations are low or variable	Incorrect homogenization or resuspension of the sample	Make sure that the homogenization and the resuspension step(s) are performed correctly and consider increasing the amount of starting material
20	Protein separation during PAGE does not appear homogeneous	Defective cable connections, power supply or buffer formulation	Check the cable connections of the NuPAGE power pack; check that the power supply is functional; make sure that the appropriate buffers have been used
24	Inefficient solvent removal from 96-well microtiter filter plate	Defective connection of the vacuum pump or of the tubing	Ensure that the kit for the 96-well microtiter filter plates is correctly mounted and connected to the vacuum pump as suggested by the manufacturer
60	Congested analytical column, over-pressure, or electrospray ionization (ESI) spray issues Inefficient MS2 identification	Sample containing particulates or excessive loading Inappropriate instrument settings	Inject lower volumes; clean the ion-transfer tube and needle; wash the pre-column and/or replace the pre-column and/or analytical column Choose the number of precursors for MS2 depending on the instrument speed. For Q-Exactive HF, the optimal Top N for crude lysate is 20 or 30. Increasing or decreasing the AGC target value markedly might result in a very low number of protein identifications
66	Inefficient trypsinization	Poor enzyme batch or insufficient incubation time	Check the digestion effectiveness for all the samples by including peptides with up to seven miscleavages during MaxQuant search analysis. Change enzyme batch or incubation time accordingly
70	Few proteins are identified in the MaxQuant search Frequent MaxQuant search crashes	Wrong database, incorrect search settings or corrupted file Incorrect data storage or data retrieval	Check that the UniProt Database used corresponds to the correct organism under investigation. Double-check that the search settings are correctly entered. Check that the RAW files are complete and make sure that they are readable and not corrupted Try storing the data locally (not on a server). It is also recommended to keep enough free space (generally four times the data size) on the hard drive during MaxQuant searches. The MaxQuant Google group is a useful support community for other issues; for any other problem, consider joining MaxQuant Google groups: http://www.coxdocs.org/doku.php?id=maxquant:common:google_groups
71	MATLAB scripts do not work correctly	Incorrect MATLAB configuration	Make sure that you have installed and correctly configured the MATLAB program (v.2014b or more recent) with the Optimization and Statistics toolboxes installed. Make sure the MATLAB search path includes the location of all called scripts
81	The fitting does not converge toward a bi-exponential curve	Only a few or noisy data points are given in the early time region	Try a lower threshold in Step 79 to increase the number of data points for a better fit. Alternatively, use the initially given pool parameters, bypass the pool fit and continue with the individual protein lifetimes (Step 85)

Table continued

Table 1 (continued)

Step	Problem	Possible reason	Solution
84	The lysine pool fitting among different conditions is substantially different	Protein turnover was measured in tissues mainly composed of mitotic cells	Turnover measurements in tissues mainly composed of mitotic cells is confounded by the fact that the turnover of cells is often faster than the turnover of the proteins that compose those cells. A three-compartment model function fitting with four rate constants, proposed by Guan et al., might be more appropriate ¹⁶ in this case
	No pool parameter set has satisfactory (<2) residuals for all datasets	Different experimental conditions lead to different free-lysine pool dynamics	Split the dataset into subsets for which there are pool parameters with low fit residuals, saving the respective datasets in different .xlsx files. Evaluate the tables individually (from Step 79)
93B(i)	Peptides are over-digested for miscleaved analysis	Over-trypsinization	Decrease trypsin digestion time or enzyme concentration for this step. Alternatively, use other proteolytic enzymes (such as GluC or chymotrypsin, which do not cleave lysines)
93D(v)	The amount of the induced protein is under the detection limit	Poor induction or inefficient promoter	Increase tamoxifen concentration or consider enriching the protein by immunoprecipitation, if an antibody is available, as previously described ⁴

Timing

Steps 1–10, metabolic mouse labeling and tissue collection: variable, depending on the experimental design; from a few days to months

Step 11, protein concentration determination: 1.5 h

Steps 12–14, protein precipitation: ~13 h, including overnight incubation

Steps 15–20, gel electrophoresis: ~4 h, followed by overnight incubation

Steps 21–41, in-gel digestion: 1 d

Steps 42–51, extraction of peptides: 8–10 h

Steps 52–55, sample preparation for LC: ~4 h, depending on sample number

Steps 56–60, reverse-phase HPLC–MS: ~2 d for each lane or sample

Steps 61–70, mass spectrometry data analysis: variable, ideally 0.5–2 d

Steps 71–78, data import: 10 min

Steps 79–84, fitting of pool parameters: 10 min to days depending on the number of proteins

