University of Groningen

Mass spectrometric analysis of PTM dynamics using stable isotope labeled metabolic precursors in cell culture

van Pijkeren, Alienke; Bischoff, Rainer; Kwiatkowski, Marcel

Published in:
Analyst

DOI:
[10.1039/c9an01258c](https://doi.org/10.1039/c9an01258c)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2019

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

van Pijkeren, A., Bischoff, R., & Kwiatkowski, M. (2019). Mass spectrometric analysis of PTM dynamics using stable isotope labeled metabolic precursors in cell culture. *Analyst*, 144(23), 6812-6833.
<https://doi.org/10.1039/c9an01258c>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Cite this: *Analyst*, 2019, **144**, 6812

Mass spectrometric analysis of PTM dynamics using stable isotope labeled metabolic precursors in cell culture

Alienke van Pijkeren,^a Rainer Bischoff ^a and Marcel Kwiatkowski ^{a,b}

Received 5th July 2019,
Accepted 13th October 2019
DOI: 10.1039/c9an01258c
rsc.li/analyst

Biological organisms represent highly dynamic systems, which are continually exposed to environmental factors and always strive to restore steady-state homeostasis. Posttranslational modifications are key regulators with which biological systems respond to external stimuli. To understand how homeostasis is restored, it is important to study the kinetics of posttranslational modifications. In this review we discuss proteomic approaches using stable isotope labeled metabolic precursors to study dynamics of posttranslational modifications in cell culture.

Introduction

In the 1930s Rudolph Schoenheimer and David Rittenberg developed a methodology called the “isotope tracer technique”,¹ where they used stable isotope enriched molecules such as deuterated water (²H₂O) or ¹⁵N labeled amino acids in the nutrition of animals to study the fate of these “tracer” molecules, thus investigating the dynamic nature of metabolism. For this, the group of Schoenheimer first had to develop new methods to synthesize physiologically relevant stable isotope enriched tracer molecules such as deuterated water,² fatty acids or steroids.³ In one of their groundbreaking studies, Schönheimer and Rittenberg synthesized deuterated linseed oil that they used as a part of the nutrition fed to mice.⁴ Deuterium containing organic substances were com-

^aDepartment of Analytical Biochemistry, Groningen Research Institute of Pharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

^bMass Spectrometric Proteomics and Metabolomics, Lab of Metabolic Signaling, Institute of Biochemistry, University of Innsbruck, Innrain 80-82, 6020 Innsbruck, Austria. E-mail: marcel.kwiatkowski@uibk.ac.at

Alienke van Pijkeren

Alienke van Pijkeren received her master's degree in Biomedical Sciences from the University of Groningen (The Netherlands). She is currently doing her PhD in the Department of Analytical Biochemistry at the University of Groningen (The Netherlands) and the Institute of Biochemistry at the University of Innsbruck (Austria). In her PhD project, she investigates the role of changes in energy metabolism and PTM dynamics in age-related inflammatory diseases.

Rainer Bischoff

Rainer Bischoff received his PhD in Chemistry from the University of Göttingen (Germany) followed by postdoctoral positions at the Max-Planck-Institute for Experimental Medicine (Göttingen) and the Department of Biochemistry at Purdue University (West-Lafayette, USA). He started his career in industry at the biotechnology company Transgene (Strasbourg, France) in 1987 and continued at AstraZeneca R&D (Lund, Sweden) before joining the University of Groningen (The Netherlands) in 2001. His research interests comprise biomarkers, therapeutic proteins and the development of novel analytical techniques. Rainer Bischoff authored more than 250 peer-reviewed publications, a number of book chapters and is inventor on 14 patents.

busted to accurately measure the deuterium content by densitometry, refractive index or the falling drop method,^{2,5} thus investigating the fate of the deuterium. Schoenheimer and Rittenberg showed that the ingested deuterated fat was used as an immediate energy source stored in fat deposits, that could be utilized for energy production in times of need. This study provided for the first time experimental evidence that fat metabolism is a dynamic rather than a static process. Soon after Urey successfully isolated and concentrated the stable nitrogen isotope ^{15}N by using mass spectrometry,⁶ Schoenheimer and Rittenberg used stable ^{15}N isotope enriched tracer molecules such as ammonium citrate and amino acids to study the incorporation of ^{15}N in amino acids⁷ and proteins.⁸ To quantify elemental ^{15}N , nitrogen containing organic substances were incubated in a micro-Kjedahl flask with concentrated sulfuric acid.⁹ The carbon in the organic material was oxidized to carbon dioxide (CO_2) and the sulfuric acid was reduced to sulfur dioxide (SO_2). The nitrogen was reduced and released as ammonium sulfate $((\text{NH}_4)_2\text{SO}_4)$ from the organic substances. $(\text{NH}_4)_2\text{SO}_4$ was incubated with an excess of a strongly alkaline hypobromite (NaOBr) solution. The developed nitrogen isotopes were analyzed using a mass spectrometer described by Bleakney,^{10,11} equipped with an electron ionization source and a magnetic field to deflect the ion beam through an arc of 180° . In another study, Schoenheimer and Rittenberg were able to show that after feeding rats with ^{15}N containing tyrosine, only half of the ^{15}N enriched molecules were excreted and detected in urine, whereas the other half of the ^{15}N isotopes was incorporated into newly synthesized proteins.¹² These results on amino acid and protein metabolism were groundbreaking. The isotope tracer technique provided the first experimental proof that proteins do not exist in a static state but in a state of continual flux (turnover). The development of the isotope tracer tech-

nique and the initial work of Schoenheimer and Rittenberg represents a milestone in biochemistry and analytical chemistry as well as in the research of intermediary metabolism and protein metabolism. Soon after the introduction of the isotope tracer technique, Rittenberg used ^{15}N -labeled glycine and deuterated water to study protein turnover and determined turnover rates in rats, human and *E. coli*.¹³

During this time, Ruben and Kamen observed that the long-lived radioisotope ^{14}C can be generated by bombarding a graphite probe with deuterons, the deuterium nucleus containing a proton and a neutron.^{14,15} The generation of ^{14}C radioisotopes represented another milestone for the isotope tracer technology. The carbon atom represents the central atom for all metabolic and biochemical processes, thus the study of carbon metabolism was essential for the understanding of protein turnover and metabolism in general. The long half-life time of the ^{14}C radioisotope and its accurate and sensitive detection using scintillation counters led to an increased use of ^{14}C -isotope enriched tracer molecules in the field of protein turnover research from the 1950s to the beginning of the 21st century.^{16,17} Although there was a decline in the use of stable isotopes to study protein metabolism and protein turnover during this time because of the use of radioisotopes such as ^{14}C , ^3H and ^{35}S , the use of ^{15}N in combination with mass spectrometry was still essential to study nitrogen metabolism representing as an important part of protein metabolism. The reason for the continuous use of ^{15}N enriched tracer molecules was very simple, because no radioisotope of nitrogen exists that could be used to study nitrogen and protein metabolism, since the ^{13}N radioisotope has a half-life of less than ten minutes (9.97 min).¹⁸ Therefore, ^{15}N stable isotope labeling in combination with mass spectrometry remained the method of choice to study nitrogen metabolism including amino acid and protein metabolism.^{19,20}

In the last 20–30 years, the use of stable isotopes including ^{13}C and deuterium has undergone a renaissance to study protein metabolism and protein turnover for two main reasons. First (1), the potential health risks associated with the use of radioisotopes and second (2), pioneering developments in the field of mass spectrometry (MS). During the 1980s, Karas and Hillenkamp developed MALDI (matrix-assisted laser desorption/ionization),^{21,22} and Fenn introduced ESI (electrospray ionization).²³ These soft ionization techniques revolutionized the MS analysis of biomolecules and especially the analysis of intact proteins and peptides paving the way towards “proteomics”. Further key innovations such as the development of nano-ESI,^{24,25} nanoLC-MS,²⁶ search algorithms for peptide and protein identification^{27,28} and the development of modern Q-TOF^{29,30} and orbitrap mass spectrometers^{31,32} that combine high sensitivity and high resolution with a fast duty cycle, enable comprehensive proteome analysis by identifying and quantifying thousands of proteins and posttranslational modification sites.

Preproteomic protein turnover studies provided mainly information about total protein pool (bulk protein) turnover rather than individual proteins. MS based proteomics opened

Marcel Kwiatkowski

Marcel Kwiatkowski received his doctorate (Dr rer. nat.) in Chemistry from the University of Hamburg (Germany) followed by a postdoctoral position at the University Medical Center Hamburg-Eppendorf and the University of Groningen (The Netherlands) as a Marie Skłodowska-Curie postdoctoral fellow. In 2019 he moved to the University of Innsbruck (Austria) to head the Mass Spectrometric Proteomics and Metabolomics

unit of the Institute of Biochemistry. His research interests lie in the development of novel analytical techniques for combinatorial analysis of PTM, protein and metabolite dynamics, and in the development of hyphenated mass spectrometry techniques to analyze intact protein species.

not only the opportunity to study individual protein turnover rates at a global scale, but even to study the dynamics of post-translational modifications (PTMs) using metabolic precursors enriched with stable isotopes.

The analysis of PTM dynamics and turnover rates adds an extra dimension to static abundance measurements and is critical for systems-wide computational modeling of signaling dynamics³³ and to distinguish between modification sites that are stable and those that have a biological role in regulating cellular dynamics.

This review focuses on the use of metabolic precursors enriched with stable isotopes to study dynamics of posttranslational modifications in cell culture systems. Although we provide a brief introduction to protein turnover analysis using stable isotope labeled amino acids in cell culture (SILAC), we would like to refer to the reviews of Beynon³⁴ and Hellerstein³⁵ for a more comprehensive overview about the use of stable isotopes to study protein turnover *in vitro* and *in vivo*.

MS based analysis of protein turnover using stable isotope labeled amino acids

There have been numerous recent advances in MS instruments, including scanning speed, resolution, mass accuracy and sensitivity,^{36–38} the separation of complex peptide mixtures^{39–41} and improved algorithms⁴² for both data acquisition and data analysis, which together made it possible to achieve a proteome depth of up to 10 000 reproducibly identified and quantified proteins.⁴³ These technological improvements have a significant impact on studying turnover of individual proteins at a global proteomic scale. Most protein turnover studies in mammalian cell lines use metabolic precursors enriched with stable isotopes such as ¹³C and ¹⁵N. The most frequently used metabolic precursors are stable isotope labeled amino acids, especially lysine and arginine, as trypsin and Lys-C are the enzymes of choice to generate peptides for the shotgun proteomics approach. Trypsin cleaves C-terminal of arginine and lysine and Lys-C cleaves C-terminal of lysine. The use of stable isotope labeled amino acids in cell culture is widely known in the field of proteomics under the term “SILAC”⁴⁴ and represents one of the most commonly used methods in differential proteomics. In general differential proteomics, SILAC is used to compare the abundance of proteins between different experimental conditions. The cells are either grown in a “normal” (light) medium (unlabeled protein pool) or in a medium that contains stable isotope labeled lysine and/or arginine (heavy media) until the protein pool is (ideally) completely labeled. The stable isotope labeled amino acids are incorporated into the protein pool by a combination of turnover and pool expansion by cell growth resulting in stable isotope labeled proteins. While pre-existing “light” proteins are degraded intracellularly and/or removed by processes such as secretion from the cell, the abundance of stable

isotope labeled proteins continuously increases until the protein pool is almost completely labeled, thus having a “relative isotopic abundance (RIA)” close to 1. A complete labeling of the protein pool is generally achieved after six to seven cell divisions.^{45–47} The lysates of the light and heavy cells are combined and the proteolytic peptides are measured by MS. Due to the mass shift introduced by the stable isotope labeled amino acids, the individual peptide/protein abundance levels/ratios can be determined by MS in the precursor mass spectra.

Compared to the conventional SILAC approach described above, the appearance and disappearance of a stable isotope label can be investigated over time by MS in a “pulsed” SILAC experiment giving information about the synthesis and degradation rates of individual proteins (Fig. 1). In principle, pulsed SILAC experiments can be carried out in two different ways – a “labeling” experiment (1) or a “label loss” experiment (2). In a labeling experiment, the cells are cultured first in a normal “light” media and pulsed with a stable isotope enriched amino acid containing media (e.g. ¹³C-labeled lysine and/or ¹³C-labeled arginine) (Fig. 1A). The measurement of label incorporation provides information about protein synthesis. In a label loss experiment, the cells are first cultured in a media that contains stable isotope enriched amino acids. Subsequently, the medium containing stable isotope labeled amino acids is

Fig. 1 Strategies for protein turnover analysis using stable isotope labeled amino acids as metabolic precursors. Turnover studies follow either the strategy to measure the incorporation of a stable isotope (“labelling” experiment, A) or the loss of a stable isotope (“loss of label” experiment, B) from the protein pool providing information about protein synthesis (A) or protein degradation (B). P_x: Protein with a fast turnover, P_y: Protein with an intermediate turnover, P_z: Protein with a slow turnover.

changed to a medium containing the light amino acid counterpart (Fig. 1B). The measurement of the loss of the label provides information about protein degradation.

The group of Beynon was one of the first that used a stable isotope enriched amino acid as a metabolic precursor to investigate turnover rates of individual proteins at a larger scale.⁴⁸ In their study, Pratt *et al.* carried out a “label loss” experiment using deuterated leucine ($[D_6]-[^2H_{10}]\text{-leucine}$) to determine protein turnover rates in a leucine auxotroph yeast cell line. After the yeast protein pool was almost completely labeled, they exchanged the media containing deuterated leucine by a media containing non-labeled leucine. Samples were collected at multiple time points over 51 hours, separated by two-dimensional gel electrophoresis and a defined number of spots were analyzed by MALDI-TOF MS and peptide mass finger printing to monitor the transition from stable isotope labeled leucine containing peptides to light-leucine containing peptides. The relative isotopic abundance (RIA) was determined for each time point (RIA_t) by calculating the ratio of the monoisotopic peak intensities of the heavy peptide (I_H) and the sum of the monoisotopic peak intensities of the heavy peptide and the light peptide (I_L):

$$RIA_t = I_H / (I_H + I_L) \quad (1)$$

In growing cells, label loss (k_{loss}) occurs due to a combination of two exponential processes, protein dilution into daughter cells (k_{dil}) and protein degradation (k_{deg}):

$$k_{\text{loss}} = k_{\text{deg}} + k_{\text{dil}} \quad (2)$$

In their study, Pratt *et al.* used non-linear curve fitting of RIA_t at the different time points to calculate k_{loss} , where $t = 0$ represented the RIA immediately before the label switch and $t = \infty$ represented the RIA at the end of the experiment.⁴⁸

$$RIA_t = RIA_{t=\infty} + (RIA_{t=0} - RIA_{t=\infty}) \exp(-k_{\text{loss}} \times t). \quad (3)$$

By using an appropriate and constant correction factor for label-loss through dilution, the degradation rates k_{deg} for the individual proteins were obtained by subtracting the constant of dilution from k_{loss} . Pratt *et al.* showed in their study that the majority of the proteins had protein degradation rates of 2.2% per hour. Some of the proteins had very fast degradation rates of more than 10% per hour. For some proteins degradation rates could not be determined as they were lower or equal to the dilution rate and label was lost due to cell growth. The protein degradation rates were very heterogeneous and did not correlate with the overall abundance of the corresponding protein. This early protein turnover study at a larger scale indicated that protein turnover is an important parameter and needs to be considered if protein abundances are compared to the abundance of correlated mRNA species.⁴⁹

In 2009, the group of Beynon introduced the “dynamic SILAC” approach where they used stable isotope labeled arginine to determine the degradation rates for 600 proteins in an adenocarcinoma cell line in a “label loss” experiment.⁵⁰ In the same year, Schwannhäusser *et al.* introduced a variation of the

“dynamic SILAC” approach where they analyzed protein turnover dynamics in a “labeling” experiment.⁵¹ Compared to the studies of Pratt *et al.*,⁴⁸ they first cultured the cells in a light medium followed by incubation in a medium that contained stable isotope labeled lysine or arginine residues. At the different time points, the differently labeled samples were combined and analyzed by LC-MS. The pair of the light peptide and the heavy peptide in the mass spectrum gives information about the abundance of the “old” protein (light signal in the mass spectrum) and the abundance of the “newly synthesized” protein (heavy signal in the mass spectrum). Schwannhäusser *et al.* used this approach to determine protein synthesis rates in a model of iron homeostasis to identify proteins which synthesis is regulated upon iron availability.

