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Opinion

Determining and interpreting protein lifetimes in mammalian tissues

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The orchestration of protein production and degradation, and the regulation of protein lifetimes, play a central role in the majority of biological processes. Recent advances in proteomics have enabled the estimation of protein half-lives for thousands of proteins *in vivo*. What is the utility of these measurements, and how can they be leveraged to interpret the proteome changes occurring during development, aging, and disease? This opinion article summarizes leading technical approaches and highlights their strengths and weaknesses. We also disambiguate frequently used terminology, illustrate recent mechanistic insights, and provide guidance for interpreting and validating protein turnover measurements. Overall, protein lifetimes, coupled to estimates of protein levels, are essential for obtaining a deep understanding of mammalian biology and the basic processes defining life itself.

Relevance of studying protein turnover *in vivo* in whole mammals

The complex nature of mammalian tissues presents several analytical challenges for studying *in vivo* **protein turnover** (see Glossary). However, recent advances in **liquid chromatography with tandem mass spectrometry (LC-MS/MS)** and proteomic data analysis have made high-throughput studies of protein turnover *in vivo* a reality [1–8]. The results of these studies have begun to revolutionize our understanding of proteome fidelity and **proteostasis**. In this opinion, we highlight the importance of measuring protein turnover and **protein half-lives** *in vivo* in whole mammals and why correctly interpreting these results is crucial for advancing the field. Specifically, we summarize leading analytical strategies, discuss recent discoveries, and disambiguate terms used to describe proteome-wide measurements of protein turnover. The strengths and weaknesses associated with commonly used experimental designs are also presented while highlighting recent mechanistic insights gained from studying protein turnover *in vivo*.

Efficient protein degradation and robust protein turnover are crucial for maintaining organ homeostasis. Accordingly, impaired protein turnover plays a key role in numerous human disorders, diseases, and during aging [9]. Historically, most early studies of protein half-lives have focused on tracking individual proteins, but currently a constellation of large-scale approaches can be used to monitor the turnover rate of several thousand proteins in a single experiment [10] (Table 1). Although these are exciting times for large-scale studies of protein turnover, an under-appreciation of what is being measured *in vivo* and the impact of these findings has emerged. In our opinion, it is crucial to emphasize the importance of careful experimental design, precise terminology, and accurate data interpretation.

A cohesive terminology to define protein renewal parameters

Proteostasis refers to the processes that ensure the delicate balance of protein production, maintenance, and degradation that are vital for cellular and tissue function. Although the general concepts underlying protein synthesis and degradation are well understood, the terms describing

Highlights

Robust proteome homeostasis (i.e., proteostasis) is crucial for organismal health because proteome imbalance and the accumulation of damaged molecules have negative effects on nearly all biological processes.

It has become clear that protein half-life data in mammals provide vital information at the whole-proteome level for understanding dynamic phenotypic changes across scales.

Although methods and analysis frameworks for determining protein half-lives *in vivo* at the whole-proteome level are becoming more popular, they require careful customization depending on the biological question.

Samples obtained from metabolic labeling schemes can be used to provide spatial turnover information through mass spectrometry imaging technologies such as matrix-assisted laser desorption ionization (MALDI) and nanoscale secondary ion mass spectrometry (NanoSIMS).

Protein abundance and turnover can be measured using similar mass spectrometry-based approaches but are fundamentally different and provide valuable and complementary insights.

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these processes are sometimes used ambiguously. In this section we would like to bring clarity by providing a common set of terms for the field.

Cells contain proteins with abundances that roughly vary from thousands to tens of millions of copies [11]. For an individual protein, the protein lifetime encompasses the entire time from synthesis (i.e., birth) to degradation (i.e., death). Historically, protein renewal (i.e., the replacement of old proteins) has been quantified in terms of average protein half-life. However, protein half-lives in mammals cannot be reliably calculated for long-lived proteins (LLPs). Because for some LLPs the **mean protein lifetime** is years or even decades, even small differences in labeling ratio will greatly influence these values.

Often the term meaning 'protein lifetime' is used interchangeably with 'protein half-life' and, because this is not strictly correct, we advise to use the latter whenever possible. In a steady-state situation, protein half-life is the point in time when the degradation of a population of old proteins is equal to the newly synthesized population of proteins. Thus, by definition, at steady-state, the mean protein lifetime is the same as the protein half-life (Box 1). Caution needs to be taken when non-steady-state conditions are the subject of investigation because the two measurements diverge. It is also important to emphasize that this terminology, in the context of LC-MS/MS measurements, reflects an average process (e.g., the mean protein lifetime, half-life, or turnover) of a pool of proteins with the same amino acid sequence. This is because these technologies typically do not achieve measurements at the level of single molecules and instead measure a population of peptides after protein extraction and trypsin digestion (Box 2).

Notably, the previously mentioned measurements are currently limited because proteins often exist in multiple independent pools. Unfortunately, we currently lack a sufficient repertoire of probes or analytical tools to distinguish the turnover of different protein subpopulations in mammals *in vivo*. The same protein is frequently present in multiple protein complexes localizing to various organelles with dissimilar turnover rates. To be more accurate, we thus propose to refer to proteins with multiple binding partners and functions (i.e., present in distinct pools) that show different turnover rates in a single cell type as multi-lifetime proteins (Figure 1A, Key figure). Currently, in whole mammals, and to properly analyze multi-lifetime proteins, we need to combine bulk measurements with biochemical purification of intact protein complexes and organelles so as to accurately determine their half-lives and distinguish protein subpopulations [12]. This is mainly due to technical limitations because, for whole animals, we lack simple and reliable experimental workflows for independently determining protein **degradation and synthesis rate constants** such as those available for cell culture [13].

Metabolic labeling with stable isotopes for studying protein lifetimes

The standard experimental strategy to study protein lifetimes at a proteome-wide scale uses **metabolic labeling** with heavy **stable isotopes** (i.e., ^{2}H, ^{13}C, or ^{15}N) coupled to LC-MS/MS-based proteomic analysis. Variations of this experimental paradigm have been reported, including the use of light isotope labeling (^{12}C) [14], but the general concept is simple and can be summarized as follows: small mammals, typically rodents, are metabolically labeled through chow or water enriched with select stable heavy isotopes that have extremely low levels in nature. As the animals consume the food provided, the supplemented isotopes are gradually incorporated into newly synthesized proteins during labeling periods that can span from days to months at a rate reflecting protein turnover (Figure 1 and Box 2). In practice, a short period of metabolic labeling for a few weeks is sufficient to measure the levels of incorporation in several organs using LC-MS/MS-based proteomics. However, to achieve the near-complete labeling (99%) that is required for some experimental workflows, labeling of mice for two generations is

Glossary

Degradation and synthesis rate constants: values that quantify the relative change in concentration of a particular molecule over time. Following metabolic labeling in animals, in a steady-state situation these constants can be obtained from relative labeling at the level of single proteins.

Liquid chromatography with tandem mass spectrometry (LC-MS/MS): the most commonly used analytical strategy for proteomic studies. LC-MS/MS ultimately provides information about peptide sequence, abundance, and isotopic distribution that are all key aspects for quantifying protein turnover.

Matrix-assisted laser desorption/ionization (MALDI): an ionization technique that allows *in situ* analysis of proteins and peptides within biological samples and provides valuable spatial information that is usually lost in conventional LC-MS/MS.