Steps 85 and 86, fitting all proteins of interest: up to several minutes

Steps 87–92, statistical analysis comparing different conditions: 10 min

Step 93A, evaluation of lysine labeling in plasma through GC–MS: ~3 d

Step 93B, validation through miscleaved peptide analysis: a few hours to weeks, depending on trypsinization efficiency

Step 93C, validation through pulse-and-chase approaches: for the estimation, only a few minutes, but several days if new data need to be gathered

Step 93D, validation by expression of an exogenous inducible protein: a few weeks to some months, including MS evaluation and analysis

Anticipated results

This protocol allows the user to obtain, for each protein, several heavy-versus-light ratios in Steps 1–70 that can be used to calculate protein lifetimes in Steps 71–92. As a reference, after feeding mice for three different time periods⁴, we reliably identified 79,995 peptides—of which 73,791 were unique—corresponding to ~5,500 protein groups, for a total of 4,737 major protein IDs, for several types of samples, including cortex, cerebellum, synaptosomes and synaptic vesicles. The median number of identified peptides for these 4,737 proteins was 12 (average 16.89 ± 0.26 s.e.m.). As a reference for the user, the average number of peptides detected for a single biological replicate (animal) at a unique time point was ~26,000, corresponding to ~12,000 heavy-versus-light-peptide ratios per replicate. In a single time point of a single biological replicate, the number of proteins yielding heavy-versus-light ratios was on average ~1,050. This relatively low value is expected, because not all of the identified proteins could be detected in both their light and heavy versions (because of the complexity of the MS

spectrum) and will therefore not yield reliable ratios. Because we combine three biological replicates and three machine replicates for three different time points for each type of sample (e.g., cortex), the coverage scales up to ~53,000 peptides, from which ~26,000 heavy-versus-light peptide ratios were measured, corresponding to the determination of lifetimes for ~3,300 proteins. In each condition, ~2,100 of the calculated lifetimes yielded a reliable confidence interval, which serves as a measure of the lifetime precision.

As expected, we could see a correlation between the accuracy of the turnover determination (confidence interval) and the number of peptides identified per protein. Proteins that were identified