The “labelling” and “label loss” approach provides either information about protein synthesis (appearance of a label) or protein degradation (disappearance of a label). To overcome this limitation, Boisvert *et al.* introduced the use of triple SILAC to investigate protein degradation, protein synthesis and net protein turnover by MS.⁵² HeLa cells were grown in either a normal medium containing “light (L) (^{12}C , ^{14}N)” lysine and arginine residues or in a medium containing “medium heavy (M) (^{13}C , ^{14}N)” lysine and arginine residues until the labeled amino acids were fully incorporated into the protein pool. The culture medium containing “M” amino acids was replaced by a culture medium containing “heavy (H) (^{13}C , ^{15}N)” lysine and arginine residues (pulsed sample), while the cells cultured in the normal medium were further grown in the medium containing “L” amino acids (light sample). At each time point the pulsed sample was mixed with an equal amount of the light sample and analyzed by LC-MS after tryptic digestion. The peptides from the light sample are an internal control and, together with the signals from the medium and heavy peptides, allow to determine the protein degradation rate, protein synthesis rate and net protein turnover. The ratio between the light and the medium peptide over time is used to determine protein degradation rates, whereas the ratio between the heavy and the light peptide is used to calculate protein synthesis rates, and the heavy/medium peptide ratios allows to determine net protein turnover. Boisvert *et al.* quantified over 8000 proteins and determined their spatial distribution and protein turnover rates, indicating that the turnover rate of a protein depends on its cellular localisation.

The above mentioned approaches rely on the ratio of the abundance of isotopically labeled peptides and are sensitive towards changes in overall protein amount.⁵³ The approaches are useful to study protein turnover rates in systems under steady state conditions where synthesis and degradation rates are balanced and cellular protein concentrations are constant, or calculating conversion rates assuming that the amount of protein remains constant. Protein turnover analysis under non-steady state conditions requires normalization at the level of individual proteins to compensate for differences in protein abundance. This can be achieved by spike-in SILAC⁵⁴ in combination with pulsed SILAC.⁵³ Spike-in samples are a mixture of

cells or proteins that are representative of the cell system to be studied. The spike-in sample is used as an internal control between the experiments to perform rate measurements, thus allow to determine protein turnover dynamics independent of differences in protein abundance. Jayapal *et al.* developed a multitagging proteomic approach employing dynamic SILAC (label loss experiment) and iTRAQ (isobaric tag for relative and absolute quantitation) to study protein turnover rates in dynamic systems under non-steady state conditions.⁵⁵ The iTRAQ channels were used to correct for changes in total protein abundance, thus eliminating the need for assuming steady state conditions.

Over the last ten years, protein turnover analysis using stable isotope labeled amino acids as metabolic precursors has become an integral part of proteome analysis with the turnover rates of several thousand proteins being determined under different conditions.^{56,57} It is important to point out that protein turnover is not a fixed value, but a dynamic process that strongly depends on the cell system and the experimental conditions. Capturing these dynamics is the power of measuring protein dynamics compared to static levels obtained by classical differential proteomics approaches.

Turnover analysis of posttranslational modifications using stable isotope labeled metabolic precursors

Most posttranslational modifications (PTMs) are based on metabolic precursors that are generated *via* major metabolic pathways such as acetyl-coenzyme A in case of protein acetylation. Stable isotope labeled metabolic precursors can therefore be used to study PTM turnover dynamics in cell culture systems. Compared to the use of stable isotope labeled amino acids to analyze protein turnover in cell culture, the choice of the appropriate metabolic precursor is somewhat more complex. In many cases, it is not possible to use the direct precursor molecule as a media component, for example, when the metabolic precursor does not or not adequately pass through the cell membrane. On the other hand, the addition of a medium component may affect cell response or metabolic precursors may result in an insufficient relative isotopic abundance of the direct precursor molecule. Although there are hundreds of different PTMs, only a few of them have been studied so far with stable isotope labeled metabolic precursors. The following section provides an overview of the PTMs that have been successfully analyzed in this way (Table 1).

Protein acetylation

Protein lysine acetylation is a reversible PTM regulated by lysine acetyltransferases (KATs, formerly known as histone acetyltransferases (HATs)) and lysine deacetylases (KDACs, formerly known as histone deacetylases (HDACs)) regulating the

transfer of the acetyl group from an acetyl-coenzyme A (acetyl-CoA) molecule to the ϵ -amino group of a lysine residue (KATs) or its removal (KDACs).⁵⁸ However, it is interesting to note that the high pH (7.9–8.0) in the mitochondrion, as well as the high steady-state concentrations of acetyl-CoA, ranging between 0.1 and 1.5 mM are sufficient to acetylate proteins non-enzymatic.⁵⁹ The first discovered proteins being acetylated were histones,⁶⁰ nuclear proteins around which the DNA is wrapped. Acetylation neutralizes the positive charge of lysine residues, thus weakening charge-dependent interactions between the histone protein and DNA molecules. This increases the accessibility of DNA to the transcription machinery and therefore regulates gene expression.^{61,62} Histone acetylation typically correlates with transcriptional activation, whereas histone deacetylation correlates with transcriptional repression. Besides histone proteins, many other proteins can be acetylated. Acetylation can play a role in a variety of processes including protein stability,^{63,64} DNA binding,⁶⁵ gene transcription,^{66,67} cell cycle,⁶⁸ protein–protein interaction,^{69,70} protein localization^{71–73} as well as RNA processing and stability.^{74,75} Moreover, recent research indicates that acetylation plays an important role in enzyme activity. It was shown that nearly all enzymes involved in the metabolism of glucose and fatty acids are acetylated.^{76,77} For example, the enzyme acetyl-CoA synthetase 2 (ACSS2), which is responsible for the production of acetyl-CoA from acetate, is activated by deacetylation of lysine residue 642 (ACSS2-K642).⁷⁸

Metabolic precursors of acetyl-CoA

Acetyl-CoA is the substrate used by KATs to acetylate lysine residues in proteins. Acetyl-CoA can be synthesized through all major metabolic pathways including carbohydrate metabolism (glycolysis, TCA cycle), lipid metabolism (beta-oxidation), and amino acid metabolism.⁷⁹ Due to the different metabolic pathways, several molecules can be used as metabolic precursors to label the two C-atoms of acetyl-CoA with stable ¹³C-isotopes to study lysine acetylation turnover. In mammals, glucose is the major C-atom donor for acetyl-CoA.⁸⁰ Glucose is metabolized by glycolysis into two molecules of pyruvate (Fig. 2), each composed of three C-atoms. Pyruvate is transported from the cytosol into the mitochondria where it is converted into acetyl-CoA *via* oxidative decarboxylation by the pyruvate dehydrogenase complex (PDC). In the TCA cycle, acetyl-CoA is converted into citrate by citrate synthase in a condensation reaction with oxaloacetate. During this reaction, the two glucose derived C-atoms of the acetyl-CoA molecule are incorporated into the citrate molecule (Fig. 2). Citrate is transported from the mitochondria to the cytosol *via* the mitochondrial tricarboxylate transporter (SLC25A1)⁸¹ and can freely diffuse into the nucleus where it is converted by ATP-citrate lyase (ACL) into acetyl-CoA.⁸⁰ During this reaction, the two glucose derived C-atoms are transferred to the acetyl moiety of acetyl-CoA and are further used by KATs to acetylate nuclear proteins including histone proteins (Fig. 2). By using uniformly labeled ¹³C-

Table 1 Studies using stable isotope labeled metabolic precursors to investigate PTM dynamics

Experimental model	Metabolic precursor	Strategy	Time points	MS method	Ref.
Acetylation					
Human embryonic kidney cells (HEK293)	[U- ¹³ C]-Glucose	Labeling experiment	0, 10, 20, 60, 120, 1440 minutes	Bottom-up MS, LC-ESI-MS, LTQ-Orbitrap-XL	93
HeLa cells	[U- ¹³ C]-Glucose, [¹³ C ₂]-pyruvate	Labeling experiment	0, 0.75, 1.5, 3, 6, 30 hours	Targeted-SRM, TSQ Quantum	99
HeLa cells	[U- ¹³ C]-Glucose	Labeling experiment	0, 0.5, 1, 4, 8, 12, 16, 24 hours	Bottom-up MS, LC-ESI-MS, Orbitrap Fusion Tribrid	110
Methylation					
HeLa cells	[¹³ C ² H ₃]-Methionine	Labeling experiment	Sampling every 10 hours, up to 100 hours	Top-down MS, ESI-FT-ICR-MS	138
HeLa cells	[¹³ C ² H ₃]-Methionine	Labeling experiment	0, 24, 48, 72, 96, 120, 144 hours	Bottom-up MS, LC-ESI-MS, LTQ-Orbitrap	113
HeLa cells	[¹³ C ² H ₃]-Methionine	Label loss experiment	0, 4, 12, 30, 52 hours	Bottom-up MS, LC-ESI-MS, LTQ-FT 12T	140
HeLa cells	[¹³ C ² H ₃]-Methionine	Labeling experiment	Sampling at 24, 48, and 72 hours	Bottom-up MS, LC-ESI-MS, LTQ-Orbitrap	173
HeLa cells	[¹³ C ² H ₃]-Methionine	Labeling experiment	Sampling at 0, 3, 6, 8, 10, 12, and 14 hours	Bottom-up MS, LC-ESI-MS, RP-LC coupled to a hybrid linear-quadrupole Orbitrap	146
Multiple myeloma (MM)-patient derived cell line (KMS11)	[¹³ C ² H ₃]-Methionine	Label loss experiment	0, 5, 10, 25, 50 hours	Targeted-SRM, TSQ Quantum	144
Primary human embryonic lung fibroblasts (MRC5), infected by the human cytomegalovirus (HCMV)	[¹³ C ² H ₃]-Methionine	Labeling experiment	12, 24, 48, 72, 96 hours	Bottom-up MS, LC-ESI-MS, LTQ-Orbitrap XL	147
<i>Saccharomyces cerevisiae</i> yeast strain BY4741	[¹³ C ² H ₃]-Methionine	Labeling experiment	0, 15, 30, 90, 150, 210, 270 minutes	Bottom-up MS, LC-ESI-MS, linear quadrupole ion trap-Orbitrap	148
THP1 (human monocytic) cells	[² H ₃]-Serine	Labeling experiment	0, 24, 48, 72, 96 hours	Bottom-up MS, LC-ESI-MS, Orbitrap QExactive	149
NMuMG cells (epithelial)	[¹³ C ² H ₃]-Methionine	Labeling experiment	0, 24, 48 hours	Bottom-up MS, LC-ESI-MS, Orbitrap Q-Exactive Middle-down MS, LC-ESI-MS, Orbitrap Fusion Tribrid	141
Phosphorylation					
Human embryonic kidney cells (HEK293) and HeLa cells	[γ- ¹⁸ O ₄]-ATP	Labeling experiment	5, 15, 30, 60, 120, 240 minutes	Bottom-up MS, LC-ESI-MS LTQ-Orbitrap XL, LTQ-Orbitrap Elite and Q-Exactive	152
O-GlcNAcylation					
HeLa cells	[U- ¹³ C]-Glucose	Labeling experiment	0, 0.5, 2, 5, 9, 24, 32, 48 hours	Bottom-up MS, LC-ESI-MS, Orbitrap Fusion Tribrid	153

glucose ([U-¹³C]-glucose) as a metabolic precursor, two ¹³C atoms are incorporated into acetyl-CoA and finally into the acetyl-group of acetyllysine residues. Next to glucose, glutamine is another C-atom donor for acetyl-CoA.⁸² Glutamine is hydrolyzed to glutamate and further metabolized to α-ketoglutarate, which enters the TCA cycle where it is ultimately oxidized to acetyl-CoA. Glutamine-derived α-ketoglutarate can also be directly metabolized to citrate by reductive carboxylation which can be further metabolized to acetyl-CoA as indicated above. The reductive carboxylation pathway is active in most cell lines under normal culture conditions but it is particularly important under conditions of hypoxia or mitochondrial dysfunction.^{83–86}

Acetate is another source of acetyl-CoA. Acetate is primarily generated through the breakdown of dietary fiber by the gut microbiota⁸⁷ and has a concentration of 50–200 μM in circulation.^{88,89} In addition, acetate is produced during KDAC mediated hydrolysis of acetyllysine residues.⁸⁶ Acetate is converted to acetyl-CoA by the enzyme ACS2 (acyl-CoA synthetase short-chain family member 2, also known as ACECS1) which is localized in the cytosol and nucleus.⁹⁰

Another source for mitochondrial acetyl-CoA is β-oxidation of fatty acids. McDonnell *et al.* have recently shown that metabolic reprogramming of hepatocytes results in increased acetyl-CoA synthesis *via* β-oxidation of medium chain fatty acids under conditions of low glucose and glutamine concentration.⁹¹ Under these conditions, fatty acid derived C-atoms were mainly used by KATs to acetylate histone proteins. The contribution of the different C-atom sources for acetyl-CoA synthesis and protein acetylation depends on various factors, such as the availability of the respective substrates as well as the physiological and metabolic conditions of the cell culture system under investigation. In most cell culture systems and under most metabolic conditions, glucose represents the major metabolic precursor for acetyl-CoA that is used for protein acetylation.⁹² [U-¹³C]-Glucose represents the metabolic precursor of choice to study histone acetylation turnover by mass spectrometry. The incorporation of the stable isotope labeled acetyl moiety results in a mass increase of 2 Da that can be resolved by modern mass spectrometers. However, as described above and shown in different publications, glutamine, acetate and fatty acids can become major sources for

Fig. 2 Flow of ^{13}C atoms from glucose to acetyllysine residues in histone proteins through glycolysis and the TCA cycle. ACL: ATP citrate lyase. KAT: Lysine acetyltransferase.

acetyl-CoA production and protein acetylation. When investigating acetylation dynamics with stable isotope labeled metabolic precursors, it is necessary to determine which precursor contributes most to acetyl-CoA production in the cell culture system under investigation.

