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REVIEW

Applications of stable isotope dimethyl labeling in quantitative proteomics

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Abstract Mass spectrometry has proven to be an indispensable tool for protein identification, characterization, and quantification. Among the possible methods in quantitative proteomics, stable isotope labeling by using reductive dimethylation has emerged as a cost-effective, simple, but powerful method able to compete at any level with the present alternatives. In this review, we briefly introduce experimental and software methods for proteome analysis using dimethyl labeling and provide a comprehensive overview of reported applications in the analysis of (1) differential protein expression, (2) posttranslational modifications, and (3) protein interactions.

Keywords Dimethyl labeling · Stable isotope labeling · Quantitative proteomics

Introduction

Robust and accurate quantification of protein expression levels is essential for deciphering the dynamics of proteomes. Mass spectrometry (MS) provides an excellent platform for quantitative proteomics [1–4], with the most common and precise quantitative approaches using stable isotopes [5, 6]. In recent years several quantitative MS-based technologies have evolved to interrogate the complexity, interconnectivity, and dynamic nature of proteomes. Isotope incorporation can occur metabolically in cell culture [7, 8], by labeling whole organisms [9], or via chemical labeling at the protein [10] or peptide [11–13] level. A relative newcomer in the latter category is dimethyl labeling, sometimes also referred to as reductive dimethylation. In this review we focus solely on this method, and describe its history, mechanism, procedures, and applications in the proteomics field.

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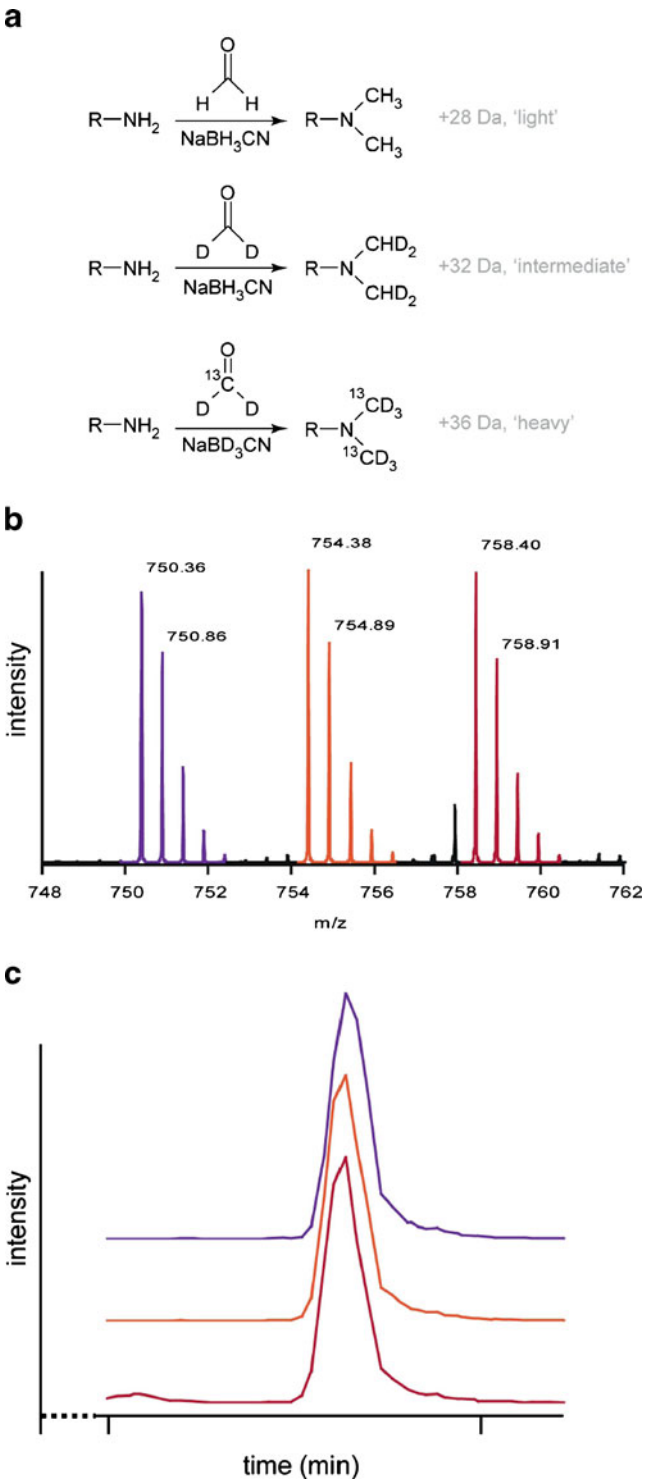
Stable isotope dimethyl labeling