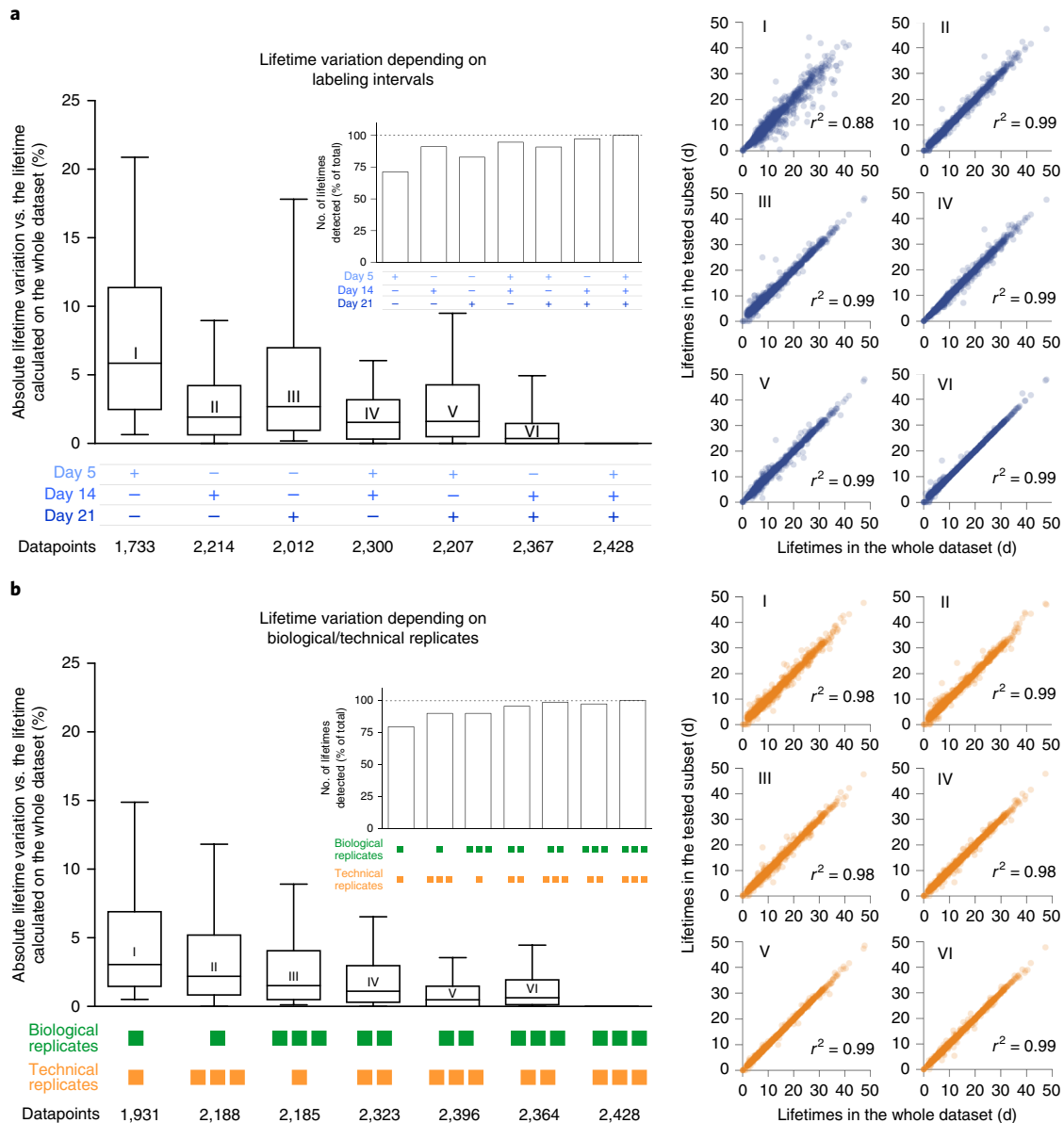


Fig. 12 | Variation in the determination of protein lifetimes depending on labeling intervals, biological samples and technical replicates. (a) Effects of the labeling intervals on the determination of lifetimes. The box plots represent the distribution of the absolute lifetime difference versus the lifetime calculated on the whole dataset, expressed as a percentage. The whiskers indicate the 10–90th percentile, the box the 25–75th percentile, and the line within each box the median. The inset in the upper right part of the panel indicates the number of lifetimes detected in the samples. The number of data points represented in each box plot is indicated under the graph. Note the smaller variation with increased number of labeling intervals used for the analysis. Likewise, the coverage increases with the number of samples included in the analysis. The scatter plots at right detail the data summarized in the box plots, and the roman numerals indicate the correspondence between graphs. **b**, Effects of the number of biological and technical replicates on the determination of lifetimes. The box plot is constructed as in **a** and the inset also indicates the number of lifetimes detected in the samples. The number of data points represented in the box plot is indicated under the graph. Increasing the number of technical and biological replicates increases the precision in the determination of the lifetimes, as well as the number of proteins measured. The scatter plots at right detail the data summarized in the box plots, and the roman numerals indicate the correspondence between graphs.

with ≤ 10 peptides had a median confidence interval of their lifetimes corresponding to 24% of their respective lifetimes. This value decreased up to 7.5% (corresponding to higher precision) for proteins identified with 60 or more peptides. This suggests that the protein coverage directly scales with the precision of the determination of the lifetime.

As a reference, in the brain, the average heavy-versus-light ratios after 5, 14 and 21 d of labeling are ~ 0.14 , ~ 0.19 and ~ 0.23 , respectively. Overall, the turnover of the brain is relatively low, and higher ratios should be expected for many other tissues.