However, it has to be pointed out that the monoisotopic peak of the ^{13}C -labeled acetylated peptide species convolutes with the third isotopic peak of the ^{12}C -containing acetylated peptide species. Deconvolution algorithms are therefore necessary to correct for the isotopic distribution of the different isotopologues to achieve an accurate quantification and turnover determination.

Histone acetylation and protein acetylation turnover

Everts *et al.* were the first to use stable isotope labeled metabolic precursors to study acetylation turnover rates of histone proteins in human embryonic kidney cells.⁹³ They initially investigated which stable isotope labeled metabolic precursor ($[\text{U}-^{13}\text{C}]$ -glucose, $[\text{U}-^{13}\text{C}]$ -glutamine, $[\text{U}-^{13}\text{C}]$ -acetate) was most suitable for labeling acetyllysine residues in histone proteins. The cells were first grown in a medium containing light glucose $[\text{U}-^{12}\text{C}]$ -glucose, followed by a switch to media containing either $[\text{U}-^{13}\text{C}]$ -glucose, $[\text{U}-^{13}\text{C}]$ -glutamine or $[\text{U}-^{13}\text{C}]$ -acetate (Fig. 3). The cells were cultured for 24 hours in the

Fig. 3 Experimental scheme for the study of histone acetylation turnover using $[\text{U}-^{13}\text{C}]$ -glucose according to Everts *et al.*⁹³

presence of the different stable isotope labeled metabolic precursors. At different time points, histone proteins were extracted with sulfuric acid and the primary amines of non-acetylated lysines were propionylated prior to tryptic digestion. Propionylation of lysine residues prevents trypsin from digesting after lysine residues and results in more hydrophobic peptides which are more suitable for reversed-phase chromatography. The histone peptides were analyzed by LC-MS with an

orbitrap mass spectrometer and label incorporation was assessed by the ratio of $^{13}\text{C}/^{12}\text{C}$ -labeled acetylated histone peptides. With $[\text{U}-^{13}\text{C}]$ -glucose, around 80% of the acetylated histone peptide species were labeled with two ^{13}C -atoms, whereas the usage of $[\text{U}-^{13}\text{C}]$ -labeled glutamine and $[\text{U}-^{13}\text{C}]$ -labeled acetate resulted in a label incorporation of approximately 20%. To determine histone acetylation turnover rates, Everitts *et al.* cultured the cells in the presence of $[\text{U}-^{13}\text{C}]$ -glucose and took samples after 10, 20, 60, 120, and 1440 min. The H3 peptide containing the lysine residues K9 and K14 showed a label incorporation after 10 minutes. Already after 2 h the stable isotope labeled species showed a higher abundance than the light species. LC-MS raw data was processed with an in-house developed pipeline⁹⁴ that can be requested and downloaded from the homepage of the Garcia group from the University of Pennsylvania (<https://hosting.med.upenn.edu/garcialab/resources/>). The pipeline includes an algorithm to deconvolute the overlapping isotopic distributions of the unlabeled and labeled isotopologues. The fraction of the ^{13}C -labeled acetylated peptides is determined by calculating the ratio of the abundance of the ^{13}C -labeled acetylated peptide (H) and the sum of abundances of the ^{12}C -labeled (L) and ^{13}C -labeled (H) acetylated peptide. The fraction of the ^{13}C -labeled peptide at different time points was fitted to an exponential decay model where A represents the RIA for each acetylation site and k represents the turnover rate:

$$\text{H}/(\text{H} + \text{L}) = A \times (1 - e^{-k \times t}) \quad (4)$$

Half-lives were calculated by $\ln 2/k$. Everitts *et al.* determined half-lives for six different acetylated peptides of the histone H3 protein and for one peptide from the histone H4 protein. The observed half-lives ranged from 53 to 87 min depending on the acetylated lysine residues and nearby modifications. PTM cross-talk is best exemplified for histone proteins in context of chromatin regulation.^{95,96} Not only do PTMs compete for the same site, a modification can affect additional PTMs at a nearby location including their kinetics. Direct measurement of site-specific PTM dynamics using LC-MS and stable isotope labeled precursors provides a powerful method for analyzing PTM crosstalk. The observed half-lives were faster compared to protein methylation⁹⁷ and slower compared to protein phosphorylation,⁹⁸ which was investigated by LC-MS in combination with stable isotope labeled methionine and differential phosphoproteomics, respectively. In addition, Everitts *et al.* studied the effect of the KDAC inhibitor butyrate on the half-life of the acetylation sites of the seven histone peptides. For some acetylation sites, the half-lives were increased by a factor of 1.9 indicating that the incorporation of $[\text{U}-^{13}\text{C}]$ -glucose derived carbons into acetyl sites equally depended on acetylation and deacetylation reactions. Some other acetylation sites showed a minor increase in half-lives and for the histone H4 acetylation site, half-life was not affected by butyrate treatment indicating that butyrate has only a very mild or no effect on the KDACs that are responsible for deacetylating the corresponding acetylation sites.

In another study, Zheng *et al.* used $[\text{U}-^{13}\text{C}]$ -glucose to study histone acetylation turnover and half-lives in HeLa cells.⁹⁹ Compared to the initial work of Everitts *et al.*,⁹³ a targeted LC-MS/MS method using selected reaction monitoring (SRM) was used. In line with the results of Everitts *et al.*, Zheng *et al.* observed a label incorporation of approximately 80% for the acetyl-group of the acetylated histone peptides. They cultured the HeLa cells in a media that contained $^{13}\text{C}_6$ -labeled arginine to label the histone pool, followed by a switch to media containing $^{12}\text{C}_6$ -arginine and $[\text{U}-^{13}\text{C}]$ -glucose. The switch towards a medium containing light arginine enabled distinction between “old” and “new” histone proteins that were synthesized after the media switch. Acetylation turnover rates were determined for 19 lysine residues of histone proteins H2, H3 and H4. The turnover rates and half-lives were calculated as described above⁹³ with the modification that value for the baseline background signal of the triple quadrupole LC-MS analysis (n) was taken into account.

$$\text{H}/(\text{H} + \text{L}) = A \times (1 - e^{-k \times t}) + n \quad (5)$$

12 out of the 19 acetyllysine residues showed a turnover with half-lives ranging from 0.8 h to 2.3 h. Wang *et al.* have shown by chromatin immunoprecipitation (ChIP) that all acetylation sites except H4K20ac were located in promoter/enhancer regions or active transcribed gene bodies that correlated positively with transcriptional activity.¹⁰⁰ Interestingly, Zheng *et al.* were able to show with their method that modifications of adjacent lysine residues strongly regulated the turnover of the acetylation group of an acetyllysine residue. For H3K9ac and H3K14ac, the turnover rate was about two times faster when the adjacent lysine was acetylated, but 50% slower when the adjacent lysine was methylated. This information is difficult, if not impossible, to extract from conventional differential proteome studies. Combination of the isotope tracer technique, using stable isotope labeled metabolic precursors, and mass spectrometry provides unique insights into the site-specific regulation of PTM dynamics. This additional level of information is critical to understand which PTM sites may have a biological role in regulating cellular systems, how they are regulated by PTM crosstalk and how they can be affected, for example, by small molecule inhibitors.

The almost direct link between citrate derived nuclear acetyl-CoA used by KATs to acetylate histone proteins make the turnover analysis of histone acetylation comparatively straightforward. Histones can be isolated from cell lysates by an acid-based extraction^{101,102} and acetylated histone peptides can be analyzed by LC-MS without any further enrichment.^{103–105} However, lysine acetylation turnover of non-histone proteins can be also investigated with stable isotope labeled metabolic precursors. In comparison, acetylated peptides from non-histone proteins must be enriched by immunoaffinity purification. Hitherto anti-acetyllysine antibodies are the method of choice to enrich acetyllysine-containing peptides from complex proteomes^{106–109} making analysis of acetylation dynamics at the proteomic scale not only time consuming but

also rather expensive, since starting material in the range of several milligrams are usually required.

Kori *et al.* recently used [U-¹³C]-glucose and D₃-acetate to study proteome-wide acetylation dynamics in HeLa cells.¹¹⁰ Cells were cultured for 24 h in the presence of stable isotope labeled metabolic precursors and harvested at different time points. After tryptic digestion, the acetyllysine containing peptides were enriched by immunoaffinity chromatography using anti-acetyllysine antibodies and the enriched peptides were analyzed by LC-MS using an orbitrap tribrid mass spectrometer. Acetylation turnover rates were calculated as described by Everitts *et al.* by fitting the ratios of the heavy (H) and light (L) acetylated peptides at each time point to eqn (4). The calculated turnover rate (*k*) was used to determine half-lives (ln 2/*k*) of the acetylation sites. Kori *et al.* reproducibly identified and quantified 1821 acetylated peptides with [U-¹³C]-glucose derived acetyl groups between the independent experiments and 2100 acetylated peptides from the cells labeled with ²H₃-acetate. The significance of label incorporation was determined by Mann–Kendall trend test across the different time points. For [U-¹³C]-glucose, 901 peptides showed a significant trend in label incorporation, whereas only 126 for the ²H₃-acetate labeling. The half-lives for glucose labeling ranged from 0.2 h to 811 h and the half-lives from acetate labeling ranged from 0.02 h to 1349 h, indicating that glucose was the major contributor to acetyl-CoA synthesis. To confirm this, they further analyzed stable isotope incorporation into acetyl-CoA upon labeling with [U-¹³C]-glucose and ²H₃-acetate. The metabolites were extracted with ice-cold methanol and analyzed by reversed phase LC-MS using tributylamine as an ion-pairing reagent for separation. Label incorporation into the acetyl-CoA pool exceeded 50% after 8 hours and 80% after 12 h of incubation with [U-¹³C]-glucose. In contrast, the label incorporation started to exceed 50% after 24 h incubation with D₃-acetate. The slow labeling of the acetyl-CoA pool explains the observed slower incorporation of the stable isotopes into the acetyl group of the acetyllysine residues using D₃-acetate compared to [U-¹³C]-glucose. Although, Kori *et al.* measured label incorporation into the acetyl-CoA pool, they did not include acetyl-CoA labeling kinetics for calculating acetylation turnover rates as recommended by Fan *et al.*¹¹¹ Fan *et al.* described in detail how to measure acetyl-CoA labeling kinetics and how to include this in a mathematical model to quantify acetylation turnover rates. Including the labeling kinetics of the precursor metabolites is important for an accurate quantification of PTM turnover rates, especially when the metabolic precursors show a relatively slow labeling of the metabolite substrate that is used to modify the protein. In their study, Kori *et al.* showed that histone proteins and proteins associated with chromatin organization showed a fast acetylation turnover, followed by proteins involved in RNA metabolism, whereas proteins involved in lipid metabolism had comparatively long half-lives. Non-enzymatic acetylation sites had the longest half-lives. However, half-lives were not defined more precisely for the different groups. Interestingly, sites with a fast acetylation turnover rate, such as K105ac and K10ac of

SMC3 (structural maintenance of chromosomes protein 3), are key targets of KDAC inhibitors like the KDAC8 inhibitor PCI34051.¹¹² Comparing turnover rates, the results of Kori *et al.* showed that non-histone protein acetylation has, on average, longer half-lives (~69.5 h)¹¹⁰ than histone methylation (~5 hours),¹¹³ histone acetylation (~53–87 min)^{93,99} and protein phosphorylation (~20 minutes).⁹⁸

Protein methylation

Next to protein acetylation, protein methylation is another important PTM regulating many cellular processes ranging from gene transcription and epigenetics to metabolism.¹¹⁴ Protein methylation is a reversible PTM occurring on the nitrogen containing amino acids lysine and arginine. Protein lysine (PKMTs) or arginine methyltransferases (PRMTs) catalyze the transfer of a methyl group from *S*-adenosylmethionine (SAM) to a lysine or arginine residue. Lysine residues can be either mono-(me1), di-(me2), or trimethylated (me3).¹¹⁵ Arginines can be monomethylated or dimethylated where arginine dimethylation can occur in symmetric¹¹⁶ or asymmetric¹¹⁷ configuration. Methylation, and especially di- and trimethylation increases the steric hindrance at the methylated site, thereby possibly affecting interaction of methylated proteins with other cellular substrates.¹¹⁸ Moreover, histone methylation is thought to play an important role in regulating gene expression. The consequence of histone methylation on gene expression is dependent on both the specific lysine/arginine residue and whether the amino acid carries me1, me2 or me3 modifications. For instance, general H4R3 methylation¹¹⁹ and H3R2me1 are associated with active transcription, whereas H3R2me2 is associated with gene silencing.¹²⁰

Metabolic donors for protein methylation

The universal methyl donor for protein methylation is *S*-adenosylmethionine (SAM). SAM is generated during the methionine cycle (Fig. 4). In the methionine cycle, the methyl group of methionine becomes activated by *S*-adenosylmethionine synthetase, also known as methionine adenosyltransferase (MAT), which catalyzes the addition of an adenosine group to the sulfur atom of methionine directly adjacent to its methyl group, resulting in the formation of SAM.¹²¹ The methyl group of SAM is used by PKMTs or PRMTs for protein methylation resulting in *S*-adenosyl homocysteine (SAH). SAH is subsequently hydrolyzed to homocysteine by removing the adenosine group, a reaction catalyzed by *S*-adenosyl homocysteine hydrolase (SAHH).¹²² Homocysteine can be converted back to methionine in a transmethylation reaction. This reaction is catalyzed by methionine synthase (MeSe) and connects the methionine cycle with the folate cycle. MeSe catalyzes the conversion of 5-methyl-tetrahydrofolate (N⁵-MeTHF) to tetrahydrofolate (THF) in the

Fig. 4 Metabolism of methionine. Methionine is metabolized in the methionine cycle. In the methionine cycle, *S*-adenosylmethionine (SAM) is formed, which is the universal methyl group donor, which is used by protein methyltransferases (PMT) to methylate lysine (protein lysine methyltransferases, PKMTs) and arginine (protein arginine methyltransferases, PRMTs) residues. MAT: methionine adenosyltransferase, SAH: *S*-adenosyl homocysteine, SAHH: *S*-adenosyl homocysteine hydrolase, N5-MeTHF: 5-methyltetrahydrofolate, THF: tetrahydrofolate, MeSe: methionine synthase, B12: cofactor vitamin B12.

presence of the cofactor vitamin B12, thereby transferring a methyl group to homocysteine leading to the formation of methionine. For non-ruminant mammals such as humans, methionine is an essential amino acid and has to be taken up by the diet.¹²³ Besides intake from the diet, methionine can be regenerated from *L*-cysteine and *L*-aspartate,¹²⁴ protein degradation and synthesis *via* the one-carbon metabolic pathway.^{125,126} A one-carbon unit from serine or glycine is transferred in the folate cycle to tetrahydrofolate to form 5-methylene-THF. Methionine can be synthesized by transmethylation from 5-meTHF as described above, and subsequently converted to SAM. In principle, SAM would be the ideal molecule to be used as a stable isotope labeled metabolic precursor to study protein methylation. However, the poor membrane permeability of SAM¹²⁷ does not allow its direct use as precursor for labelling methyl groups, thus stable isotope labeled methionine represents the metabolic precursor of choice.

improve proteome-wide identification and quantification of methylated protein species. Similar to SILAC, cells are grown in media containing stable isotope labeled methionine, bearing one ¹³C atom and three deuterium atoms (*L*-methionine-¹³C²H₃). After incubating cells with the stable isotope labeled methionine, cells metabolically convert the labeled methionine to [¹³C²H₃]-SAM. The [¹³C²H₃]-methyl group is used for protein methylation by protein methyltransferases. Incorporation of [¹³C²H₃]-methyl groups results in a mass shift of +4 Da compared to proteins containing unlabeled methyl groups. A mass shift of 4 Da can be easily detected with high-resolution mass spectrometers, and the isotopic distributions of the methylated peptide species overlap only very slightly. The observation of peptide pairs with mass shifts of 4 Da, 8 Da, or 12 Da at the MS1-level indicate the presence of mono-, di-, and trimethylated peptide species.