Mean protein lifetime: the average length of time a protein species persists, often formally defined as the time required for a protein to be reduced to 1/e of its initial quantity.

Metabolic labeling: a type of labeling of cells or tissues based on introducing one or more tracer isotopes into the cell culture media or the animal diet. This approach allows a stable isotope that is present in protein precursor molecules to be processed into labeled amino acid(s) and to become incorporated into the proteome during mRNA translation.

Nanoscale secondary ion mass spectrometry (NanoSIMS): also known as multi-isotope imaging mass spectrometry (MIMS), NanoSIMS is an analytical strategy that facilitates nanoscale resolution measurements of the isotopic composition of biological molecules *in situ*.

Protein half-life: the average time required for a protein species to be reduced to half of its initial number of molecules.

Protein turnover: a dynamic process by which old proteins are degraded into amino acids and are subsequently replaced by new versions through protein synthesis within cells.

Proteostasis: the term comprises the integrated cellular activities that determine/regulate protein homeostasis including synthesis, folding, trafficking, and degradation aimed at ensuring proteome fidelity and function.

necessary. Following the measure of the metabolic labeling *in vivo*, several strategies can be used for the analysis of data to extract protein turnover measurements that consider or do not consider the reuse of the isotopic labels. Because detailed data analysis of metabolic labeling largely exceeds the purpose of this opinion, readers might refer to several excellent works covering this aspect in detail [4,6,15–18].

Amino acids or amino acid precursor molecules enriched with heavy atoms are now the most common chemical tracers used to study protein turnover [1,4,5,19–21]. Alternative chemical strategies for measuring protein turnover leverage biorthogonal labeling and can be performed with amino acid analogs. These can be either directly incorporated instead of methionine, such as in the case of L-azidohomoalanine (AHA) [22], or through the expression of a modified methionyl-tRNA synthetase, such as in the case of azidonorleucine (ANL) [23]. Although strategies based on click-chemistry provide an opportunity to enrich the labeled proteome and measure the newly synthesized proteins, they can be toxic to animals at high concentrations, limiting their applicability to studies of protein turnover. By contrast, stable isotopes are particularly powerful for metabolic labeling because they are almost indistinguishable from naturally occurring atoms and provide the rare opportunity to confidently measure protein half-lives under nearly endogenous conditions [24]. Furthermore, protein labeling with these minimal tracers surmounts the limitations associated with the introduction of exogenous over-expressed fluorescent proteins or epitopes that may alter natural protein production, folding, complex formation, and degradation.

Pulse-chase analysis: a technique used for examining changes in abundance and labeling over a defined time-period. Organisms or cells are exposed to a labeled compound (i.e., pulse), the labeled compound is then removed and replaced with an unlabeled version (i.e., chase). By monitoring the level of the labeled compound over time, one can determine its degradation dynamics and half-life.

Stable isotopes: atoms generally containing extra neutrons that are stable and non-radioactive, and hence are often present in nature and not dangerous for cells and animals. Examples of the practical use of stable isotopes for protein metabolic labeling include essential amino acids containing one or more stable isotopes (e.g., $^{13}C_6$-lysine containing six atoms of ^{13}C).

Stable isotope labeling in mammals (SILAM): a method to metabolically label rodents with stable isotopes.

Stable isotope labeling with amino acids in culture (SILAC): an LC-MS/MS-based proteomic technique using stable isotopes supplied in cell culture media to measure relative differences in protein abundance.

Studying protein lifetimes in mammals accelerates biological discoveries

Proteomics provides a rare opportunity to probe relationships between groups of proteins with similar turnover rates and thus extract biological information about complex biogenesis and degradation directly from mammalian tissues. For example, it has been established which subunits of several large protein complexes, such as the core of the nuclear pore and parts of the mitochondrial oxidative phosphorylation chain, show similarly exceptionally long lifetimes [2,4,25]. It has also been discovered that these LLPs are maintained together as single units for months and even years whereas several other components of the same complexes are turned over on much shorter timeframes [2,4,25–27]. These discoveries represent the basis of new research avenues addressing differences in macromolecular complexes occurring during aging and pathologies that might affect their stability. Moreover, selectively impaired protein turnover can be observed after stress or by the expression of mutant proteins that become misfolded and accumulate [28–30]. Changes in protein half-lives can also reflect physiological responses, which modulate protein turnover [4,31]. For example, changes in subcellular localization and post-translational modifications (PTMs) can influence protein stability. For this reason measurements of protein half-life can be used for discovery purposes to reveal previously unknown interactors and molecular mechanisms (Figure 1C).

In the context of aging, the study of protein lifetimes has shown that different tissues may affect protein turnover in slightly dissimilar manners, although the relative half-lives of mitochondrial components are very precisely coordinated across tissues [32]. The brain of aged animals specifically shows reduced protein turnover, increased half-lives, and specific alterations that might point to metabolic differences that affect proteome composition. Interestingly, these alterations are also linked to biological processes that are observed in neurodegenerative diseases [33]. These studies only start to address the involvement of different pathways that regulate protein stability, and more systematic approaches will be necessary to understand the precise molecular causes of these observations and the involvement of proteasomal and lysosomal mechanisms.

Table 1. Compendium of turnover studies of particular relevance to mammalian tissues and whole animals, organized from the oldest to the most recent studies[a]

Number	First author and year	Label	Animal	Labeling paradigm(s)	Data analysis strategy	Considerations	Biological relevance	Refs
1	Wu 2004	^{15}N	*Rattus norvegicus*	Short pulses	^{15}N absolute quantification	Pioneering work that introduced ^{15}N for metabolic labeling of mammals	Feasibility study	[19]
2	McClatchy 2007	^{15}N	*R. norvegicus*	Short and generational pulses	^{15}N enrichment	Pioneering work for slow-turnover proteins Analysis is limited to unlabeled proteins	Tissue comparison	[62]
3	Bateman 2007	$^{13}C_6$-leucine $^{13}C_6$-phenylalanine	*Homo sapiens*	Short pulses	SILT	Pioneering work for the analysis of turnover in humans	Method for quantifying the turnover of low-abundance proteins	[63]
4	Price 2010	^{15}N	*Mus musculus*	Several short pulses	Exponential fitting	Pioneering work designed to define the half-lives of rapid-turnover proteins	Tissue comparison, half-life determination	[1]
5	Kasumov 2011	$^{2}H_2O$	*R. norvegicus*	Several short pulses	Exponential fitting	Pioneering work that introduced $^{2}H_2O$ labeling for protein turnover studies	Effects of feeding on albumin synthesis	[56]
6	Savas 2012	^{15}N	*R. norvegicus*	Generational pulse and chase	$^{15}N/^{14}N$ abundance	Pioneering work to identify intracellular ELLPs	Identification of NUPs and histones as ELLPs	[27]
7	Price 2012	$^{2}H_2O$	*H. sapiens*	Drinking $^{2}H_20$ administration (single pulse)	Kinetic model to account for precursor enrichment	Pioneering work for the analysis of protein lifetimes in humans	Protein turnover values in human plasma	[51]
8	Guan 2012	^{15}N	Modeling/data analysis	Several short pulses	Several computational approaches	Pioneering work dealing with compartment modeling for mammalian turnover studies	Simple exponential decays are not appropriate for whole animals	[15]
9	Toyama 2013	^{15}N	*R. norvegicus*	Generational pulse and chase	$^{15}N/^{14}N$ abundance	Detailed work characterizing the long-lived proteome	Detailed analysis of nuclear pore complexes	[26]
10	Lam 2014	$^{2}H_2O$	*M. musculus* *H. sapiens*	Several short pulses	Exponential fitting	Extensive work that also provided data on turnover of the human plasma proteome	Study of isoproterenol effects on heart remodeling	[64]
11	McClatchy 2015	AHA	*M. musculus*	Short pulses	Enrichment of AHA (click and biotinylation)	Work aimed at identifying the newly synthesized proteome	Introduces a method for newly synthesized proteins in tissues	[22]
12	Karunadharma 2015	$^{2}H_3$-leucine	*M. musculus*	Several short pulses	Exponential fitting	Several conditions and tissues were analyzed in parallel (respiratory chain)	Tissue comparison for mitochondrial proteins	[32]
13	Hammond 2016	$^{13}C_6$-lysine	*Myodes glareolus* (bank vole)	Several short pulses	Exponential fitting	Turnover in a small rodent (bank vole) by cross-species matching to mouse	Analytical methodology may contribute to variance in turnover	[6]
14	Lau 2016	$^{2}H_2O$	*M. musculus*	Several short pulses	Exponential fitting	Comprehensive dataset of half-lives in the heart	Heart hypertrophy studied across six mouse strains	[58]
15	Rahman 2016	^{15}N and $^{2}H_2O$	Modeling/data analysis	Several short pulses	Several approaches including a stochastic model	One- and two-compartment models were used to analyze data from other studies	Models can be independent of the labeling isotope	[16]