Overall, in regard to the quality of the lifetime measurements and the data reproducibility, the user should always find a compromise between the precision in the lifetime determination and a number of practical matters, which include the cost of the labeled food, the number of the analyzed animals, the machine time and the desired proteome coverage. Along these lines, we include here the analysis of the influence of labeling intervals and biological and/or technical replicates on the lifetimes measured (Fig. 12; see also Supplementary Table 2 for additional details). Briefly, we found that, owing to the overall median distribution of lifetimes and the effect of the delayed protein labeling exerted by the kinetics of the lysine pool, the most relevant time point for lifetime determination corresponds to a pulse of 14 d (Fig. 12a). In practical terms, this suggests that in a minimal design, a unique 14-d pulse might be useful for estimating up to 90% of lifetimes with a median variation in their determination of $\sim 2.5\%$ with respect to a design including three time pulses. As for the effects of biological or technical replicates, using three labeling intervals and only one biological replicate and one technical replicate, the coverage is $\sim 80\%$ of the possible lifetimes, with a median variation of $\sim 3\%$ with respect to a design with three biological and three technical replicates (Fig. 12b). The coverage is extended to 90% of all detected lifetimes by including two additional technical replicates (bringing the experiment to one biological and three technical replicates).

As an additional measure of the reproducibility of this method, we also evaluated the lifetime in a subset of the data that was not used to calculate the lysine pool (Fig. 13). For this purpose, we have subdivided the original data into a 'training' set and a 'testing' set (corresponding to 2/3 and 1/3 of one of our original datasets). We have calculated the lysine pool and the protein lifetimes on the training set and compared them to the lifetimes calculated on the testing set (Fig. 13b). The lifetimes measured are extremely similar ($r^2 = 0.97$), suggesting that the approach is highly reproducible. Importantly, if a comparison between different conditions is the purpose of the study, the number of statistically significant differences detected will also depend on the robustness of the results and on the confidence intervals of the measurements, which is an important consideration in choosing the experimental design.

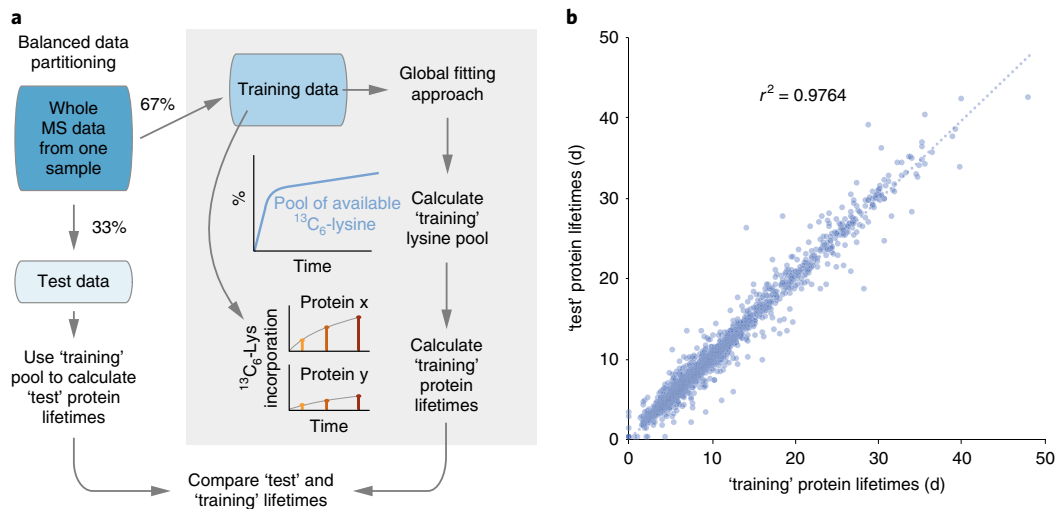


Fig. 13 | Evaluation of lifetime determination precision in a subset of the data that is not used to calculate the lysine pool. **a**, Schematic representation of the approach. First, we have subdivided the original data into a 'training' set and a 'testing' set (corresponding, respectively, to 2/3 and 1/3 of the original dataset). Second, we have calculated the lysine pool and the protein lifetimes on the training set (leaving the 'testing' data untouched). Third, we have calculated the protein lifetimes in the 'testing' data, based on the lysine pool from the 'training' data. Finally, we compared the lifetimes from the two datasets in a scatter plot (panel **b**). For the initial subdivision of the data, we randomly selected 2/3 of the available data for each pulsing time so that both subsets contained a balanced distribution of data across the entire labeling procedure. **b**, Results of the analysis introduced in **a**. The lifetimes of 1,908 proteins are represented.

These results are in line with the observation that the turnover measurements are overall quite reproducible. As an example, the average coefficient of determination (r^2) between technical replicates is 0.95 ± 0.01 (s.e.m.) and is 0.91 ± 0.03 for biological replicates, highlighting the small variation among experiments. It is likely that the high reproducibility of these measurements arises from the fact that each turnover measure is based on a ratio that arises from the detection of 'heavy' and 'light' values, which is much less error prone than, for example, absolute quantification of protein amounts.