After the introduction of the heavy methyl SILAC approach, it was used for differential protein methylation analysis.^{133–137}

Heavy methyl SILAC – stable isotope labeled SAM as a metabolic precursor

The substoichiometric nature of protein methylation, the complex fragmentation behaviour in collision induced dissociation, especially for (di)methylated arginine species,^{128–130} and similar mass shifts to other PTMs like acetylation in case of trimethylation¹³¹ were and still are reasons why proteome-wide analysis of protein methylation is challenging. Ong *et al.* 2004 introduced the 'heavy methyl SILAC' approach¹³² to

Histone methylation turnover using stable isotope labeled methionine as a metabolic precursor

While the above mentioned studies provided a static snapshot of protein methylation, stable isotope labeled methionine can also be used to study site-specific protein methylation dynamics.

Pesavento *et al.* 2008 used top-down mass spectrometry (TD-MS) in combination with stable isotope labeled arginine,

valine and methionine as metabolic precursor to analyze histone protein 4 lysine 20 (H4K20) methylation during cell cycle progression in human HeLa S3 cells.¹³⁸ They isolated histone proteins by sulfuric acid extraction and purified H4 protein species by RP-HPLC. Intact H4 species were analyzed by a quadrupole Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR-MS) with a custom build 8.5 tesla magnet and electron capture dissociation (ECD). TD-MS provides direct access to the H4 species distribution and allows to quantify the combinatorially modified protein species, since the ionization efficiency of intact protein species is not appreciably affected by the comparably small modification.¹³⁹ The study showed that newly synthesized, non-methylated H4 species are incorporated into chromatin during S phase. The unmethylated species are monomethylated at K20 between the G2 phase and the end of mitosis or early G1 phase, respectively. Between the G2 phase and the G1 phase of the next cell cycle, the monomethylated H4 species are successively dimethylated by the addition of a second methyl group, and a small portion of the H4 species is then trimethylated by the insertion of another methyl group. TD-MS analysis revealed that approximately 98% of the H4 species become dimethylated after three cell cycles. Pesavento *et al.* further showed that unmethylated H4 species are methylated independent of acetylation at adjacent lysine residues, and that H4 species carrying a K20 dimethylation were predominantly acetylated. These results disproved the hypothesis that methylation of K20 in H4 species antagonizes lysine acetylation. This first analysis of methylation turnover by MS further showed that dimethylation of K20 in H4 species is a very stable modification, since almost no turnover was detected over 50 h. In addition to [¹³C²H₃]-methionine, Pesavento *et al.* has very elegantly used a pulse with stable isotope labeled valine to distinguish methylation turnover between already existing and newly synthesized proteins by TD-MS. However, the TD-MS approach is still largely confined to isolated protein species and requires high-resolution mass spectrometers, ideally equipped with different fragmentation options including radical driven ECD as used by Pesavento *et al.* or electron transfer dissociation (ETD).

In another study Zee *et al.* used stable isotope labeled methionine and bottom-up proteomics to analyze histone methylation dynamics in HeLa cells.¹¹³ To quantitatively assess dynamics of different methylation states (me1, me2, me3) at individual lysine and arginine residues, two models were developed to calculate methylation turnover and half-life times. The two models differ in what was considered to be the "total methylation pool". In one model, the total pool referred to all intermediates for a specific methylation (*e.g.* monomethylation), and was used to determine turnover-rates for a specific methylated peptide species. The second model considered all methylation states and intermediates (unmethylated (me0), me1, me2, me3) for a given tryptic peptide. This model provided a more accurate comparison between the different methylated and intermediate species. Turnover kinetics were calculated by a set of first order differential equations (for further details, we refer to the supplemental material of

the published study).¹¹³ Parameters included in the differential equations were the sequential addition of a labeled methyl group to an unmodified peptide ($k_{me0 \rightarrow me1}$, $k_{me1 \rightarrow me2}$, $k_{me2 \rightarrow me3}$), sequential demethylation ($k_{me3 \rightarrow me2}$, $k_{me2 \rightarrow me1}$, $k_{me1 \rightarrow me0}$), degradation of methylated peptides and dilution of the stable-isotope-labeled methionine and methyl group pool with unlabeled methionine and methyl groups due to protein degradation and amino acid recycling. The group emphasized that the turnover rates derived by their model do not reflect the activity of specific protein methyltransferases or demethylases. It is an idealized model based on sequential methylation and demethylation reflecting the average activity of all methyltransferases and demethylases. They determined turnover rates for nine methylated histone species and reported progressive methylation similar to Pesavento *et al.*¹³⁸ They further showed that different methylation states (me1, me2, me3) have considerably different half live times. For example, the histone H3 protein showed the following half-live times for lysine 9 methylation: H3K9me1: $t_{1/2} = 0.342$ days, H3K9me2: $t_{1/2} = 1.031$ days, H3K9me3: $t_{1/2} = 1.344$ days. The detected half-live times for histone methylation were considerably longer compared to histone acetylation (half-lives ~53–87 min).^{93,99} Interestingly, Zee *et al.* showed different methylation kinetics for lysine and arginine methylation. After categorizing the investigated residues by their epigenetic function, Zee *et al.* observed that methylations associated with active genes had significantly faster half-maximal times than methylations associated with silent genes. Moreover, other PTMs residing on a methylated peptide could influence the methylation turnover rate of a specific residue. Zee *et al.* showed that the H3K9me1 turnover rate was increased by more than 50% if H3K14 was acetylated. These results are in line with the results of Zheng *et al.*⁹⁹

Compared to the previous studies, Sweet *et al.* used an "label loss" experiment following the disappearance of the stable isotopes to study dynamics of H3K79me1 and H3K79me2 in old and newly synthesized histone H3 variants, H3.1, H3.2 and H3.3.¹⁴⁰ HeLa cells were first incubated with stable isotope labeled methionine and arginine until an almost complete labeling was achieved. Subsequently, the medium was exchanged against a medium containing unlabeled methionine and arginine to study methylation dynamics in old and newly synthesized H3 variants. Different H3 variants were purified by RP-HPLC, digested with Arg-C and analyzed by LC-ESI-FT-ICR-MS. Although the abundance of K79me1 was different between the different H3 variants, similar methylation turnover rates were observed. The formation of dimethylated species from unmodified ones (H3K79me0 $\rightarrow$ H3K79me2) was significantly slower compared to the formation of monomethylated species (H3K79me0 $\rightarrow$ H3K79me1). However, taking the intermediate dimethylated species (H3K79me2:1) into account, turnover rates for newly synthesized di-, and monomethylated K79 species were not significantly different. Interestingly, Sweet *et al.* were able to show, by using stable isotope labeled metabolic precursors for methylation and protein turnover analysis, that pre-existing

and newly synthesized H3 variants showed similar turnover rates for K79me1 and K79me2.

Recently, Sidoli *et al.* compared a middle-down (MD) MS with the bottom-up (BU) MS to investigate whether the MD-MS can be combined with metabolic labeling for an accurate quantification of PTM dynamics.¹⁴¹ The MD approach is a compromise between the TD and BU approach, where either intact proteins or proteolytic peptides with a length of 5–20 amino acids are analyzed by MS. In MD-MS, histones are digested with GluC that have less frequent cleavage sites compared to trypsin. This results in polypeptides of 50–60 amino acids in length, and most importantly, the histone N-terminal tails that carry most of the biologically relevant PTMs remain intact.^{142,165} Hereby combinatorial PTM effects can be investigated with MD-MS, which is not possible with the BU approach, since direct access to the intact histone-tail species is lost. In their study, Sidoli *et al.* used [¹³C²H₃]-methionine to follow label incorporation in methyl groups (“labeling” experiment) to investigate methylation dynamics in epithelial cells transitioning into mesenchymal cells. NMuMG cells were cultured in [¹³C²H₃]-methionine and treated with TGFβ to induce epithelial to mesenchymal transition (EMT). Samples were taken at day 0, 1 and 2. Histones were isolated by sulfuric acid extraction and separated by RP-HPLC. The purified H3 protein species were digested with either trypsin or GluC for BU or MD analysis, respectively. For BU analysis, tryptic peptides were analyzed by reversed phase nano-LC-ESI-MS using HCD fragmentation. For MD, GluC derived polypeptides were analyzed by nano-LC-ESI-MS using ETD fragmentation and salt-less pH-gradient hybrid weak cation exchange–hydrophilic interaction (WCX-HILIC) chromatography developed by Young *et al.*¹⁴³ During the pH gradient, the stationary phase is protonated and cationic interactions are reduced, resulting in an effective separation of intact histone N-terminal tail species. Acetylated and trimethylated species, as well as mono-, di- and trimethylated species can be separated from each other chromatographically, which is difficult to achieve with the reversed phase separation. The results of the study show that the BU and MD approach provide very similar information about histone methylation dynamics. However, Sidoli *et al.* were able to show by MD-MS that H3K27me3 was a critical player during the EMT process as a consistent portion of histones modified at H3K27me2K36me2 in epithelial cells were converted into H3K27me3K36me2 in mesenchymal cells. Moreover, they showed by MD-MS that this conversion occurred most frequently on histone tails where H3K27me2 is flanked by H3K36me2 and no modifications on H3K9. These results could be obtained with MD-MS, as there was direct access to the intact histone tail species. This allowed the combinatorial effect of the PTMs to be investigated, which was not possible with the BU approach, as direct access to the intact histone-tail species was no longer present.

Compared to the above mentioned studies, Zheng *et al.* developed a targeted SRM-method to investigate combinatorial methylation dynamics on K27 and K36 of histone H3 in a multiple myeloma-patient derived cell line (KMS11).¹⁴⁴ Similar to

Sweet *et al.*¹⁴⁰ they used a “label loss” strategy with stable isotope labeled arginine and methionine. After 50 hours, 0.7% and 3.4% of overall monomethylation on new histones at K27 and K36 was derived from stable isotope labeled methionine. Based on this observation, they concluded that the contribution of the old pool to methylation was negligible. Measured abundances of the detected methylated species were used to build a kinetic model to calculate rate constants of the interconversions of methylated states. To this end, Zheng *et al.* used the model previously reported by Zee *et al.*¹¹³ and adapted it to their kinetic scheme of methylation considering sequential methylation and demethylation steps. The kinetics for H3K27 methylation were in line with the results reported by Zee *et al.*¹¹³ The rate constant for the formation of K27me1 was higher compared to the formation of K27me2, and the formation rate of K27me2 was higher compared to K27me3. This was the case both when K36 was unmodified or monomethylated, which is in line with the fact that EZH2, the major K27 histone methyltransferase,¹⁴⁵ prefers H3K27 in lower methylation states as substrates. Further investigation of combinatorial K27 and K36 methylation revealed that the rate constants for the formation of K27me1 and K27me2 decreased significantly when K36me2 or K36me3 was present. A similar decrease of rate constants was observed for K36me1 and K26me2 formation when K27 was di- or trimethylated. The conversion rates of K36me0 to K36me1 and K36me1 to K36me2 were increased in the presence of increased levels of the histone methyltransferase MMSET when compared to MMSET low-cells, while the rate for the conversion of K36me2 to K36me3 was not increased, suggesting that MMSET serves as a H3K36 dimethyltransferase. Moreover, in MMSET-overexpressing NTKO cells, the formation of K36me1 and me2 is much faster than the methylation rate of K27. This data show that this type of analysis can be used to investigate the specificity of methyltransferases with respect to the conversion of methylation states.

The work described so far has investigated methylation dynamics in proliferating cells. Xu *et al.* used a labeling strategy with [¹³C²H₃]-methionine in combination with a bottom-up approach to investigate H3 methylation turnover in cell cycle arrested HeLa cells.¹⁰¹ By investigating cell cycle arrested cells, the turnover of methyl groups could be analyzed in the absence of *de novo* histone synthesis. For H3K9me2, a clear shift from light to stable isotope labeled methyl groups was observed after 24 hours. About 34% was H3K9me2:2 (2 labeled methyl groups), 29% H3K9me2:1 (1 unlabeled methyl group, 1 labeled methyl group) and 37% H3K9me2:0 (2 unlabeled methyl groups). After 72 hours, about 61% was H3K9me2:2, 24% H3K9me2:1 and 15% H3K9me2:0, indicating an active turnover (demethylation and subsequent remethylation) of H3K9me2. Without using stable isotope labeled precursors, this active turnover could not be detected, highlighting the relevance of using metabolic labeling with stable isotope precursors to investigate PTM dynamics. The turnover rate of H3K9me3 was considerably slower (H3K9me3:0/3:1/3:2/3:3 levels changed from 81%:11%:4%:4% at 24 hours to

69%:12%:9%:10% at 72 hours). Consistent with the data from Sweet *et al.*, H3K79me2 formation from unmodified species was slower compared to the formation of monomethylated species.

In addition, Zee *et al.*,¹⁴⁶ O'Connor *et al.*¹⁴⁷ and Mews *et al.*¹⁴⁸ analyzed histone methylation dynamics. They all used a bottom-up approach to follow the incorporation of the stable isotopes upon labeling with [¹³C²H₃]-methionine.

Zee *et al.* included analysis of the intracellular level of the methionine-derived methyl-group donor [¹²CH₃]-SAM and [¹³C²H₃]-SAM by HILIC-SRM-MS. The intracellular [¹³C²H₃]-SAM pool was saturated after 3 hours with a labeling efficiency of 94%. Dilution by light methyl groups may be negligible for the calculation of the methylation turnover rates, because of the rapid labeling of the intracellular SAM pool compared to methylation turnover. Zee *et al.* observed that trimethylation of H3K9 and H3K27 in HeLa cells begins already in S phase, while steady-state levels were only reached in G1 phase of the next cell cycle. In contrast to H3K9me3, H3K9me2 levels reached steady-state levels within one cell cycle. Moreover, they showed that H4K20 methylation occurs at specific cell cycle stages, whereas H3K36 methylation occurs independently of the cell cycle.