(continued on next page)

Table 1. (continued)

Number	First author and year	Label	Animal	Labeling paradigm(s)	Data analysis strategy	Considerations	Biological relevance	Refs
16	Naylor 2017	$^{2}H_2O$	Modeling/data analysis	Short pulses	Exponential fitting	The described software platform simplifies analysis	Turnover rates are consistent across studies	[60]
17	Fornasiero 2018	$^{13}C_6$-lysine $^{13}C_6$-$^{15}N_4$-arginine	*M. musculus*	Several pulses including pulse and chase	Exponential fitting/global modeling	Comprehensive dataset of protein half-lives in the brain and in other tissues, including cell sorting and fractionation	Proteins have a reduced turnover at synapses Environmental enrichment changes specific lifetimes	[4]
18	Basisty 2018	$^{2}H_3$-leucine	*M. musculus*	Several short pulses	Exponential fitting	Analysis of the antibody-enriched ubiquitinome at different mouse ages and fed with different diets	Aging increases bulk protein ubiquitination Aggregated proteins are older	[7]
19	Heo 2018	$^{13}C_6$-lysine	*M. musculus*	Short pulse and chase	Turnover ratios	Analysis of brain synaptic proteins	Proteins have reduced turnover at synapses	[31]
20	Lau 2018	$^{2}H_2O$	*M. musculus*	Several short pulses	Exponential fitting	Integrated omics: transcript abundance; protein abundance and turnover	Integrated omics provides several gene candidates for heart hypertrophy	[35]
21	Sadygov 2018	$^{2}H_2O$	*M. musculus* and modeling/data analysis	Short pulses	Nonlinear fitting with outlier detection and removal	The software platform simplifies the analysis of protein lifetimes	Analysis of fatty liver disease reveals changes in ribosomal proteins	[59]
22	Ko 2018	^{15}N	*M. musculus*	Short pulse	Difference in labeling ratios	Aimed at understanding the effects of peripheral nerve injury on protein turnover	Peripheral nerve injury induces faster turnover of defined synaptic proteins	[37]
23	Alevra 2019	$^{13}C_6$-lysine	Protocol with data analysis	Short pulses	Exponential fitting/global modeling	Detailed indications for determining protein lifetimes	Protocol covering aspects of lifetime measurements	[3]
24	McClatchy 2020	AHA	*M. musculus*	Short pulse and chase	Exponential fitting	Use of AHA for determining degradation dynamics in different tissues	Subcellular localization and activity influence protein stability	[8]
25	Bomba-Warczak 2021	^{15}N	*M. musculus*	Several pulses (up to 4 months)	$^{15}N/^{14}N$ abundance	Pulse labeling shows that some mitochondrial proteins are exceptionally long-lived	Oxidative phosphorylation complexes are preserved with low subunit exchange	[2]
26	Krishna 2021	^{15}N	*M. musculus*	Pulse and chase	$^{15}N/^{14}N$ abundance	NanoSIMS confirmation that some mitochondrial proteins are exceptionally long-lived	COX7C contributes to oxidative phosphorylation complex assembly	[25]
27	Hark 2021	^{15}N	*M. musculus*	Generational pulse and chase	$^{15}N/^{14}N$ abundance	Three genetic models of AD were analyzed	The turnover of synaptic vesicle-associated proteins is altered in AD	[34]
28	Chepyala 2021	$^{13}C_6$-lysine	*M. musculus*	Several short pulses	Exponential fitting	Three settings can be used for calculating lifetimes	Direct measurements of lysine pools improve the data	[17]
29	Rolfs 2021	$^{13}C_6$-lysine	*M. musculus*	Several short pulses	Exponential fitting	Analysis of half-lives across five mouse tissues	Postnatal tissue development complicates the analysis of results	[65]
30	Kluever 2022	$^{13}C_6$-lysine	*M. musculus*	Several short pulses	Exponential fitting	Analysis of mean protein lifetimes in aged mouse brain	Aged brain proteins last longer than young proteins	[33]
31	Hammond 2022	$^{2}H_2O$ and $^{13}C_6$-lysine	*M. musculus*	Several short pulses	Exponential fitting	Comparison of different labeling strategies	The amino acid labels tested are suited for turnover studies	[44]

[a]Abbreviations: AD, Alzheimer's disease; AHA, L-azidohomoalanine; ELLPs, extremely long-lived proteins; NanoSIMS, nanoscale secondary ion mass spectrometry; NUPs, nucleoporins; SILT, stable isotope labeling tandem MS.

Box 1. Analyzing and interpreting *in vivo* measurements of protein kinetics

In a steady-state situation, protein kinetics can be approximated by simple exponential decays (Figure I) in which the degradation and synthesis rate constants are equal. In reality, in a mammal the availability of precursor molecule might depend on the modalities by which the labels are provided (Box 2), further complicating half-life calculations.

When interpreting the results of metabolic labeling, it is crucial that the age of the animal and the period of labeling are carefully considered. This is exemplified by the fact that some long-lived proteins (LLPs; such as extracellular matrix components and nuclear scaffolds in postmitotic cells) are synthesized at a specific age of the animal development [52,53]. If the goal is to study protein half-lives, using relatively short labeling periods with heavy amino acids is often appropriate, especially for measuring the components of the proteome that are replaced frequently. However, if the goal is to identify LLPs, then multi-generation labeling followed by a chase with unlabeled food is probably more appropriate.

Protein turnover measurements in isotopic labeling experiments are typically fractional, and the intact heavy and light labeled peptides are captured in the same MS1 scan. Thus, they are internally normalized within individual samples analyses and are not impacted by sample-to-sample technical variation. Attention must be exerted for the interpretation of these calculations because, depending on the workflow used, the fractional abundance of isotopically labeled proteins can reflect either an 'older' or a 'newer' population of proteins. However, bottom-up proteomic analysis depends on the ability to identify the peptide sequence in the MS2 scan. For some studies, both the heavy and light peptide pairs are identified, whereas in others only one isotopolog is selected for MS2 and the abundance is solely based on inferring its sequence based on the *m/z* values and peak intensities. MS-based imaging of metabolically labeled tissue sections can be achieved with MALDI and NanoSIMS, providing spatial information.