Finally, in this protocol, we describe four different validation experiments (Step 93A–D), which can be used as internal quality controls or as tools to extend the use of this protocol to specific tissues or experimental organisms. Despite the increased complexity of applying them, these validation steps are extremely beneficial for evaluating the reliability of the lifetime measurements. In particular, the measurement of the lysine pool from the reference biological fluid is the most valuable for testing the reliability of the pool used for defining the lifetimes through fitting. A certain variability is to be expected at the beginning of the feeding protocol (during the first few days). This depends on the variable feeding behavior of animals. To reduce this variability, we advise beginning each labeling experiment at precisely the same hour when possible. The variability in the feeding behavior, in our experience, should not be a problem, as long as the overall behavior of the lysine pool overlaps with the pool of lysines that can be estimated through the global fitting approach, and as long as the correlation coefficient of repeated heavy-versus-light measures is >0.85 .

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data and code availability

The dataset presented in this protocol was originally generated in ref. ⁴. All data are available from the corresponding authors on reasonable request. All code from Supplementary Data 1 is also publicly available at GitHub: <https://github.com/malevra/protein-turnover>.

References

1. Labbadia, J. & Morimoto, R. I. The biology of proteostasis in aging and disease. *Annu. Rev. Biochem.* **84**, 435–464 (2015).
2. Ong, S.-E. & Mann, M. A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). *Nat. Protoc.* **1**, 2650–2660 (2006).
3. Schoenheimer, R. *The Dynamic State of Body Constituents* (Harvard University Press, 1946).
4. Fornasiero, E. F. et al. Precisely measured protein lifetimes in the mouse brain reveal differences across tissues and subcellular fractions. *Nat. Commun.* **9**, 4230 (2018).
5. Chan, X. C. Y., Black, C. M., Lin, A. J., Ping, P. & Lau, E. Mitochondrial protein turnover: Methods to measure turnover rates on a large scale. *J. Mol. Cell. Cardiol.* **78**, 54–61 (2015).
6. Busch, R. et al. Measurement of protein turnover rates by heavy water labeling of nonessential amino acids. *Biochim. Biophys. Acta* **1760**, 730–744 (2006).
7. Price, J. C., Guan, S., Burlingame, A., Prusiner, S. B. & Ghaemmaghami, S. Analysis of proteome dynamics in the mouse brain. *Proc. Natl. Acad. Sci. USA* **107**, 14508–14513 (2010).
8. McClatchy, D. B., Dong, M., Wu, C. C., Venable, J. D. & Yates, J. R. 15N metabolic labeling of mammalian tissue with slow protein turnover. *J. Proteome Res.* **6**, 2005–2010 (2007).
9. Savas, J. N., Toyama, B. H., Xu, T., Yates, J. R. & Hetzer, M. W. Extremely long-lived nuclear pore proteins in the rat brain. *Science* **335**, 942 (2012).
10. Toyama, B. H. et al. Identification of long-lived proteins reveals exceptional stability of essential cellular structures. *Cell* **154**, 971–982 (2013).
11. Heo, S. et al. Identification of long-lived synaptic proteins by proteomic analysis of synaptosome protein turnover. *Proc. Natl. Acad. Sci. USA* **115**, E3827–E3836 (2018).
12. Claydon, A. J. & Beynon, R. J. Proteome dynamics: revisiting turnover with a global perspective. *Mol. Cell. Proteom.* **11**, 1551–1565 (2012).
13. Basisty, N., Meyer, J. G. & Schilling, B. Protein turnover in aging and longevity. *Proteomics* **18**, e1700108 (2018).
14. Claydon, A. J. et al. Heterogenous turnover of sperm and seminal vesicle proteins in the mouse revealed by dynamic metabolic labeling. *Mol. Cell. Proteom.* **11**, M111.014993 (2012).
15. Claydon, A. J., Thom, M. D., Hurst, J. L. & Beynon, R. J. Protein turnover: measurement of proteome dynamics by whole animal metabolic labelling with stable isotope labelled amino acids. *Proteomics* **12**, 1194–1206 (2012).
16. Guan, S., Price, J. C., Ghaemmaghami, S., Prusiner, S. B. & Burlingame, A. L. Compartment modeling for mammalian protein turnover studies by stable isotope metabolic labeling. *Anal. Chem.* **84**, 4014–4021 (2012).