O'Connor *et al.* studied the effect of virus infection on histone H3 and H4 methylation dynamics.¹⁴⁷ Primary human embryonic lung fibroblasts (MRC5) were infected with human cytomegalovirus (HCMV) and incubated with [¹³C²H₃]-labeled methionine. Significantly up- and downregulated methylation levels were found in HCMV infected cells compared to controls. The unlabeled H3K27me3K36me2 peptide species was less abundant in the infected cells than in the control cells. For infected cells, the highest abundance was observed for stable isotope labeled H3K27me3K36me2 peptide species with four labeled methyl groups, followed by species with three and two labeled methyl groups. These results indicate a higher methylation turnover of these sites in infected cells compared to control cells. Moreover, methylation of H3K79 was significantly increased throughout the time of infection. Fully labeled H3K79me1 (H3K79me1:1) and H3K79me2 (H3K79me2:2) were twofold increased in infected cells compared to control cells. Concurrent with the increase of H3K79me2, RNA levels of DOT1L (the H3K79 methyltransferase) were significantly increased in HCMV-infected cells. O'Connor *et al.* showed that knockdown of this specific histone methyltransferase in HCMV-infected cells resulted in a nearly 10-fold lower virus titer. This implies the importance of DOT1L-mediated H3K79 methylation for virus replication and opens the opportunity to suppress virus infection by targeting H3K79me2.

Mews *et al.* investigated the effects of an altered metabolic state on methylation dynamics in the *Saccharomyces cerevisiae* yeast strain BY4741.¹⁴⁸ Histone acetylation has been shown to respond rapidly to changes in the metabolic state of cells. To compare changes in acetylation and methylation, Mews *et al.* induced quiescence in cells by nutrient starvation. Subsequently, cells were induced to reenter the cell cycle by replacing water with a medium containing [U-¹³C]-glucose, or

[¹³C²H₃]-methionine, to track acetylation or methylation dynamics, respectively. New [U-¹³C]-glucose derived histone acetylation (H3K9/H3K14) took place within minutes, indicating a fast response of acetylation to the changed metabolic state. However, histone methylation remained highly stable during exit from quiescence, as shown for the H3K4, H3K36, and H3K79 methylation sites and their different methylation states (mono-, di-, or trimethylation). New methylation was observed around 2 hours after reentry into the cell cycle coinciding with *de novo* histone protein synthesis. Chromatin immunoprecipitation DNA sequencing analysis revealed that this new histone methylation exclusively occurred on genes associated with cell growth, resulting in a shift from genes upregulated under nutrient stress towards genes necessary to initiate cellular replication.

Histone methylation turnover using stable isotope labeled serine as a metabolic precursor

Compared to the studies described above, Tang *et al.* recently used stable isotope labeled serine (2,3,3-²H-serine) instead of [¹³C²H₃]-methionine to study histone methylation dynamics.¹⁴⁹ THP1 cells were cultured in medium containing [2,3,3-²H]-serine. During the course of incubation one deuterium is incorporated into SAM *via* the folate cycle and the methionine cycle (Fig. 4). The deuterated methyl group of SAM is used by methyltransferases to methylate histone proteins. Incorporation of deuterium atoms over time was used to investigate the methylation dynamics. Tang *et al.* focused in their study on lysine methylation turnover. For this, they used signature ions of methyllysines generated during collision induced dissociation. A mono-methylated lysine residue generates an immonium ion at *m/z* 98.0963. After incorporation of ²H from the [2,3,3-²H]-serine, the immonium ion has an *m/z* of 99.1023. A resolution of 70 000 was sufficient to resolve the two immonium ions and to quantify them separately. Similarly, trimethylation dynamics can be monitored by identifying and quantifying characteristic signature ions in the MS/MS spectra. Compared to monomethylated lysines, trimethylated lysines do not show an immonium ion, but a specific fragment generated due to the neutral-loss of a trimethyl ammonium ion [NH(CH₃)₃]⁺ at *m/z* 60.0811. After [2,3,3-²H]-serine-mediated trimethylation, this neutral-loss ion shifts from *m/z* 60.0811 to *m/z* 61.0873. With a resolution of 35 000 both signal ions could be resolved and quantified. To calculate methylation dynamics, the relative intensities of the unlabeled and labeled immonium ion (mono-methylation) and neutral-loss ion (trimethylation) were used. For further details about this calculation, we refer to the published study.¹⁴⁹ Tran *et al.* used specific fragment ions to determine methylation dynamics, because the isotopologues clusters of the different species overlap in the intact MS spectra, and even after deconvolution of the isotopic peaks,

the portion of incorporated ^2H from methylated lysine cannot be precisely determined from the MS1 spectra. Culturing THP1 cells in $[2,3,3\text{-}^2\text{H}]$ -serine for four consecutive days (96 hours) resulted in an increase of 0.1% to 3.7% of the stable isotope labeled methylated immonium ion. Although 3.7% itself is not a high number, it represents more than a 25-fold increase when compared to the natural abundance. Tang *et al.* subsequently used this method in U937 cells and investigated the effect of phorbol myristate acetate (PMA) treatment on methylation dynamics. PMA is a well-known differentiation agent, which converts monocytes into M1/M2 macrophages.¹⁵⁰ The transmethylation rate $[\text{C}^2\text{HH}_2/\text{CH}_3 (\%)]$ decreased nearly 2-fold in PMA-treated cells compared to controls for monomethylation of H4K20, H3K4, H3K9, H3K36, and H3K79. This data indicates that the one-carbon metabolism was impaired by PMA treatment. Furthermore, methylation sites in protein N-termini (H3K4, H3K9, and H4K20) had a higher methylation rate compared to sites (closely) located in the protein's structural domain (H3K36, H3K79). All together, these results show the capability of using stable isotope labeled serine to study one-carbon metabolism-mediated histone methylation.

Labeling of amino acids in newly synthesized proteins

The use of stable isotope labeled metabolic precursors does not only label the metabolite substrates of PTMs but also amino acids used for protein synthesis. For example, the use of $[\text{U-}^{13}\text{C}]$ -glucose results in labeling of several amino acids *via* intermediates of glycolysis and the TCA cycle. Serine is labeled *via* 3-phosphoglycerate and glucose derived pyruvate is the precursor for alanine, valine and leucine. The TCA cycle intermediates oxaloacetate and α -ketoglutarate are precursors for aspartate and glutamate, which can be further metabolized to other amino acids. The stable isotope labeled amino acids are used for protein synthesis and are incorporated into the backbone of newly synthesized proteins. The additional labeling of these amino acids and their incorporation into the backbone of newly synthesized proteins represents an analytical challenge in MS analysis and bioinformatics data processing. The introduction of stable isotope labeled carbons into the amino acid backbone and the PTM group leads to multiple peptide isotopologues. The different isotopologues result in very complex mass spectra due to multiple and overlapping isotopic distributions of the different isotopologues. For an accurate and reliable quantification, powerful deconvolution and feature detection algorithms considering the isotopic distribution of the different isotopologues are necessary. Although additional labeling of amino acids presents a challenge, it allows the distinction between “old” and “new” proteins so that information about the PTM dynamics of the two different subsets is obtained as well as overall protein turnover rates. However, it must be noted that the amino acid pools are diluted due to unlabeled metabolic precursors used for amino

acid synthesis and use of recycled unlabeled amino acids from degraded proteins. The actual precursor enrichment rate of the amino acids studied should be investigated by measuring and quantifying the different amino acid isotopologues *via* LC-MS or GC-MS.

Phosphorylation and O-GlcNAcylation

There are many more PTMs besides protein acetylation (acylation) and protein methylation,¹⁵¹ and most of their substrates can be labeled using stable isotope labeled metabolic precursors. To the best of our knowledge, for two additional PTMs, namely protein O-phosphorylation¹⁵² and protein O-GlcNAcylation (O-linked β -N-acetylglucosamine),¹⁵³ stable isotope labeled metabolic precursors were used to investigate their turnover.

Phosphorylation

Phosphorylation of serine, threonine and tyrosine residues (O-phosphorylation) is one of the most widely studied PTMs in the field of proteomics (phosphoproteomics).^{154,155} The PhosphoSitePlus database (<https://www.phosphosite.org/staticSiteStatistics>) listed 290 128 experimentally confirmed phosphorylation sites for the human proteome (as of 09/15/2019) and more than two-thirds of the proteins encoded by the human genome are phosphorylated.¹⁵⁶ Serine phosphorylation (59.9%) is the most abundant protein phosphorylation followed by threonine phosphorylation (24.6%) and tyrosine phosphorylation (15.5). Protein phosphorylation is a regulatory mechanism that plays an essential role in almost all cellular processes. These include processes such as metabolic signaling,¹⁵⁷ cell cycle,¹⁵⁸ protein-protein interactions,¹⁵⁹ cell growth¹⁶⁰ and development.¹⁶¹ O-Phosphorylation represents a reversible modification that is catalyzed by protein kinases and protein phosphatases. The human genome encodes 518 protein kinases and approximately 200 phosphatases.¹⁶² Adenosine triphosphate (ATP) is a nucleoside triphosphate with an intracellular concentration in the range of 1 mM to 10 mM and is the substrate used by protein kinases to transfer a phosphate group to a serine, threonine or tyrosine residue. ATP is mainly synthesized in eukaryotes by glycolysis, TCA cycle/oxidative phosphorylation and beta-oxidation of fatty acids. To investigate the turnover of individual phosphorylation sites, the phosphate group has to be enriched with isotopes. Phosphorus is a monoisotopic element with ^{31}P being the only stable isotope. All the other 22 phosphorus isotopes including ^{32}P and ^{33}P are radioisotopes. The ^{32}P radioisotope has been used to study protein phosphorylation by mass spectrometry and Edman sequencing.¹⁶³ One way to label ATP with stable isotopes is to use the stable oxygen isotope ^{18}O which has a natural abundance 0.2%.

The group of Garcia used stable isotope labeled $[\gamma\text{-}^{18}\text{O}_4]$ -ATP as a metabolic precursor to investigate nuclear protein phosphorylation turnover in human embryonic kidney cells

Fig. 5 Kinase mediated reaction of stable isotope labeled $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$ with serine, threonine or tyrosine residues results in phosphorylated species labeled with three ^{18}O atoms and ADP containing one ^{18}O atom.

(HEK293) and cell-cycle-synchronized HeLa cells.¹⁵² In analogy to SILAC, they named their method SILAP – stable isotope labeling of phosphate by $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$. The transfer of three ^{18}O atoms from stable isotope enriched ATP to the OH-group of the peptide substrate residues serine, threonine and tyrosine results in a mass shift of 6 Da for the stable isotope labeled phosphopeptide species (Fig. 5).

In their study, Molden *et al.* showed that next to the species carrying three ^{18}O atoms $\text{P}(^{18}\text{O}_3)$, the majority (53%) of the stable isotope labeled phosphopeptide species carried one ^{18}O atom and two ^{16}O atoms $\text{P}(^{18}\text{O}_1^{16}\text{O}_2)$. The $\text{P}(^{18}\text{O}_2^{16}\text{O}_1)$ species was also detected but to a lesser extent. The reason for this observation was a partially labeled ATP pool. Although $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$ is stable in buffer systems used for kinase assays in combination with purified kinases and synthesized substrate, ^{18}O atoms can be exchanged against ^{16}O atoms under physiological conditions in the nucleus due to enzymatic and metabolic processes.¹⁶⁴ Molden *et al.* measured ATP species extracted from the nuclei after $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$ incubation by LC-MS. The observed abundance of the different ATP isotopologues containing three or less ^{18}O atoms was higher than expected by the authors compared to the $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$ standard. For the ATP species $\text{ATP}(^{18}\text{O}_4)$ and $\text{ATP}(^{18}\text{O}_2^{16}\text{O}_2)$ a ratio of approximately 0.66 ($^{18}\text{O}_4/^{18}\text{O}_2^{16}\text{O}_2$) was observed and the sum of the abundancies of the ATP species containing three, two or one ^{18}O atom was higher than the abundance of the $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$ species. As long as the stable isotope labeled phosphopeptide species contained more than one ^{18}O atom Molten *et al.* considered these species as being newly synthesized. The different isotopologues of the phosphopeptides $\text{P}(^{18}\text{O}_3)$, $\text{P}(^{18}\text{O}_2^{16}\text{O}_1)$, $\text{P}(^{18}\text{O}_1^{16}\text{O}_2)$ and $\text{P}(^{16}\text{O}_3)$ generate complex isotope features in the MS1 spectrum that must be deconvoluted. In their study, Molden *et al.* calculated the theoretical isotope distribution based on the chemical formula and the measured signal intensities of the different isotopologues were partitioned between the isotopic labeling states ($\text{P}(^{18}\text{O}_3)$, $\text{P}(^{18}\text{O}_2^{16}\text{O}_1)$, $\text{P}(^{18}\text{O}_1^{16}\text{O}_2)$ and $\text{P}(^{16}\text{O}_3)$) using linear regression. To model and calculate the phosphorylation kinetics for the individual phosphorylation sites, phosphorylation progress curves were generated by

plotting the ratio of the abundance of the heavy and the light phosphopeptide (H/L) over time and fitted by nonlinear least squares approximation to the following equation to determine the rate constant (k) and extent of phosphorylation turnover ($\text{H}\infty/\text{L}$):¹⁵²

$$\text{H/L} = \text{H}\infty/\text{L} \times (1 - e^{-k \times t})$$

where H is the total extracted ion current for the ^{18}O enriched phosphopeptide isotopologue (including $\text{P}(^{18}\text{O}_3)$, $\text{P}(^{18}\text{O}_2^{16}\text{O})$, $\text{P}(^{18}\text{O}^{16}\text{O}_2)$), $\text{H}\infty$ is the maximum amount of ^{18}O enriched phosphopeptide species and L is the extracted ion current of the ^{16}O containing phosphopeptide isotopologue. Although they measured the isotope enrichment of the metabolic precursor, it was not taken into account for calculation of the turnover rate.

After incubation of isolated HeLa nuclei with $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$, Molden *et al.* identified 1123 ^{18}O -labeled phosphosites and they calculated phosphorylation turnover rates ranging from 0.34 min^{-1} (fast turnover) to 0.001 min^{-1} (slow turnover) reflecting differences in nuclear kinase activities and substrate concentration. Their SILAP approach enabled to distinguish the phosphorylation dynamics of different histone 1 protein species. The use of $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$ as a metabolic precursor provided insights in site-specific, individual turnover dynamics for multiple phosphorylated peptide species and how they affect each other. Such dynamic information is difficult or not accessible with other protein biochemical techniques.

However, because of the poor permeability of the cell membrane to ATP,¹⁶⁵ the use of $[\gamma\text{-}^{18}\text{O}_4]\text{-ATP}$ as a metabolic precursor to study phosphorylation dynamics is limited to the analysis of isolated organelles whose membranes are permeable to stable isotope enriched ATP, such as the nucleus, mitochondrion or endoplasmic reticulum.