Several labeling and analysis strategies have been deployed to investigate protein turnover and measure protein lifetimes *in vivo*. A problem that needs to be addressed when studying protein turnover *in vivo* is that amino acids (essential and non-essential) are recycled within animals to preserve energy and increase metabolic performance. All these approaches are based on theoretical predictions of kinetic influx and efflux of pools of amino acids and proteins, and several computational approaches are available [1,4,15,17,54,55]. It is important to underline that these approaches are based on assumptions which are necessary to allow mathematical modeling (see Table 1 in main text). The most common assumption is that the protein of interest does not change its level during the period analyzed, which may confound interpretation of the results.

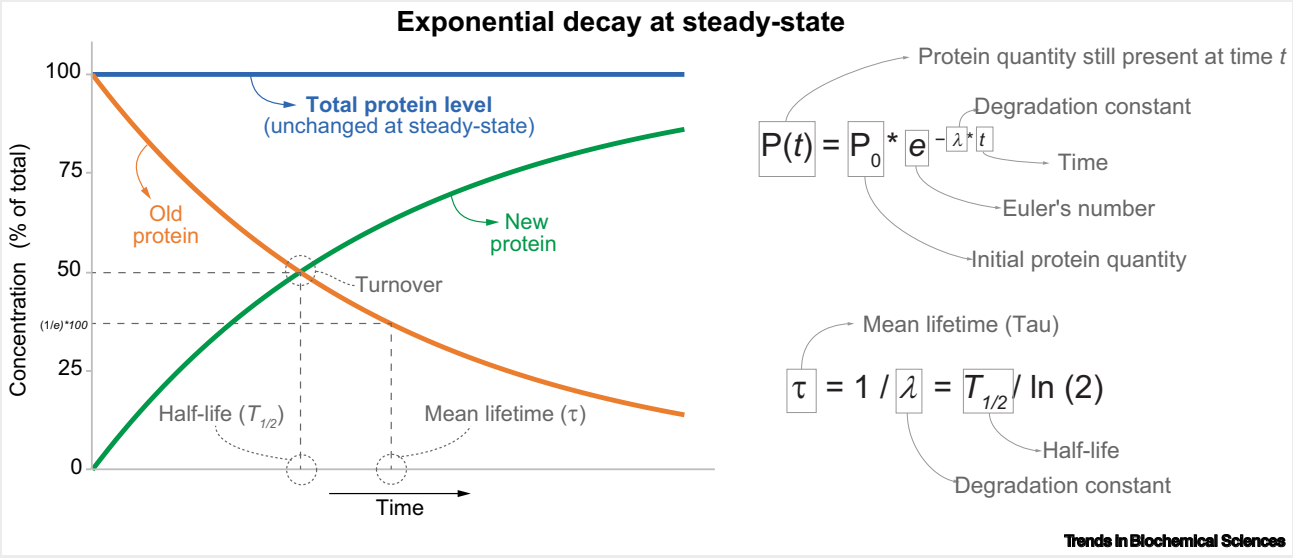

Figure I. Schematic representation of exponential decay and the basic equations at steady-state.

To demonstrate the usefulness of protein turnover measurements, we summarize some exemplary case studies. To better understand the mechanisms underlying neurodegeneration, **pulse-chase analysis** of amyloid precursor protein (*App*) knock-in mice showed that amyloid accumulates over a time-period of months [34]. The amyloidogenic processing of APP also caused a selective turnover impairment of synaptic vesicle-associated proteins. It is important to underline that there is no standardized 'protein turnover analysis workflow', and different strategies need to be tailored for each biological question. As an example, to study amyloid deposition, an initial long-term pulse was necessary to label the majority of the slowly accumulating

protein pool, and a subsequent chase was required for tracking the degradation of the aggregated long-lived pool and the non-aggregated protein that is shorter-lived. This strategy increased the signal and minimized noise, which would not be possible with a continuous labeling paradigm, and thus might be more informative for the determination of protein half-lives.

In the study of heart remodeling, protein turnover has been used in an elegant multi-omic study integrating other omic data (such as transcript and protein abundance) to reveal new disease gene candidates linked to heart hypertrophy [35]. In the same study, the integrated analysis of half-lives has confirmed that protein–protein interacting partners are coordinated in turnover, and that changes in these measurements can therefore be used to infer changes in protein complex composition.

Protein turnover has also been used for addressing changes in synaptic physiology in both the central and peripheral nervous systems [4,31,36,37]. One intriguing aspect revealed by these studies is that the localization to a specific cellular subdomain (the synapse) extends the half-lives of proteins [4,31], suggesting that local degradation mechanisms in the cell body or at the synapse are not equally active. Another intriguing aspect is that peripheral nerve injury decreases the turnover of synaptic proteins, suggesting that cellular damage could be one of the causes of inefficient neurotransmission [36].

Beyond the several examples from specific fields of biology, there are some overarching questions about biological systems that protein turnover measurements can help to solve. As an example, we still know very little about how proteome composition and homeostasis are maintained, and can only address these questions when several thousands of protein lifetimes and abundances are measured in parallel. These large-scale studies facilitate the identification of determinants of protein stability, such as biochemical parameters influencing protein turnover [38]. However addressing the role of PTMs in protein stability remains challenging [39] (see Outstanding questions).

Box 2. Labeling rodents with stable isotopes for studying protein turnover

Stable isotopes can be incorporated into rodent proteins by metabolic labeling, similarly to what is done using **stable isotope labeling with amino acids in culture (SILAC)**. In animals this is done with custom chow enriched with 'heavy' nitrogen (i.e., ^{15}N) or carbon (i.e., ^{13}C). The rodent chow is formulated without 'light' atoms for one or more defined molecular species, thus allowing specific labeling to be obtained.

There are currently two practical strategies used to label proteins for experiments requiring **stable isotope labeling in mammals (SILAM)**: (i) by providing 'heavy essential amino acids' such as lysine (e.g., $^{13}C_6$-lysine) that cannot be synthesized and are thus solely provided from the diet and incorporated during protein biosynthesis and (ii) by providing 'labeled amino acid precursors' that are incorporated into biomolecules and proteins through enzymatic reactions occurring within cells. As an example, this is what is achieved when employing ^{15}N diets. In this case ^{15}N atoms are slowly incorporated into all the nitrogen-containing molecules such as amino acid sidechains and backbones. Because mice cannot efficiently incorporate ^{15}N as a derivatized salt, historically the ^{15}N diet is based on blue-green algae (i.e., *Spirulina platensis*) which can use ^{15}N as the sole nitrogen source.

An alternative strategy is to deliver heavy atoms by subcutaneous injection or in drinking water in the form of deuterium oxide (i.e., ^2H_2O) [56]. Owing to the large difference in relative mass with respect to ^1H (protium), deuterium (^2H) is the only stable isotope which exerts a sizeable 'kinetic isotope effect' that slows enzymatic reactions and results in toxicity at concentrations higher than 30% in animals and eukaryotic cells [57]. Nevertheless, because of its relatively low price, heavy water is an attractive solution for protein turnover studies not only in rodents but also in humans [51]. In practice, the toxicity issues are mitigated by using low concentrations of heavy water and relying on robust bioinformatic approaches for data interpretation [58–60].