17. Zhang, Y. et al. Proteome scale turnover analysis in live animals using stable isotope metabolic labeling. *Anal. Chem.* **83**, 1665–1672 (2011).
18. Vogt, J. A. et al. Determination of fractional synthesis rates of mouse hepatic proteins via metabolic ¹³C-labeling, MALDI-TOF MS and analysis of relative isotopologue abundances using average masses. *Anal. Chem.* **77**, 2034–2042 (2005).
19. Rahman, M., Previs, S. F., Kasumov, T. & Sadygov, R. G. Gaussian process modeling of protein turnover. *J. Proteome Res.* **15**, 2115–2122 (2016).
20. Toyama, B. H. et al. Visualization of long-lived proteins reveals age mosaicism within nuclei of postmitotic cells. *J. Cell Biol.* **218**, 1–16 (2018).
21. Lau, E. et al. A large dataset of protein dynamics in the mammalian heart proteome. *Sci. Data* **3**, 1–15 (2016).
22. Sadygov, R. G. et al. d2ome, software for in vivo protein turnover analysis using heavy water labeling and LC-MS, reveals alterations of hepatic proteome dynamics in a mouse model of NAFLD. *J. Proteome Res.* **17**, 3740–3748 (2018).
23. Naylor, B. C. et al. DeuteRater: a tool for quantifying peptide isotope precision and kinetic proteomics. *Bioinformatics* **33**, 1514–1520 (2017).
24. Dufner, D. & Previs, S. F. Measuring in vivo metabolism using heavy water. *Curr. Opin. Clin. Nutr. Metab. Care* **6**, 511–517 (2003).
25. Krüger, M. et al. SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. *Cell* **134**, 353–364 (2008).
26. Chen, X., Wei, S., Ji, Y., Guo, X. & Yang, F. Quantitative proteomics using SILAC: principles, applications, and developments. *Proteomics* **15**, 3175–3192 (2015).
27. Hanke, S., Besir, H., Oesterhelt, D. & Mann, M. Absolute SILAC for accurate quantitation of proteins in complex mixtures down to the attomole level. *J. Proteome Res.* **7**, 1118–1130 (2008).
28. Zanivan, S., Krueger, M. & Mann, M. In vivo quantitative proteomics: the SILAC mouse. *Methods Mol. Biol.* **757**, 435–450 (2012).
29. Pena, I. A. et al. Mouse lysine catabolism to amino adipate occurs primarily through the saccharopine pathway; implications for pyridoxine dependent epilepsy (PDE). *Biochim. Biophys. Acta* **1863**, 121–128 (2017).
30. Mandad, S. et al. The codon sequences predict protein lifetimes and other parameters of the protein life cycle in the mouse brain. *Sci. Rep.* **8**, 16913 (2018).
31. Cox, J. et al. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol. Cell. Proteomics* **13**, 2513–2526 (2014).
32. Phillip, Y. & Schreiber, G. Formation of protein complexes in crowded environments—from in vitro to in vivo. *FEBS Lett.* **587**, 1046–1052 (2013).
33. Venable, J. D., Dong, M.-Q., Wohlschlegel, J., Dillin, A. & Yates, J. R. Automated approach for quantitative analysis of complex peptide mixtures from tandem mass spectra. *Nat. Methods* **1**, 39–45 (2004).
34. Collins, B. C. et al. Multi-laboratory assessment of reproducibility, qualitative and quantitative performance of SWATH-mass spectrometry. *Nat. Commun.* **8**, 1–11 (2017).
35. Meier, F., Geyer, P. E., Virreira Winter, S., Cox, J. & Mann, M. BoxCar acquisition method enables single-shot proteomics at a depth of 10,000 proteins in 100 minutes. *Nat. Methods* **15**, 440–448 (2018).
36. McShane, E. et al. Kinetic analysis of protein stability reveals age-dependent degradation. *Cell* **167**, 803–815 (2016).
37. Masters, P. M., Bada, J. L. & Zigler, J. S. Aspartic acid racemisation in the human lens during ageing and in cataract formation. *Nature* **268**, 71–73 (1977).
38. John, A. M. & Bell, J. M. Amino acid requirements of the growing mouse. *J. Nutr.* **106**, 1361–1367 (1976).
39. Overmyer, K. A. et al. Multiplexed proteome analysis with neutron-encoded stable isotope labeling in cells and mice. *Nat. Protoc.* **13**, 293–306 (2018).
40. Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V. & Mann, M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* **1**, 2856–2860 (2006).
41. Schirle, M., Heurtier, M.-A. & Kuster, B. Profiling core proteomes of human cell lines by one-dimensional PAGE and liquid chromatography-tandem mass spectrometry. *Mol. Cell. Proteom.* **2**, 1297–1305 (2003).
42. Batth, T. S., Francavilla, C. & Olsen, J. V. Off-line high-pH reversed-phase fractionation for in-depth phosphoproteomics. *J. Proteome Res.* **13**, 6176–6186 (2014).
43. Lam, M. P. Y. et al. Protein kinetic signatures of the remodeling heart following isoproterenol stimulation. *J. Clin. Invest.* **124**, 1734–1744 (2014).
44. Ahmed, S., Holt, M., Riedel, D. & Jahn, R. Small-scale isolation of synaptic vesicles from mammalian brain. *Nat. Protoc.* **8**, 998–1009 (2013).
45. Dunkley, P. R., Jarvie, P. E. & Robinson, P. J. A rapid Percoll gradient procedure for preparation of synaptosomes. *Nat. Protoc.* **3**, 1718–1728 (2008).
46. Sims, N. R. & Anderson, M. F. Isolation of mitochondria from rat brain using Percoll density gradient centrifugation. *Nat. Protoc.* **3**, 1228–1239 (2008).
47. Cox, B. & Emili, A. Tissue subcellular fractionation and protein extraction for use in mass-spectrometry-based proteomics. *Nat. Protoc.* **1**, 1872–1878 (2006).
48. Alvarez-Castelao, B., Schanzenbächer, C. T., Langer, J. D. & Schuman, E. M. Cell-type-specific metabolic labeling, detection and identification of nascent proteomes in vivo. *Nat. Protoc.* **14**, 556–575 (2019).