O-GlcNAcylation

Protein O-GlcNAc is a dynamic PTM catalysed by the O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) regulating the addition or the removal of a GlcNAc moiety to a serine or threonine residue of cytoplasmic, nuclear and mitochondrial proteins.¹⁶⁶ Protein O-GlcNAcylation regulates a number of biological processes. Protein O-GlcNAcylation is involved in the regulation of transcription,¹⁶⁷ subcellular localization,¹⁶⁸ epigenetic regulation¹⁶⁹ and regulation of cell signalling dynamics as in the case of insulin signalling.¹⁷⁰ OGTs use uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) as a substrate which is synthesised *via* the hexosamine biosynthetic pathway which integrates glucose, amino acid, fatty acid and nucleotide metabolism. The first step of the hexosamine biosynthetic pathway is the conversion of fructose-6-phosphate to glucosamine-6-phosphate (GlcN-6P) catalyzed by glutamine-fructose-6-phosphate aminotransferase I (GFAT1) (Fig. 6). GlcN-6P is subsequently acetylated to N-acetylglucosamine-6-phosphate (GlcNAc-6P). GlcNAc-6P is converted to GlcNAc-1P and further metabolized to UDP-GlcNAc. Since the hexosamine biosynthesis pathway branches off from glycolysis as part of

Fig. 6 Flow of ^{13}C atoms from glucose to O-GlcNAcylated proteins through glycolysis and the hexosamine biosynthesis pathway. OGT: O-GlcNAc transferase, OGA: O-GlcNAcase.

the glucose metabolism, $[\text{U-}^{13}\text{C}]$ -glucose is a suitable metabolic precursor to study O-GlcNAc turnover.

Wang *et al.* used $[\text{U-}^{13}\text{C}]$ -labeled glucose as a metabolic precursor to investigate protein O-GlcNAc turnover in HeLa cells.¹⁵³ LC-MS analysis of the metabolite extract showed that the majority of UDP-GlcNAc molecules contained eight ^{13}C atoms ($^{13}\text{C}_8$ -UDP-GlcNAc) followed by $^{13}\text{C}_6$ -UDP-GlcNAc. For $^{13}\text{C}_8$ -UDP-GlcNAc, the six ^{13}C atoms of the glucosamine ring are directly derived from $[\text{U-}^{13}\text{C}]$ -labeled glucose. The two additional ^{13}C atoms are incorporated into the acetyl-group of the GlcNAc moiety, which is derived from $^{13}\text{C}_2$ -acetyl-CoA (Fig. 6). The authors determined the time necessary to label 50% of the UDP-GlcNAc pool with either six or eight ^{13}C atoms ($t = 8.9$ h) and included this information to calculate protein GlcNAc turnover. For protein GlcNAc turnover analysis, Wang *et al.* cultured HeLa cells with $^{13}\text{C}_6$ -glucose and extracted nuclei at different time points. They incubated the LysC-digested extracts with PNGase F to remove N-glycosylation and the O-GlcNAc containing peptides were enriched by phenylboronic acid based solid phase extraction after PNGase F treatment, since the large excess of N-glycosylated peptides competes with O-GlcNAc species for binding to the immobilized phenyl boronic acid. The enriched O-GlcNAc peptide species were analyzed by LC-MS/MS using a combination of CID and ETD. Under CID conditions, a specific GlcNAc oxonium ion, as well as fragment ions resulting from the GlcNAc oxonium ion, were generated, which triggered an additional ETD fragmentation of the precursor ion to obtain amino acid sequence information. The O-GlcNAc modification remained stable and

attached to the modified amino acid during ETD-mediated peptide backbone fragmentation. Wang *et al.* identified 105 O-GlcNAc peptides from 42 nuclear proteins. Similar to UDP-GlcNAc labeling, the peptides carried $^{13}\text{C}_6$ -O-GlcNAc or $^{13}\text{C}_8$ -O-GlcNAc modifications. The stable isotope labeled O-GlcNAc fraction was calculated at different time points by forming the sum of the H/L ratio of the $^{13}\text{C}_6$ -O-GlcNAc and $^{13}\text{C}_8$ -O-GlcNAc species. For O-GlcNAc turnover analysis, H/L ratio at different time points were fitted to a model for stable isotope labeling under non-steady state conditions. The model took the degradation rates of the modified proteins into account, which were determined in a protein turnover experiment using pulse-labeling with $^{13}\text{C}_6$ - $^{15}\text{N}_2$ -lysine and $^{13}\text{C}_6$ - $^{15}\text{N}_4$ -arginine as well as the labeling kinetics of the metabolic precursor UDP-GlcNAc. The work of Wang *et al.* was the first and hitherto only study where the labeling kinetics of the metabolic precursor were taken into account for the calculation of PTM turnover rates. For metabolic precursors that are slowly labeled such as UDP-GlcNAc (50% UDP-GlcNAc labeling in 8.9 h) it is important to consider the labeling kinetics to accurately quantify PTM turnover rates. In their work, Wang *et al.* considered only modified peptides for turnover calculations, if the corresponding protein degradation rates were available from the pulse labeling experiment and if the peptide carried only one O-GlcNAc modification, because of the complexity of the overlapping isotopic distributions due to the multiple mass shifts. Out of the 105 identified O-GlcNAc peptides, turnover rates were determined for 20 O-GlcNAc sites in 14 proteins. The turnover rates range from 0.02 h^{-1} to 1.6 h^{-1} and

are slow compared to the turnover rates of histone lysine acetylation^{93,99} and *O*-phosphorylation.¹⁵² Interestingly, Wang *et al.* were able to show with their labeling approach that the different *O*-GlcNAc sites of the transcriptional coregulator host cell factor C1 (HCF-1), which is involved in regulation of cell cycle progression, showed different turnover rates. These results on different site-specific *O*-GlcNAc dynamics within a protein illustrate the performance of stable isotope labeled metabolic precursors for the analysis of PTM dynamics, providing a new dimension in the understanding of biological processes that may be important in HCF-1 maturation and its function.^{171,172} Although the study highlights the potential of using stable isotope labeled metabolic precursors to study *O*-GlcNAc turnover, a variety of modified peptides were not taken into account to calculate their turnover rates because they carried more than one *O*-GlcNAc residue and showed complex spectra due to multiple mass shifts. This problem highlights the need for powerful deconvolution and feature detection algorithms that consider the isotopic distribution of the different isotopologues.

Conclusion

Biological organisms are highly dynamic systems that continuously respond to changes in their environment to maintain homeostatic balance or to respond to specific stimuli. Post-translational modifications are key regulators by which biological systems respond to environmental factors and initiate specific cellular responses. Over the past 20 years, mass spectrometric proteomics has become the method of choice for studying post-translational modifications globally at the cellular level. With mass spectrometry, several thousand site-specific modifications can be identified and quantified simultaneously,^{174,175} which is currently not possible with any other protein (bio)chemical method. However, most proteomic studies provide only a static snapshot of PTM frequencies rather than dynamic information. The use of stable isotope labeled metabolic precursors to study PTM dynamics can fill this gap. Time-resolved and site-specific PTM dynamics provide an additional dimension of information that is necessary to understand how biological systems regulate and respond to environmental factors. Information on PTM dynamics and turnover can help distinguish tightly regulated PTMs from those that have been non-enzymatically through chemical reactions, as in the case of the mitochondrial acetylome.^{176,177} Compared to the analysis of metabolic fluxes by LC-MS or GC-MS or the LC-MS based analysis of protein turnover dynamics, the analysis of PTM dynamics using stable isotope labeled metabolic precursors is still in its infancy and limited to a few PTMs mainly histone acetylation and methylation. However, in particular the work on the dynamics of histone acetylation and methylation illustrates the potential of dynamic PTM analysis using stable isotope labeled metabolic precursors. Information about site-specific acetylation and methylation dynamics was obtained, how they can affect each

other and their regulatory influence on chromatin formation, cell cycle and gene expression.

In principle, the necessary analytical technologies and fundamentals are available for the analysis of PTM dynamics to become established as an integral part of proteome analysis in the coming years. The LC-MS systems have the necessary sensitivity, resolution and fragmentation options to study PTM dynamics *via* bottom-up, middle-down and top-down approaches. The current bottlenecks are mainly in sample preparation and data analysis.

For data analysis, powerful algorithms are needed in particular for evaluation of the complex spectra, for the identification of different peptide and protein species, for the accurate quantification of the different isotopologues of the peptide and protein species and the calculation of their labeling kinetics. This includes especially algorithms for peak and feature detection and spectral deconvolution taking theoretical and practical isotope distributions into account. Although a number of algorithms and software programs are available, in-house algorithms and software solutions often have to be developed for the respective experimental problem. In some cases, the complex spectra must be interpreted and evaluated manually, which is not feasible for a proteome-wide application of the methodology.

Suitable metabolic precursors are crucial to be able to analyze PTM dynamics. For some PTMs, such as protein phosphorylation, limited options are available. For glucose derived metabolites that serve as PTM substrates, it is possible to use uniformly labeled glucose as a metabolic precursor to study PTM dynamics. However, it is crucial that the substrate metabolites can be labeled to a sufficiently high level and with kinetics that are considerably faster than the PTM dynamics under study. The study of Wang *et al.* investigating *O*-GlcNAc dynamics using uniformly labeled glucose showed that it is possible to obtain accurate kinetic data if the labeling kinetics of the metabolite substrate are taken into account in calculations of the turnover rates.¹⁵³

This methodology may be further extended in the future, for example, by combining metabolic precursors for various modifications such as uniformly labeled glucose and stable isotope labeled methionine in order to study the dynamic cross-talk between acetylation and methylation.

Future perspectives are to use this methodology to study PTM and protein dynamics together as well as the metabolic flux through certain metabolic pathways in one and the same sample using approaches to simultaneously extract metabolites and proteins. Such dynamic multi-OMICS approaches would be of great interest, for example, in cases where metabolic changes are likely to produce changes in PTMs, which in turn has regulatory effects on metabolism, such as in protein acetylation and energy metabolism.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

M. K. thanks the European Respiratory Society (ERS, RESPIRE3, project reference: R3201703-00121) for financial support.

References

- R. Schoenheimer and D. Rittenberg, *Science*, 1935, **82**, 156–157.
- D. Rittenberg and R. Schoenheimer, *J. Biol. Chem.*, 1935, **111**, 169–174.
- R. Schoenheimer and D. Rittenberg, *J. Biol. Chem.*, 1935, **111**, 163–168.
- R. Schoenheimer and D. Rittenberg, *J. Biol. Chem.*, 1935, **111**, 175–181.
- K. Fenger-Eriksen and H. Ussing, *Biochem. J.*, 1936, **30**, 1264–1269.
- H. C. Urey, J. R. Huffmann, H. G. Thode and M. Fox, *J. Chem. Phys.*, 1937, **5**, 856–868.
- R. Schoenheimer, D. Rittenberg, G. L. Foster, A. S. Keston and S. Ratner, *Science*, 1938, **88**, 599–600.
- R. Schoenheimer, S. Ratner and D. Rittenberg, *Science*, 1939, **89**, 272–273.
- D. Rittenberg, A. Keston, F. Rosebury and R. Schoenheimer, *J. Biol. Chem.*, 1939, **127**, 291–299.
- W. Bleakney, *Phys. Rev.*, 1929, **34**, 157–160.
- W. Bleakney, *Phys. Rev.*, 1932, **40**, 496–501.
- R. Schoenheimer, S. Ratner and D. Rittenberg, *J. Biol. Chem.*, 1939, **127**, 333–343.
- E. Borek, L. Ponticorvo and D. Rittenberg, *Proc. Natl. Acad. Sci. U. S. A.*, 1958, **44**, 369–374.
- S. Ruben and M. D. Kamen, *Phys. Rev.*, 1940, **57**, 549–549.
- S. Ruben and M. D. Kamen, *Phys. Rev.*, 1941, **59**, 349–354.
- R. Dische and D. Rittenberg, *J. Biol. Chem.*, 1954, **211**, 199–212.
- J. Mandelstam, *Nature*, 1957, **179**, 1179–1181.
- S. Ishii, N. Suzui, S. Ito, N. S. Ishioka, N. Kawachi, N. Ohtake, T. Ohshima and S. Fujimaki, *Soil Sci. Plant Nutr.*, 2009, **50**, 660–666.
- H. Wu and C. W. Bishop, *J. Appl. Physiol.*, 1959, **14**, 1–5.
- C. L. Kien, V. R. Young, D. K. Rohrbaugh and J. F. Burke, *Metab., Clin. Exp.*, 1978, **27**, 27–34.
- M. Karas, D. Bachmann and F. Hillenkamp, *Anal. Chem.*, 1985, **14**, 2935–2939.
- M. Karas, D. Bachmann, U. Bahr and F. Hillenkamp, *Int. J. Mass Spectrom. Ion Processes*, 1987, **78**, 53–68.
- J. B. Fenn, M. Mann, C. K. Meng, S. F. Wong and C. M. Whitehouse, *Science*, 1989, **246**, 64–71.
- M. Wilm and M. Mann, *Anal. Chem.*, 1996, **68**, 1–8.
- U. Bahr, A. Pfenninger, M. Karas and B. Stahl, *Anal. Chem.*, 1997, **69**, 4530–4535.
- A. J. Oosterkamp, E. Gelpi and J. Abian, *J. Mass Spectrom.*, 1998, **33**, 976–983.
- J. R. Yates 3rd, S. Speicher, P. R. Griffin and T. Hunkapiller, *Anal. Biochem.*, 1993, **214**, 397–408.
- M. Mann and M. Wilm, *Anal. Chem.*, 1994, **66**, 4390–4399.
- S. Beck, A. Michalski, O. Raether, M. Lubeck, S. Kaspar, N. Goedecke, C. Baessmann, D. Hornburg, F. Meier, I. Paron, N. A. Kulak, J. Cox and M. Mann, *Mol. Cell. Proteomics*, 2015, **14**, 2014–2029.
- F. Meier, S. Beck, N. Grassl, M. Lubeck, M. A. Park, O. Raether and M. Mann, *J. Proteome Res.*, 2015, **14**, 5378–5387.
- A. Makarov, *Anal. Chem.*, 2000, **72**, 1156–1162.
- M. W. Senko, P. M. Remes, J. D. Canterbury, R. Mathur, Q. Song, S. M. Eliuk, C. Mullen, L. Earley, M. Hardman, J. D. Blethrow, H. Bui, A. Specht, O. Lange, E. Denisov, A. Makarov, S. Horning and V. Zabrouskov, *Anal. Chem.*, 2013, **85**, 11710–11714.
- B. B. Aldridge, J. M. Burke, D. A. Lauffenburger and P. K. Sorger, *Nat. Cell Biol.*, 2006, **8**, 1195–1203.
- A. J. Claydon and R. Beynon, *Mol. Cell. Proteomics*, 2012, **11**, 1551–1565.
- W. E. Holmes, T. E. Angel, K. W. Li and M. K. Hellerstein, *Methods Enzymol.*, 2015, **561**, 219–276.
- A. S. Hebert, A. L. Richards, D. J. Bailey, A. Ulbrich, E. E. Coughlin, M. S. Westphall and J. J. Coon, *Mol. Cell. Proteomics*, 2014, **13**, 339–347.
- R. A. Scheltema, J. P. Hauschild, O. Lange, D. Hornburg, E. Denisov, E. Damoc, A. Kuehn, A. Makarov and M. Mann, *Mol. Cell. Proteomics*, 2014, **13**, 3698–3708.
- F. Meier, A. D. Brunner, S. Koch, H. Koch, M. Lubeck, M. Krause, N. Goedecke, J. Decker, T. Kosinski, M. A. Park, N. Bache, O. Hoerning, J. Cox, O. Raether and M. Mann, *Mol. Cell. Proteomics*, 2018, **17**, 2534–2545.
- N. A. Kulak, P. E. Geyer and M. Mann, *Mol. Cell. Proteomics*, 2017, **16**, 694–705.
- M. Z. Zhu, N. Li, Y. T. Wang, N. Liu, M. Q. Guo, B. Q. Sun, H. Zhou, L. Liu and J. L. Wu, *J. Proteome Res.*, 2017, **16**, 3470–3475.
- M. Kwiatkowski, D. Krosser, M. Wurlitzer, P. Steffen, A. Barcaru, C. Krisp, P. Horvatovich, R. Bischoff and H. Schluter, *Anal. Chem.*, 2018, **90**, 9951–9958.
- A. S. Hebert, C. Thoing, N. M. Riley, N. W. Kwiecien, E. Shiskova, R. Huguet, H. L. Cardasis, A. Kuehn, S. Eliuk, V. Zabrouskov, M. S. Westphall, G. C. McAlister and J. J. Coon, *Anal. Chem.*, 2018, **90**, 2333–2340.
- F. Meier, P. E. Geyer, S. Virreira Winter, J. Cox and M. Mann, *Nat. Methods*, 2018, **15**, 440–448.
- S. E. Ong, B. Blagoev, I. Kratchmarova, D. B. Kristensen, H. Steen, A. Pandey and M. Mann, *Mol. Cell. Proteomics*, 2002, **1**, 376–386.
- K. Bartkowiak, M. Kwiatkowski, F. Buck, T. M. Gorges, L. Nilse, V. Assmann, A. Andreas, V. Muller, H. Wikman, S. Riethdorf, H. Schluter and K. Pantel, *Cancer Res.*, 2015, **75**, 5367–5377.
- L. Myllynen, M. Kwiatkowski, L. Gleissner, B. Riepen, K. Hoffer, M. Wurlitzer, C. Petersen, E. Dikomey,