There are advantages and disadvantages to using global isotopic labeling (e.g., ^{15}N-labeled essential amino acids; Figure I). Briefly, although atom-based tracers can provide reliable measurements of relative labeling, analysis of the MS spectra is challenging due to the presence of heterogeneous populations of peptides of the same chemical composition that differ only by their isotopic composition (i.e., isotopologs). It is important to mention that this aspect has recently been addressed to simplify the analysis of the isotopolog distribution either by forcing a light isotope labeling shift or by using water labeling and considering only the enrichment of two mass isotopomers [18,61]. The use of heavy essential amino acids simplifies the analysis but only allows protein turnover measurements for peptides containing the heavy

amino acids. At the same time, because these have predictable mass shifts which can be precisely separated and accurately measured by MS, their modeling is easier to handle.

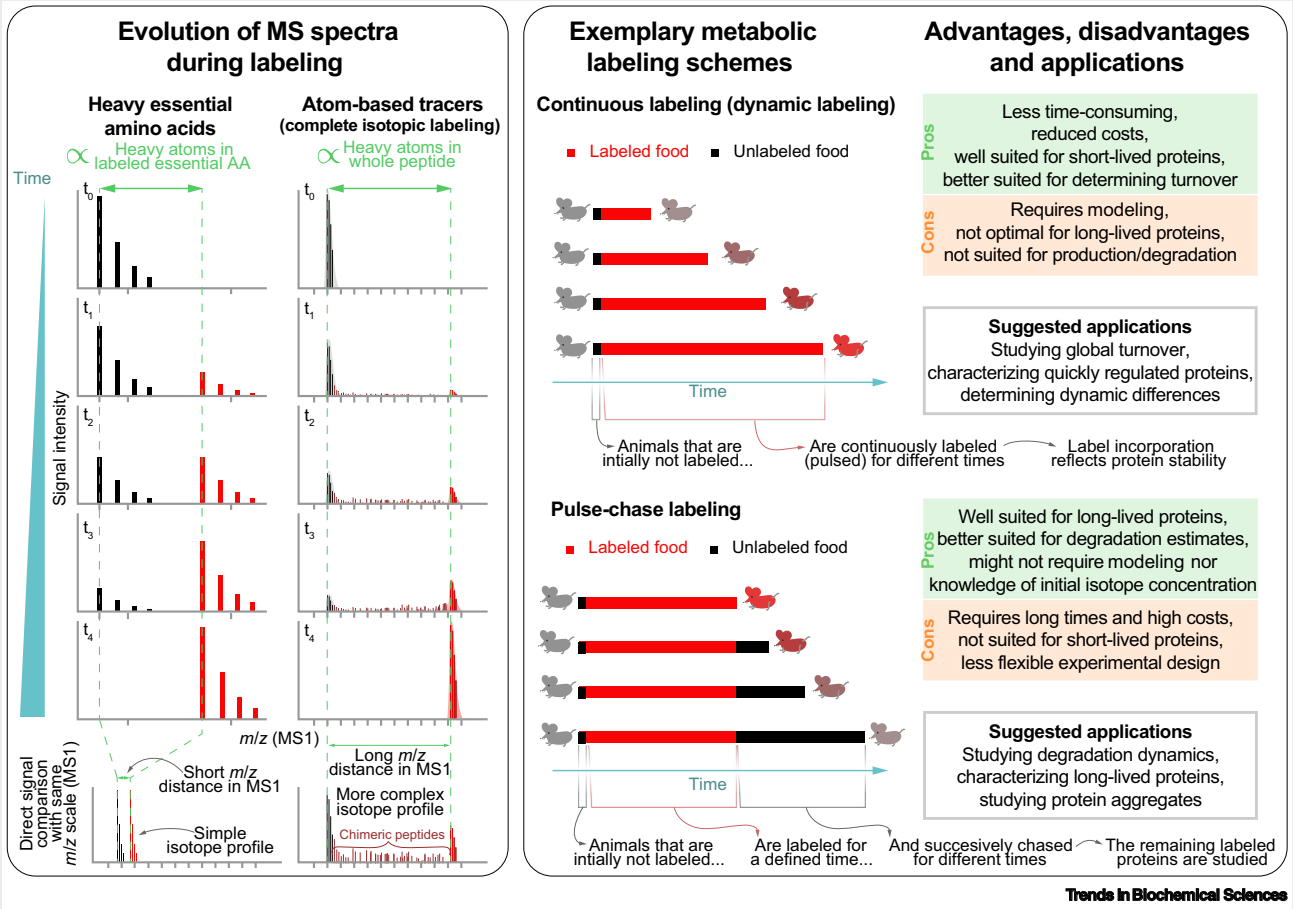

Figure I. Theoretical peptide mass spectra from a metabolic labeling experiment (left), commonly used experimental labeling schemes, and a list of applications (right). What is the main difference between 'continuous labeling' versus 'pulse-chase' labeling? In the 'continuous labeling' strategy, because there is only one type of measurement, more complex trajectories in protein degradation dynamics are not captured. At the same time, it is simpler to analyze and model the data for quantitative purposes if they approximate to first-order degradation kinetics. For proteins that are long-lived and possibly stabilized in aggregates, a pulse-chase approach might become useful to decrease the background noise arising from the short-lived (non-aggregated) counterparts. In this case a chase allows washout of the short-lived proteins and reveals more reliably the longer-lived species. Both strategies can be combined to obtain different sets of data and for checking possible labeling inconsistencies. Abbreviation: AA, amino acid.

Protein half-life and protein abundance: two orthogonal parameters

What is the difference between measuring protein levels (i.e., protein abundances) and protein half-lives? In simple terms, classical protein abundance measurements provide information about the relative quantity of protein, which in principle could be the consequence of changes in gene expression, in protein degradation, or in both processes. At steady-state, protein production and degradation are equal by definition, and protein levels therefore do not change (Figure 1B). Protein half-lives at steady-state provide a substantially different set of data than protein abundance. For example, if both production and degradation are doubled (and thus turnover is faster and the half-life becomes shorter), protein abundance would not change and protein abundance measurements would not be informative. One could still foresee that, in such a situation, a protein could be used much faster and thus be more rapidly degraded