49. Tu, R. Comparison between confidence intervals of linear regression models with and without restriction. *Commun. Stat. Theory Methods* **28**, 2879–2898 (1999).
50. Feil, R., Brocard, J., Mascrez, B. & Lemeur, M. Ligand-activated site-specific recombination in mice. *Proc. Natl. Acad. Sci. USA* **93**, 10887–10890 (1996).
51. Erdmann, G., Schütz, G. & Berger, S. Inducible gene inactivation in neurons of the adult mouse forebrain. *BMC Neurosci.* **8**, 63 (2007).
52. Gage, G. J., Kipke, D. R. & Shain, W. Whole animal perfusion fixation for rodents. *J. Vis. Exp.* **65**, 3564 (2012).
53. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* **26**, 1367–1372 (2008).
54. Cox, J. et al. Andromeda: a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res.* **10**, 1794–1805 (2011).
55. Tyanova, S., Temu, T. & Cox, J. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat. Protoc.* **11**, 2301–2319 (2016).

Acknowledgements

We thank S. Truckenbrodt (IST, Austria) for initial suggestions on manuscript preparation. We thank S. Reshetniak (University Medical Center Göttingen), T. Dankovich (University Medical Center Göttingen) and I. Atanassov (Max Planck Institute for Biology of Ageing, Germany) for carefully reading and commenting on the final version of the manuscript. We thank U. Plessmann (University Medical Center Göttingen) for setting up and maintaining the LC-MS configuration. The work was supported by grants to S. O. R. from the European Research Council (ERC-2013-CoG NeuroMolAnatomy) and the Deutsche Forschungsgemeinschaft (DFG 1967/7-1, SFB1286/A3, ExC 2067/Multiscale Bioimaging) and to HU from the SFB-1286/A10.

Author contributions

Conceptualization: E.F.F. and S.O.R. Methodology: E.F.F., S.M., T.I. and H.U. Software: M.A. Formal analysis: M.A., E.F.F. and S.O.R. Resources and funding acquisition: S.O.R. Writing—initial draft: E.F.F. and S.O.R. Writing—final draft, review and editing: M.A., S.M., T.I., H.U., S.O.R. and E.F.F. Supervision: E.F.F.

Competing interests

The authors declare no competing interests.