- K. Rothkamm, H. Schluter and M. Kriegs, *Radiother. Oncol.*, 2015, **116**, 423–430.
- 47 L. Gleissner, M. Kwiatkowski, L. Myllynen, P. Steffen, C. Petersen, K. Rothkamm, H. Schluter and M. Kriegs, *Oncotarget*, 2017, **8**, 110983–110993.
 - 48 J. M. Pratt, J. Petty, I. Riba-Garcia, D. H. Robertson, S. J. Gaskell, S. G. Oliver and R. J. Beynon, *Mol. Cell. Proteomics*, 2002, **1**, 579–591.
 - 49 P. R. Housley, E. R. Sanchez, M. Danielsen, G. M. Ringold and W. B. Pratt, *J. Biol. Chem.*, 1990, **265**, 12778–12781.
 - 50 M. K. Doherty, D. E. Hammond, M. J. Clague, S. J. Gaskell and R. J. Beynon, *J. Proteome Res.*, 2009, **8**, 104–112.
 - 51 B. Schwanhäusser, M. Gossen, G. Dittmar and M. Selbach, *Proteomics*, 2009, **9**, 205–209.
 - 52 F. M. Boisvert, Y. Ahmad, M. Gierlinski, F. Charriere, D. Lamont, M. Scott, G. Barton and A. I. Lamond, *Mol. Cell. Proteomics*, 2012, **11**, M111.011429.
 - 53 C. Hughes and J. Krijgsveld, *Trends Biotechnol.*, 2012, **30**, 668–676.
 - 54 M. Looso, C. S. Michel, A. Konzer, M. Bruckskotten, T. Borchardt, M. Kruger and T. Braun, *J. Proteome Res.*, 2012, **11**, 4693–4704.
 - 55 K. P. Jayapal, S. Sui, R. J. Philp, Y. J. Kok, M. G. Yap, T. J. Griffin and W. S. Hu, *J. Proteome Res.*, 2010, **9**, 2087–2097.
 - 56 T. Mathieson, H. Franken, J. Kosinski, N. Kurzawa, N. Zinn, G. Sweetman, D. Poeckel, V. S. Ratnu, M. Schramm, I. Becher, M. Steidel, K. M. Noh, G. Bergamini, M. Beck, M. Bantscheff and M. M. Savitski, *Nat. Commun.*, 2018, **9**, 689.
 - 57 J. Zecha, C. Meng, D. P. Zolg, P. Samaras, M. Wilhelm and B. Kuster, *Mol. Cell. Proteomics*, 2018, **17**, 974–992.
 - 58 T. Narita, B. T. Weinert and C. Choudhary, *Nat. Rev. Mol. Cell Biol.*, 2019, **20**, 156–174.
 - 59 G. R. Wagner and R. M. Payne, *J. Biol. Chem.*, 2013, **288**, 29036–29045.
 - 60 V. G. Allfrey, R. Faulkner and A. E. Mirsky, *Proc. Natl. Acad. Sci. U. S. A.*, 1964, **51**, 786–794.
 - 61 R. B. Selvi and T. K. Kundu, *Biotechnol. J.*, 2009, **4**, 375–390.
 - 62 G. E. Zentner and S. Henikoff, *Nat. Struct. Mol. Biol.*, 2013, **20**, 259–266.
 - 63 T. J. Cohen, A. W. Hwang, C. R. Restrepo, C. X. Yuan, J. Q. Trojanowski and V. M. Lee, *Nat. Commun.*, 2015, **6**, 5845.
 - 64 S. W. Min, X. Chen, T. E. Tracy, Y. Li, Y. Zhou, C. Wang, K. Shirakawa, S. S. Minami, E. Defensor, S. A. Mok, P. D. Sohn, B. Schilling, X. Cong, L. Ellerby, B. W. Gibson, J. Johnson, N. Krogan, M. Shamloo, J. Gestwicki, E. Masliah, E. Verdin and L. Gan, *Nat. Med.*, 2015, **21**, 1154–1162.
 - 65 J. Luo, M. Li, Y. Tang, M. Laszkowska, R. G. Roeder and W. Gu, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 2259–2264.
 - 66 W. Gu and R. G. Roeder, *Cell*, 1997, **90**, 595–606.
 - 67 S. M. Reed and D. E. Quelle, *Cancers*, 2014, **7**, 30–69.
 - 68 M. A. Deardorff, M. Bando, R. Nakato, E. Watrin, T. Itoh, M. Minamino, K. Saitoh, M. Komata, Y. Katou, D. Clark, K. E. Cole, E. De Baere, C. Decroos, N. Di Donato, S. Ernst, L. J. Francey, Y. Gyftodimou, K. Hirashima, M. Hullings, Y. Ishikawa, C. Jaulin, M. Kaur, T. Kiyono, P. M. Lombardi, L. Magnaghi-Jaulin, G. R. Mortier, N. Nozaki, M. B. Petersen, H. Seimiya, V. M. Siu, Y. Suzuki, K. Takagaki, J. J. Wilde, P. J. Willems, C. Prigent, G. Gillesen-Kaesbach, D. W. Christianson, F. J. Kaiser, L. G. Jackson, T. Hirota, I. D. Krantz and K. Shirahige, *Nature*, 2012, **489**, 313–317.
 - 69 J. Shi, Y. Wang, L. Zeng, Y. Wu, J. Deng, Q. Zhang, Y. Lin, J. Li, T. Kang, M. Tao, E. Rusinova, G. Zhang, C. Wang, H. Zhu, J. Yao, Y. X. Zeng, B. M. Evers, M. M. Zhou and B. P. Zhou, *Cancer Cell*, 2014, **25**, 210–225.
 - 70 T. Fujisawa and P. Filippakopoulos, *Nat. Rev. Mol. Cell Biol.*, 2017, **18**, 246–262.
 - 71 G. A. Baltus, M. P. Kowalski, H. Zhai, A. V. Tutter, D. Quinn, D. Wall and S. Kadam, *Stem Cells*, 2009, **27**, 2175–2184.
 - 72 H. Inuzuka, D. Gao, L. W. Finley, W. Yang, L. Wan, H. Fukushima, Y. R. Chin, B. Zhai, S. Shaik, A. W. Lau, Z. Wang, S. P. Gygi, K. Nakayama, J. Teruya-Feldstein, A. Toker, M. C. Haigis, P. P. Pandolfi and W. Wei, *Cell*, 2012, **150**, 179–193.
 - 73 T. Li, B. A. Diner, J. Chen and I. M. Cristea, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 10558–10563.
 - 74 T. Shimazu, S. Horinouchi and M. Yoshida, *J. Biol. Chem.*, 2007, **282**, 4470–4478.
 - 75 S. Sharma, F. Poetz, M. Bruer, T. B. Ly-Hartig, J. Schott, B. Seraphin and G. Stoecklin, *Mol. Cell*, 2016, **63**, 927–938.
 - 76 S. C. Kim, R. Sprung, Y. Chen, Y. Xu, H. Ball, J. Pei, T. Cheng, Y. Kho, H. Xiao, L. Xiao, N. V. Grishin, M. White, X. J. Yang and Y. Zhao, *Mol. Cell*, 2006, **23**, 607–618.
 - 77 S. Zhao, W. Xu, W. Jiang, W. Yu, Y. Lin, T. Zhang, J. Yao, L. Zhou, Y. Zeng, H. Li, Y. Li, J. Shi, W. An, S. M. Hancock, F. He, L. Qin, J. Chin, P. Yang, X. Chen, Q. Lei, Y. Xiong and K. L. Guan, *Science*, 2010, **327**, 1000–1004.
 - 78 B. Schwer, J. Bunkenborg, R. O. Verdin, J. S. Andersen and E. Verdin, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 10224–10229.
 - 79 A. Drazic, L. M. Myklebust, R. Ree and T. Arnesen, *Biochim. Biophys. Acta*, 2016, **1864**, 1372–1401.
 - 80 K. E. Wellen, G. Hatzivassiliou, U. M. Sachdeva, T. V. Bui, J. R. Cross and C. B. Thompson, *Science*, 2009, **324**, 1076–1080.
 - 81 S. Sivanand, I. Viney and K. E. Wellen, *Trends Biochem. Sci.*, 2018, **43**, 61–74.
 - 82 C. Yang, B. Ko, C. T. Hensley, L. Jiang, A. T. Wasti, J. Kim, J. Sudderth, M. A. Calvaruso, L. Lumata, M. Mitsche, J. Rutter, M. E. Merritt and R. J. DeBerardinis, *Mol. Cell*, 2014, **56**, 414–424.
 - 83 C. M. Metallo, P. A. Gameiro, E. L. Bell, K. R. Mattaini, J. Yang, K. Hiller, C. M. Jewell, Z. R. Johnson, D. J. Irvine,

- L. Guarente, J. K. Kelleher, M. G. Vander Heiden, O. Iliopoulos and G. Stephanopoulos, *Nature*, 2011, **481**, 380–384.
- 84 A. R. Mullen, W. W. Wheaton, E. S. Jin, P. H. Chen, L. B. Sullivan, T. Cheng, Y. Yang, W. M. Linehan, N. S. Chandel and R. J. DeBerardinis, *Nature*, 2011, **481**, 385–388.
- 85 D. R. Wise, P. S. Ward, J. E. Shay, J. R. Cross, J. J. Gruber, U. M. Sachdeva, J. M. Platt, R. G. DeMatteo, M. C. Simon and C. B. Thompson, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 19611–19616.
- 86 V. Bulusu, S. Tumanov, E. Michalopoulou, N. J. van den Broek, G. MacKay, C. Nixon, S. Dhayade, Z. T. Schug, J. Vande Voorde, K. Blyth, E. Gottlieb, A. Vazquez and J. J. Kamphorst, *Cell Rep.*, 2017, **18**, 647–658.
- 87 Z. T. Schug, J. Vande Voorde and E. Gottlieb, *Nat. Rev. Cancer*, 2016, **16**, 708–717.
- 88 C. L. Skutches, C. P. Holroyde, R. N. Myers, P. Paul and G. A. Reichard, *J. Clin. Invest.*, 1979, **64**, 708–713.
- 89 C. D. Tollinger, H. J. Vreman and M. W. Weiner, *Clin. Chem.*, 1979, **25**, 1787–1790.
- 90 A. Luong, V. C. Hannah, M. S. Brown and J. L. Goldstein, *J. Biol. Chem.*, 2000, **275**, 26458–26466.
- 91 E. McDonnell, S. B. Crown, D. B. Fox, B. Kitir, O. R. Ilkayeva, C. A. Olsen, P. A. Grimsrud and M. D. Hirschey, *Cell Rep.*, 2016, **17**, 1463–1472.
- 92 F. Pietrocola, L. Galluzzi, J. M. Bravo-San Pedro, F. Madeo and G. Kroemer, *Cell Metab.*, 2015, **21**, 805–821.
- 93 A. G. Everitts, B. M. Zee, P. A. DiMaggio, M. Gonzales-Cope, H. A. Collier and B. A. Garcia, *J. Biol. Chem.*, 2013, **288**, 12142–12151.
- 94 R. C. Baliban, P. A. DiMaggio, M. D. Plazas-Mayorca, N. L. Young, B. A. Garcia and C. A. Floudas, *Mol. Cell. Proteomics*, 2010, **9**, 764–779.
- 95 S. L. Berger, *Nature*, 2007, **447**, 407–412.
- 96 J. S. Lee, E. Smith and A. Shilatifard, *Cell*, 2010, **142**, 682–685.
- 97 B. M. Zee, R. S. Levin, P. A. DiMaggio and B. A. Garcia, *Epigenet. Chromatin*, 2010, **3**, 22.
- 98 M. V. Bennetzen, D. H. Larsen, J. Bunkenborg, J. Bartek, J. Lukas and J. S. Andersen, *Mol. Cell. Proteomics*, 2010, **9**, 1314–1323.
- 99 Y. Zheng, P. M. Thomas and N. L. Kelleher, *Nat. Commun.*, 2013, **4**, 2203.
- 100 Z. Wang, C. Zang, J. A. Rosenfeld, D. E. Schones, A. Barski, S. Cuddapah, K. Cui, T. Y. Roh, W. Peng, M. Q. Zhang and K. Zhao, *Nat. Genet.*, 2008, **40**, 897–903.
- 101 M. Xu, S. Chen and B. Zhu, *Methods Enzymol.*, 2012, **512**, 29–55.
- 102 A. D'Urzo, A. P. Boichenko, T. van den Bosch, J. Hermans, F. Dekker, V. Andrisano and R. Bischoff, *Anal. Bioanal. Chem.*, 2016, **408**, 3547–3553.
- 103 C. Feller, I. Forne, A. Imhof and P. B. Becker, *Mol. Cell*, 2015, **57**, 559–571.
- 104 N. G. Leus, P. E. van der Wouden, T. van den Bosch, W. T. R. Hooghiemstra, M. E. Ourailidou, L. E. Kistemaker, R. Bischoff, R. Gosens, H. J. Haisma and F. J. Dekker, *Biochem. Pharmacol.*, 2016, **108**, 58–74.
- 105 N. G. Leus, T. van den Bosch, P. E. van der Wouden, K. Krist, M. E. Ourailidou, N. Eleftheriadis, L. E. Kistemaker, S. Bos, R. A. Gjaltema, S. A. Mekonnen, R. Bischoff, R. Gosens, H. J. Haisma and F. J. Dekker, *Sci. Rep.*, 2017, **7**, 45047.
- 106 C. Choudhary, C. Kumar, F. Gnad, M. L. Nielsen, M. Rehman, T. C. Walther, J. V. Olsen and M. Mann, *Science*, 2009, **325**, 834–840.
- 107 B. L. Parker, N. E. Shepherd, S. Trefely, N. J. Hoffman, M. Y. White, K. Engholm-Keller, B. D. Hambly, M. R. Larsen, D. E. James and S. J. Cordwell, *J. Biol. Chem.*, 2014, **289**, 25890–25906.
- 108 C. Scholz, B. T. Weinert, S. A. Wagner, P. Beli, Y. Miyake, J. Qi, L. J. Jensen, W. Streicher, A. R. McCarthy, N. J. Westwood, S. Lain, J. Cox, P. Matthias, M. Mann, J. E. Bradner and C. Choudhary, *Nat. Biotechnol.*, 2015, **33**, 415–423.
- 109 B. T. Weinert, T. Narita, S. Satpathy, B. Srinivasan, B. K. Hansen, C. Scholz, W. B. Hamilton, B. E. Zuconi, W. W. Wang, W. R. Liu, J. M. Brickman, E. A. Kesicki, A. Lai, K. D. Bromberg, P. A. Cole and C. Choudhary, *Cell*, 2018, **174**, 231–244.
- 110 Y. Kori, S. Sidoli, Z. F. Yuan, P. J. Lund, X. Zhao and B. A. Garcia, *Sci. Rep.*, 2017, **7**, 10296.
- 111 J. Fan, J. Baeza and J. M. Denu, *Methods Enzymol.*, 2016, **574**, 125–148.
- 112 S. Balasubramanian, J. Ramos, W. Luo, M. Sirisawad, E. Verner and J. J. Buggy, *Leukemia*, 2008, **22**, 1026–1034.
- 113 B. M. Zee, R. S. Levin, B. Xu, G. LeRoy, N. S. Wingreen and B. A. Garcia, *J. Biol. Chem.*, 2010, **285**, 3341–3350.
- 114 J. Murn and Y. Shi, *Nat. Rev. Mol. Cell Biol.*, 2017, **18**, 517–527.
- 115 Z. Wu, J. Connolly and K. K. Biggar, *FEBS J.*, 2017, **284**, 2732–2744.
- 116 L. Sun, M. Wang, Z. Lv, N. Yang, Y. Liu, S. Bao, W. Gong and R. M. Xu, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 20538–20543.
- 117 A. Reintjes, J. E. Fuchs, L. Kremser, H. H. Lindner, K. R. Liedl, L. A. Huber and T. Valovka, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, 4326–4331.
- 118 B. Raman, C. Guarnaccia, K. Nadassy, S. Zakhariev, A. Pintar, F. Zanuttin, D. Frigyes, C. Acatrinei, A. Vindigni, G. Pongor and S. Pongor, *Nucleic Acids Res.*, 2001, **29**, 3377–3384.
- 119 H. Wang, Z. Q. Huang, L. Xia, Q. Feng, H. Erdjument-Bromage, B. D. Strahl, S. D. Briggs, C. D. Allis, J. Wong, P. Tempst and Y. Zhang, *Science*, 2001, **293**, 853–857.
- 120 A. Kirmizis, H. Santos-Rosa, C. J. Penkett, M. A. Singer, R. D. Green and T. Kouzarides, *Nat. Struct. Mol. Biol.*, 2009, **16**, 449–451.
- 121 S. Horikawa, J. Sasuga, K. Shimizu, H. Ozasa and K. Tsukada, *J. Biol. Chem.*, 1990, **265**, 13683–13686.