Key figure

Assessing and understanding *in vivo* protein turnover at multiple scales

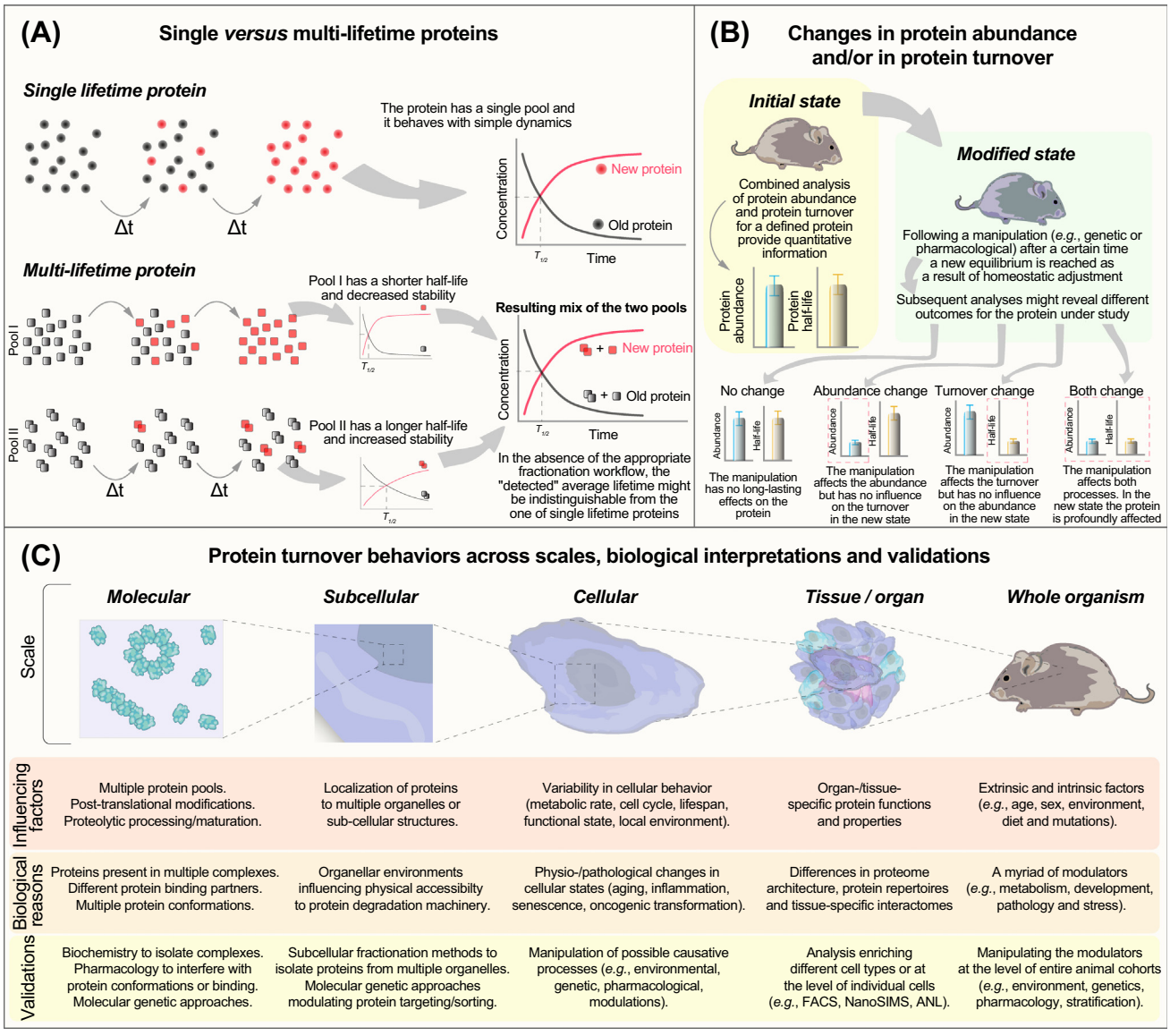

Figure 1. (A) Theoretical cartoon depiction of single- and multi-lifetime proteins and how these factors impact on global measurements of protein turnover. (B) Cartoon depiction of possible changes in protein abundance and turnover upon manipulation *in vivo* (C) Bulk measurements of protein turnover are influenced at multiple scales during *in vivo* experiments. This biological complexity can occur at the molecular, organelle, cellular, organ, or organismal (i.e., individual) level. Although many of these validations discussed are straightforward, they are rarely performed and we encourage their inclusion whenever possible.

because of increased molecular damage. If the levels of that protein need to be kept constant for the optimal function of the organism, homeostatic mechanisms will continuously counterbalance the increase in degradation with higher protein production, very quickly reaching a new steady-state situation where the overall protein levels are not changed.

In non-steady-state situations, following metabolic labeling for a given period, half-life measurements are more complex to investigate, especially in whole mammals, where formal analysis of protein turnover trajectories is not as simple as in cell culture [13]. In any case, associated with protein abundance measurements, protein turnover provides additional information about the dynamic changes in protein expression. In this context, we would like to stress that, from a practical point of view, protein abundance measurements when changes are small (<5–10% difference) are often too noisy to provide reliable information. In these cases, measurements of the metabolic incorporation of stable isotopes can be extremely quantitative because they often include an 'internal control' – the unlabeled version of the same protein – across multiple biological replicates. In other words, the fractional abundance [i.e., 'old'/('new' + 'old')] is a direct way to capture the protein properties [26]. In practice, this allows one to ascertain changes in protein renewal rates with high precision that cannot be currently obtained from conventional protein abundance data. Ideally for discovery purposes, metabolic labeling dynamics and protein abundance should be monitored in parallel, allowing investigators to obtain a more complete picture of changes following perturbations.

Are protein lifetimes similar *in vitro* and *in vivo*?

Although simplified models are instrumental for addressing the regulation of protein lifetimes [40], recent data suggest that protein lifetimes measured in cultured cells underestimate those obtained from *in vivo* studies [4,41,42]. Immortalized cells have abbreviated cell cycles and may have lost key regulatory mechanisms necessary to ensure proper coordination of protein complex assembly and proteome stoichiometry. Ultimately, this situation may accelerate cellular processes and protein turnover. In addition, proteins which have a lifetime exceeding the duration of the cell cycle will complicate the interpretation.

In some cases we can overcome this limitation by studying primary cells, with the caveat that they frequently represent model systems that reflect developmental processes *in vivo* that require robust protein synthesis. This is problematic because it can result in misestimating the protein turnover rates observed *in vivo*. On the other hand, there are cases where cells in culture do show very long lifetimes, for example, senescent cells undergoing contact inhibition [43]. These cells have minimal protein synthesis and show extended protein lifetimes that only capture minimal aspects of the dynamic situations observed *in vivo*.

Although all these cellular models have their strengths and weaknesses, we suggest using them only when there is no *in vivo* alternative. In general, we wish to emphasize that researchers need to understand that, to obtain the most biologically meaningful results, the biological question should match the protein labeling paradigm (Figure 1C). If the goal is to determine absolute protein lifetimes, one needs to be very careful in considering the experimental limitations. However, determining relative changes in protein lifetimes between two conditions (i.e., genetic, pharmacological) is generally straightforward provided that the protein measurements are precise.

Possible strategies to troubleshoot and validate protein turnover measurements

Validation of changes in protein turnover poses a challenge even if there are several possible strategies. First, there is a need to discern technical validations from biological validations, which ultimately serve as a confirmation that the observed alterations of the biological process under investigation are reliable and ultimately correspond to a meaningful biological change.

In terms of technical validations, we would like to delineate several categories. One category addresses the rigor of the fractional abundance measurements. In this case, a spike-in

experiment can be run in parallel with the biological samples to provide an idea of the sensitivity of the actual measurements. This is obtained by mixing tissue extracts from unlabeled and fully labeled animals and measuring different ratios of incorporation in the mixed samples [4]. Although the mixing is performed *in vitro*, the tissues that will be measured are the same used for the actual turnover measurement, thus providing useful information about the reliability of the data. One other category addresses the reproducibility across cohorts of animals and should be addressed either by performing the same measurements with different animals (and potentially in different laboratories) to ensure that the measurements are reproducible (although this is often practically unfeasible owing to the relevant costs of these experiments). One additional technical validation addresses the influence of a defined isotope or of a precise diet formulation on the measurements. To solve this issue, one option is to perform a second experiment and track protein degradation with a different isotopolog. Finally, to avoid measurement differences due to variability of the analysis workflow (typically the type of LC-MS/MS), several crossvalidation methods based on classical biochemical analysis or imaging approaches can be used, although these usually only recapitulate changes for a defined number of proteins. Crossvalidating results using orthogonal MS analysis and labeling strategies is an opportunity to integrate complementary data and reach more reliable conclusions. A recent work directly compared different strategies to analyze datasets obtained using different labeling techniques but the same mouse strains [44]. Such integrated approaches will be key for establishing shared ground-truth frameworks for analyzing and interpreting proteome-wide turnover measurements.