- 122 M. A. Turner, X. Yang, D. Yin, K. Kuczera, R. T. Borchardt and P. L. Howell, *Cell Biochem. Biophys.*, 2000, **33**, 101–125.
- 123 J. A. van der Knaap and C. P. Verrijzer, *Genes Dev.*, 2016, **30**, 2345–2369.
- 124 P. Lijnen, R. Fagard, J. Staessen, W. Lissens and A. Amery, *J. Cardiovasc. Pharmacol.*, 1989, **14**, 319–325.
- 125 J. T. Fox and P. J. Stover, *Vitam. Horm.*, 2008, **79**, 1–44.
- 126 J. W. Locasale, *Nat. Rev. Cancer*, 2013, **13**, 572–583.
- 127 Y. G. Zheng, *Epigenetic technological applications*, Elsevier, Academic Press, Amsterdam, Boston, 2015.
- 128 J. Rappsilber, W. J. Friesen, S. Paushkin, G. Dreyfuss and M. Mann, *Anal. Chem.*, 2003, **75**, 3107–3114.
- 129 P. M. Gehrig, P. E. Hunziker, S. Zahariev and S. Pongor, *J. Am. Soc. Mass Spectrom.*, 2004, **15**, 142–149.
- 130 A. P. Snijders, M. L. Hung, S. A. Wilson and M. J. Dickman, *J. Am. Soc. Mass Spectrom.*, 2010, **21**, 88–96.
- 131 M. S. Kim, J. Zhong and A. Pandey, *Proteomics*, 2016, **16**, 700–714.
- 132 S. E. Ong, G. Mittler and M. Mann, *Nat. Methods*, 2004, **1**, 119–126.
- 133 H. T. Lau, K. A. Lewis and S. E. Ong, *Methods Mol. Biol.*, 2014, **1188**, 161–175.
- 134 D. Yagoub, G. Hart-Smith, J. Moecking, M. A. Erce and M. R. Wilkins, *Proteomics*, 2015, **15**, 3209–3218.
- 135 K. E. Caslavka Zempel, A. A. Vashisht, W. D. Barshop, J. A. Wohlschlegel and S. G. Clarke, *J. Proteome Res.*, 2016, **15**, 4436–4451.
- 136 M. Zhang, J. Y. Xu, H. Hu, B. C. Ye and M. Tan, *Proteomics*, 2018, **18**, 1700300.
- 137 D. Musiani, J. Bok, E. Massignani, L. Wu, T. Tabaglio, M. R. Ippolito, A. Cuomo, U. Ozbek, H. Zorgati, U. Ghoshdastider, R. C. Robinson, E. Guccione and T. Bonaldi, *Sci. Signaling*, 2019, **12**, eaat8388.
- 138 J. J. Pesavento, H. Yang, N. L. Kelleher and C. A. Mizzen, *Mol. Cell. Biol.*, 2008, **28**, 468–486.
- 139 Z. Wu, T. N. Tiambeng, W. Cai, B. Chen, Z. Lin, Z. R. Gregorich and Y. Ge, *Anal. Chem.*, 2018, **90**, 4935–4939.
- 140 S. M. Sweet, M. Li, P. M. Thomas, K. R. Durbin and N. L. Kelleher, *J. Biol. Chem.*, 2010, **285**, 32778–32786.
- 141 S. Sidoli, C. Lu, M. Coradin, X. Wang, K. R. Karch, C. Ruminowicz and B. A. Garcia, *Epigenet. Chromatin*, 2017, **10**, 34.
- 142 S. D. Taverna, B. M. Ueberheide, Y. Liu, A. J. Tackett, R. L. Diaz, J. Shabanowitz, B. T. Chait, D. F. Hunt and C. D. Allis, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 2086–2091.
- 143 N. L. Young, P. A. DiMaggio, M. D. Plazas-Mayorca, R. C. Baliban, C. A. Floudas and B. A. Garcia, *Mol. Cell. Proteomics*, 2009, **8**, 2266–2284.
- 144 Y. Zheng, S. M. Sweet, R. Popovic, E. Martinez-Garcia, J. D. Tipton, P. M. Thomas, J. D. Licht and N. L. Kelleher, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 13549–13554.
- 145 M. T. McCabe, A. P. Graves, G. Ganji, E. Diaz, W. S. Halsey, Y. Jiang, K. N. Smitheman, H. M. Ott, M. B. Pappalardi, K. E. Allen, S. B. Chen, A. Della Pietra 3rd, E. Dul, A. M. Hughes, S. A. Gilbert, S. H. Thrall, P. J. Tummino, R. G. Kruger, M. Brandt, B. Schwartz and C. L. Creasy, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 2989–2994.
- 146 B. M. Zee, L. M. Britton, D. Wolle, D. M. Haberman and B. A. Garcia, *Mol. Cell. Biol.*, 2012, **32**, 2503–2514.
- 147 C. M. O'Connor, P. A. DiMaggio Jr., T. Shenk and B. A. Garcia, *Mol. Cell. Proteomics*, 2014, **13**, 2399–2410.
- 148 P. Mews, B. M. Zee, S. Liu, G. Donahue, B. A. Garcia and S. L. Berger, *Mol. Cell. Biol.*, 2014, **34**, 3968–3980.
- 149 H. Tang, B. Tian, A. R. Brasier, L. C. Sowers and K. Zhang, *Sci. Rep.*, 2016, **6**, 31537.
- 150 D. Nicholas, H. Tang, Q. Zhang, J. Rudra, F. Xu, W. Langridge and K. Zhang, *Mol. Cell. Proteomics*, 2015, **14**, 15–29.
- 151 C. T. Walsh, S. Garneau-Tsodikova and G. J. Gatto Jr., *Angew. Chem., Int. Ed.*, 2005, **44**, 7342–7372.
- 152 R. C. Molden, J. Goya, Z. Khan and B. A. Garcia, *Mol. Cell. Proteomics*, 2014, **13**, 1106–1118.
- 153 X. Wang, Z. F. Yuan, J. Fan, K. R. Karch, L. E. Ball, J. M. Denu and B. A. Garcia, *Mol. Cell. Proteomics*, 2016, **15**, 2462–2475.
- 154 N. Krahmer, B. Najafi, F. Schueder, F. Quagliarini, M. Steger, S. Seitz, R. Kasper, F. Salinas, J. Cox, N. H. Uhlenhaut, T. C. Walther, R. Jungmann, A. Zeigerer, G. H. H. Borner and M. Mann, *Dev. Cell*, 2018, **47**, 205–221.
- 155 S. Doll, F. Gnad and M. Mann, *Proteomics: Clin. Appl.*, 2019, **13**, e1800113.
- 156 F. Ardito, M. Giuliani, D. Perrone, G. Troiano and L. Lo Muzio, *Int. J. Mol. Med.*, 2017, **40**, 271–280.
- 157 P. Dalle Pezze, S. Ruf, A. G. Sonntag, M. Langelaar-Makkinje, P. Hall, A. M. Heberle, P. Razquin Navas, K. van Eunen, R. C. Tolle, J. J. Schwarz, H. Wiese, B. Warscheid, J. Deitersen, B. Stork, E. Fassler, S. Schauble, U. Hahn, P. Horvatovich, D. P. Shanley and K. Thedieck, *Nat. Commun.*, 2016, **7**, 13254.
- 158 D. Fisher, L. Krasinska, D. Coudreuse and B. Novak, *J. Cell Sci.*, 2012, **125**, 4703–4711.
- 159 M. J. Betts, O. Wichmann, M. Utz, T. Andre, E. Petsalaki, P. Minguez, L. Parca, F. P. Roth, A. C. Gavin, P. Bork and R. B. Russell, *PLoS Comput. Biol.*, 2017, **13**, e1005462.
- 160 K. Thedieck, P. Polak, M. L. Kim, K. D. Molle, A. Cohen, P. Jenö, C. Arrieumerlou and M. N. Hall, *PLoS One*, 2007, **2**, e1217.
- 161 E. H. Peuchen, O. F. Cox, L. Sun, A. S. Hebert, J. J. Coon, M. M. Champion, N. J. Dovichi and P. W. Huber, *Sci. Rep.*, 2017, **7**, 15647.
- 162 F. Sacco, L. Perfetto, L. Castagnoli and G. Cesareni, *FEBS Lett.*, 2012, **586**, 2732–2739.
- 163 D. G. Campbell and N. A. Morrice, *J. Biomol. Tech.*, 2002, **13**, 119–130.
- 164 V. V. Phelan, Y. Du, J. A. McLean and B. O. Bachmann, *Chem. Biol.*, 2009, **16**, 473–478.
- 165 I. H. Chaudry, *Yale J. Biol. Med.*, 1982, **55**, 1–10.

- 166 M. B. Lazarus, Y. Nam, J. Jiang, P. Sliz and S. Walker, *Nature*, 2011, **469**, 564–567.
- 167 H. B. Ruan, X. Han, M. D. Li, J. P. Singh, K. Qian, S. Azarhoush, L. Zhao, A. M. Bennett, V. T. Samuel, J. Wu, J. R. Yates 3rd and X. Yang, *Cell Metab.*, 2012, **16**, 226–237.
- 168 G. Majumdar, A. Harrington, J. Hungerford, A. Martinez-Hernandez, I. C. Gerling, R. Raghoebar and S. Solomon, *J. Biol. Chem.*, 2006, **281**, 3642–3650.
- 169 J. A. Hanover, M. W. Krause and D. C. Love, *Nat. Rev. Mol. Cell Biol.*, 2012, **13**, 312–321.
- 170 K. Vosseller, L. Wells, M. D. Lane and G. W. Hart, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 5313–5318.
- 171 F. Capotosti, S. Guernier, F. Lammers, P. Waridel, Y. Cai, J. Jin, J. W. Conaway, R. C. Conaway and W. Herr, *Cell*, 2011, **144**, 376–388.
- 172 S. Daou, N. Mashtalir, I. Hammond-Martel, H. Pak, H. Yu, G. Sui, J. L. Vogel, T. M. Kristie and B. Affar el, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 2747–2752.
- 173 M. Xu, W. Wang, S. Chen and B. Zhu, *EMBO Rep.*, 2011, **13**, 60–67.
- 174 A. Hogrebe, L. von Stechow, D. B. Bekker-Jensen, B. T. Weinert, C. D. Kelstrup and J. V. Olsen, *Nat. Commun.*, 2018, **9**, 1045.
- 175 B. K. Hansen, R. Gupta, L. Baldus, D. Lyon, T. Narita, M. Lammers, C. Choudhary and B. T. Weinert, *Nat. Commun.*, 2019, **10**, 1055.
- 176 A. S. Hebert, K. E. Dittenhafer-Reed, W. Yu, D. J. Bailey, E. S. Selen, M. D. Boersma, J. J. Carson, M. Tonelli, A. J. Balloon, A. J. Higbee, M. S. Westphall, D. J. Pagliarini, T. A. Prolla, F. Assadi-Porter, S. Roy, J. M. Denu and J. J. Coon, *Mol. Cell*, 2013, **49**, 186–199.
- 177 A. J. Still, B. J. Floyd, A. S. Hebert, C. A. Bingman, J. J. Carson, D. R. Gunderson, B. K. Dolan, P. A. Grimsrud, K. E. Dittenhafer-Reed, D. S. Stapleton, M. P. Keller, M. S. Westphall, J. M. Denu, A. D. Attie, J. J. Coon and D. J. Pagliarini, *J. Biol. Chem.*, 2013, **288**, 26209–26219.