To provide spatial information that is lost in other MS approaches, **nanoscale secondary ion mass spectrometry (NanoSIMS)** or MS imaging [i.e., **matrix-assisted laser desorption/ionization (MALDI)**] can be used to study protein replacement in the endogenous environment using tissue sections [45–49]. Biological validations are more complex and, although virtually infinite scenarios exist, we showcase a few examples in Figure 1C and discuss additional interpretative aspects in Box 2 that might be useful for planning the correct validations.

Concluding remarks

It is our opinion that the proliferation of new technologies and the development of robust analysis workflows will culminate in a wealth of *in vivo* protein turnover data that will allow researchers to address several open questions (see Outstanding questions).

The main challenge that this field will face is how to obtain a 'meaningful biological understanding' of the complex underpinnings of protein lifetimes. To address this, we will need to consider contemporarily technological aspects that can influence the biological interpretation of the results while also combining more advanced metabolic labeling-based measurements in a multi-level perspective. To provide a simple example, the influence of different cell types on these measurements is difficult to address *in vivo* and might require cell sorting or genetic labeling strategies. Tissues that harbor only a few cell types will be less challenging than more complex tissues with highly heterogeneous cellular compositions. However, even a single cell type is likely to have differences that are only addressable at single-cell resolution. Furthermore, within cells, protein pools might have dissimilar lifetimes depending on their interactors, PTMs, or subcellular localizations, reflecting an opportunity for so far unimaginable biological discoveries.

Overall, we believe that, in combination with numerous experimental manipulations, all these approaches will allow us to systematically define the dynamic properties of the proteomes which will provide new avenues for human pathophysiology and biomedical research [50,51].

Outstanding questions

Does physicochemical protein damage broadly interfere with protein turnover, and how does this mechanistically contribute to aging and pathology?

What are the molecular processes that regulate protein turnover, and can they be pharmacologically manipulated?

What is the impact of PTMs such as phosphorylation on protein turnover? Will it be possible to differentiate at the molecular level the modifications that have causative effects in protein stabilization or degradation?

Specific PTMs (such as ubiquitination) are thought to regulate protein turnover, but their role in the regulation of protein stability *in vivo* remains largely unexplored. Will it be possible to obtain a comprehensive and quantitative atlas of these protein modifications that combines information about their subtypes, abundance, and influence on protein stability?

Will the rapidly evolving field of protein turnover be able to measure turnover at the 'intact protein' level, transitioning from a peptide-centric to a more precise 'top-down' approach?

Will *in vivo* protein turnover data become more common in the clinical context and be used to guide early diagnosis and personalized medicine?

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Declaration of interests

The authors declare no conflicts of interest.

References

1. Price, J.C. *et al.* (2010) Analysis of proteome dynamics in the mouse brain. *Proc. Natl. Acad. Sci. U. S. A.* 107, 14508–14513
2. Bomba-Warczak, E. *et al.* (2021) Long-lived mitochondrial cristae proteins in mouse heart and brain. *J. Cell Biol.* 220, e202005193
3. Alevra, M. *et al.* (2019) A mass spectrometry workflow for measuring protein turnover rates in vivo. *Nat. Protoc.* 14, 3333–3365
4. Fornasiero, E.F. *et al.* (2018) Precisely measured protein lifetimes in the mouse brain reveal differences across tissues and subcellular fractions. *Nat. Commun.* 9, 4230
5. Wilkinson, D.J. *et al.* (2021) Principles of stable isotope research – with special reference to protein metabolism. *Clin. Nutr. Open Sci.* 36, 111–125
6. Hammond, D.E. *et al.* (2016) Proteome dynamics: tissue variation in the kinetics of proteostasis in intact animals. *Mol. Cell. Proteomics* 15, 1204–1219
7. Basisty, N.B. *et al.* (2018) Stable isotope labeling reveals novel insights into ubiquitin-mediated protein aggregation with age, calorie restriction, and rapamycin treatment. *J. Gerontol. A Biol. Sci. Med. Sci.* 73, 561–570
8. McClatchy, D.B. *et al.* (2020) Quantitative analysis of global protein stability rates in tissues. *Sci. Rep.* 10, 15983
9. Basisty, N. *et al.* (2018) Protein turnover in aging and longevity. *Proteomics* 18, e1700108
10. Ross, A.B. *et al.* (2021) Proteome turnover in the spotlight: approaches, applications, and perspectives. *Mol. Cell. Proteomics* 20, 100016
11. Edfors, F. *et al.* (2016) Gene-specific correlation of RNA and protein levels in human cells and tissues. *Mol. Syst. Biol.* 12, 883
12. Mathis, A.D. *et al.* (2017) Mechanisms of in vivo ribosome maintenance change in response to nutrient signals. *Mol. Cell. Proteomics* 16, 243–254
13. McShane, E. *et al.* (2016) Kinetic analysis of protein stability reveals age-dependent degradation. *Cell* 167, 803–815
14. Léger, T. *et al.* (2017) A simple light isotope metabolic labeling (SLIM-labeling) strategy: a powerful tool to address the dynamics of proteome variations in vivo. *Mol. Cell. Proteomics* 16, 2017–2031
15. Guan, S. *et al.* (2012) Compartment modeling for mammalian protein turnover studies by stable isotope metabolic labeling. *Anal. Chem.* 84, 4014–4021
16. Rahman, M. *et al.* (2016) Gaussian process modeling of protein turnover. *J. Proteome Res.* 15, 2115–2122
17. Chepyala, S.R. *et al.* (2021) JUMPt: comprehensive protein turnover modeling of in vivo pulse SILAC data by ordinary differential equations. *Anal. Chem.* 93, 13495–13504
18. Sadygov, R.G. (2020) Partial isotope profiles are sufficient for protein turnover analysis using closed-form equations of mass isotopomer dynamics. *Anal. Chem.* 92, 14747–14753
19. Wu, C.C. *et al.* (2004) Metabolic labeling of mammalian organisms with stable isotopes for quantitative proteomic analysis. *Anal. Chem.* 76, 4951–4959
20. Krüger, M. *et al.* (2008) SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. *Cell* 134, 353–364
21. Holmes, W.E. *et al.* (2015) Dynamic proteomics: in vivo proteome-wide measurement of protein kinetics using metabolic labeling. *Methods Enzymol.* 561, 219–276
22. McClatchy, D.B. *et al.* (2015) Pulsed azidohomoalanine labeling in mammals (PALM) detects changes in liver-specific LKB1 knockout mice. *J. Proteome Res.* 14, 4815–4822
23. Alvarez-Castelao, B. *et al.* (2017) Cell-type-specific metabolic labeling of nascent proteomes in vivo. *Nat. Biotechnol.* 35, 1196–1201
24. Beynon, R.J. and Pratt, J.M. (2005) Metabolic labeling of proteins for proteomics. *Mol. Cell. Proteomics* 4, 857–872
25. Krishna, S. *et al.* (2021) Identification of long-lived proteins in the mitochondria reveals increased stability of the electron transport chain. *Dev. Cell* 56, 2952–2965
26. Toyama, B.H. *et al.* (2013) Identification of long-lived proteins reveals exceptional stability of essential cellular structures. *Cell* 154, 971–982
27. Savas, J.N. *et al.* (2012) Extremely long-lived nuclear pore proteins in the rat brain. *Science* 335, 942
28. Jongkamonwiwat, N. *et al.* (2020) Noise exposures causing hearing loss generate proteotoxic stress and activate the proteostasis network. *Cell Rep.* 33, 108431
29. Hark, T.J. and Savas, J.N. (2021) Using stable isotope labeling to advance our understanding of Alzheimer's disease etiology and pathology. *J. Neurochem.* 159, 318–329
30. Michno, W. *et al.* (2021) Following spatial Aβ aggregation dynamics in evolving Alzheimer's disease pathology by imaging stable isotope labeling kinetics. *Sci. Adv.* 7, eabg4855
31. Heo, S. *et al.* (2018) Identification of long-lived synaptic proteins by proteomic analysis of synaptosome protein turnover. *Proc. Natl. Acad. Sci. U. S. A.* 115, E3827–E3836
32. Karunadharma, P.P. *et al.* (2015) Respiratory chain protein turnover rates in mice are highly heterogeneous but strikingly conserved across tissues, ages, and treatments. *FASEB J.* 29, 3582–3592
33. Kluever, V. *et al.* (2022) Protein lifetimes in aged brains reveal a proteostatic adaptation linking physiological aging to neurodegeneration. *Sci. Adv.* 8, eabn4437
34. Hark, T.J. *et al.* (2021) Pulse-chase proteomics of the App knockin mouse models of alzheimer's disease reveals that synaptic dysfunction originates in presynaptic terminals. *Cell Syst.* 12, 141–158
35. Lau, E. *et al.* (2018) Integrated omics dissection of proteome dynamics during cardiac remodeling. *Nat. Commun.* 9, 120
36. Ko, H.-G. *et al.* (2020) Proteomic analysis of synaptic protein turnover in the anterior cingulate cortex after nerve injury. *Mol. Brain* 13, 19
37. Ko, H.-G. *et al.* (2018) Rapid turnover of cortical NCAM1 regulates synaptic reorganization after peripheral nerve injury. *Cell Rep.* 22, 748–759
38. Mandad, S. *et al.* (2018) The codon sequences predict protein lifetimes and other parameters of the protein life cycle in the mouse brain. *Sci. Rep.* 8, 16913
39. Wu, C. *et al.* (2021) Global and site-specific effect of phosphorylation on protein turnover. *Dev. Cell* 56, 111–124
40. Yousefi, R. *et al.* (2021) Influence of subcellular localization and functional state on protein turnover. *Cells* 10, 1747
41. Cohen, L.L.D. *et al.* (2013) Metabolic turnover of synaptic proteins: kinetics, interdependencies and implications for synaptic maintenance. *PLoS One* 8, e63191
42. Dörrbaum, A.R. *et al.* (2018) Local and global influences on protein turnover in neurons and glia. *eLife* 7, e34202
43. Payea, M. *et al.* (2022) Measurement of protein turnover rates in senescent and non-dividing cultured cells with metabolic labeling and mass spectrometry. *J. Vis. Exp.* 182, 63835

44. Hammond, D.E. *et al.* (2022) Harmonizing labeling and analytical strategies to obtain protein turnover rates in intact adult animals. *Mol. Cell. Proteomics* 21, 100252
45. Lechene, C. *et al.* (2006) High-resolution quantitative imaging of mammalian and bacterial cells using stable isotope mass spectrometry. *J. Biol.* 5, 20
46. Bonnin, E.A. *et al.* (2021) NanoSIMS observations of mouse retinal cells reveal strict metabolic controls on nitrogen turnover. *BMC Mol. Cell Biol.* 22, 5
47. Vogt, J.A. *et al.* (2005) Determination of fractional synthesis rates of mouse hepatic proteins via metabolic ^{13}C-labeling, MALDI-TOF MS and analysis of relative isotopologue abundances using average masses. *Anal. Chem.* 77, 2034–2042
48. Narendra, D.P. *et al.* (2020) Coupling APEX labeling to imaging mass spectrometry of single organelles reveals heterogeneity in lysosomal protein turnover. *J. Cell Biol.* 219, e201901097
49. Arrojo e Drigo, R. *et al.* (2019) Age mosaicism across multiple scales in adult tissues. *Cell Metab.* 30, 343–351
50. Sato, C. *et al.* (2018) Tau kinetics in neurons and the human central nervous system. *Neuron* 97, 1284–1298
51. Price, J.C. *et al.* (2012) Measurement of human plasma proteome dynamics with $^{2}H_{2}O$ and liquid chromatography tandem mass spectrometry. *Anal. Biochem.* 420, 73–83
52. Long, K.R. and Huttner, W.B. (2019) How the extracellular matrix shapes neural development. *Open Biol.* 9, 180216
53. D'Angelo, M.A. *et al.* (2009) Age-dependent deterioration of nuclear pore complexes causes a loss of nuclear integrity in postmitotic cells. *Cell* 136, 284–295
54. Claydon, A.J. *et al.* (2012) Protein turnover: measurement of proteome dynamics by whole animal metabolic labelling with stable isotope labelled amino acids. *Proteomics* 12, 1194–1206
55. Doherty, M.K. *et al.* (2005) Proteome dynamics in complex organisms: using stable isotopes to monitor individual protein turnover rates. *Proteomics* 5, 522–533
56. Kasumov, T. *et al.* (2011) Measuring protein synthesis using metabolic ^{2}H labeling, high-resolution mass spectrometry, and an algorithm. *Anal. Biochem.* 412, 47–55
57. Kushner, D.J. *et al.* (1999) Pharmacological uses and perspectives of heavy water and deuterated compounds. *Can. J. Physiol. Pharmacol.* 77, 79–88
58. Lau, E. *et al.* (2016) A large dataset of protein dynamics in the mammalian heart proteome. *Sci. Data* 3, 160015
59. Sadygov, R.G. *et al.* (2018) 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
60. Naylor, B.C. *et al.* (2017) DeuteRater: a tool for quantifying peptide isotope precision and kinetic proteomics. *Bioinformatics* 33, 1514–1520
61. Sénécaut, N. *et al.* (2021) Novel insights into quantitative proteomics from an innovative bottom-up simple light isotope metabolic (bSLIM) labeling data processing strategy. *J. Proteome Res.* 20, 1476–1487
62. McClatchy, D.B. *et al.* (2007) ^{15}N metabolic labeling of mammalian tissue with slow protein turnover. *J. Proteome Res.* 6, 2005–2010
63. Bateman, R.J. *et al.* (2007) Stable isotope labeling tandem mass spectrometry (SILT) to quantify protein production and clearance rates. *J. Am. Soc. Mass Spectrom.* 18, 997–1006
64. Lam, M.P.Y. *et al.* (2014) Protein kinetic signatures of the remodeling heart following isoproterenol stimulation. *J. Clin. Invest.* 124, 1734–1744
65. Rolfs, Z. *et al.* (2021) An atlas of protein turnover rates in mouse tissues. *Nat. Commun.* 12, 6778