The chemical reaction employed for introducing dimethyl groups possessing heavy stable isotopes has been known for a long time, and was, for instance, used more than 30 years ago for labeling proteins to increase the sensitivity for ^{13}C NMR analysis [14]. It was only in 2003 that Hsu et al. [15] introduced stable isotope dimethyl labeling as a quantitative proteomics technology. Highly selective dimethylation of primary amines (N-termini and lysine residue side chain) is achieved through reductive amination using formaldehyde and cyanoborohydride (Fig. 1). The reaction, which is optimally performed at near-neutral pH, involves the formation of a Schiff base (reaction of formaldehyde with the amine), which is subsequently reduced by cyanoborohydride. The reaction is very fast and goes to completion in only a few minutes, does not give rise to any significant side products [14], and has no negative impact on MS/MS peptide identification. With use of dimethylation, all primary amines present in the sample are converted, with the only exception being an



◀ **Fig. 1** Basic concepts of stable isotope dimethyl labeling. **a** Labeling scheme for stable isotope dimethyl labeling. The reaction is performed at neutral pH, and involves the formation of a Schiff base via the reaction of formaldehyde with the primary amines, which is then reduced by cyanoborohydride. With use of different isotopomers of formaldehyde, the mass added per labeling event can be varied. **b** Mass spectrum and **c** extracted ion chromatogram of bovine serum albumin peptide YICDNQDTISSK triple-labeled with light (*purple*), intermediate-mass (*orange*) and heavy (*red*) dimethyl labels. The labeled peptides are shifted by 28.03, 32.06, and 36.08*m/z*, respectively, from the original *m/z* of 722.83 of the peptide, corresponding to two labeling events and the peptide ion being doubly charged. Within reason, these three peptide isotopomers are coeluted. (Adapted from [31])

N-terminal proline, in which a monomethylamine is formed [16]. This means that, when trypsin is used to digest protein samples, the peptides generated from cleavage of arginine will have a single label (at their N-terminus) and lysine-cleaved peptides will have two labels (N-terminus and lysine side chain). If one prefers the same number of dimethyl groups per peptide, then one can first protect the lysine side chains by guanidination, resulting in only N-terminal dimethylation, a method that has been termed 2MEGA [17].

The original setup used two labels and relied on the incorporation of only deuterium as a heavy isotope. The analysis of up to four different samples using only deuterium isotopes has been shown, but required the use of endoproteinase Lys-C instead of trypsin to ensure sufficient mass difference between the differentially labeled peptides, because the mass difference per labeling event in that setup was only 2 Da [18]. Boersema et al. [13] developed a labeling scheme that allowed the simultaneous analysis of three samples, while retaining a mass difference of 4 Da with the inclusion of formaldehyde containing also ^{13}C (Fig. 1). It was shown that such a scheme allows reliable quantification of trypsin-generated peptides that contain only a single labeling site (i.e., arginine-cleaved peptides). Although this triple labeling greatly reduced the amount of overlapping between isotopic peaks of different labels, it was shown very recently that the quantitation of such peptides can be further improved by in silico deconvolution of the isotope clusters [19].

Over the last couple of years, dimethyl labeling has been shown to be compatible with a variety of common peptide separation and enrichment strategies, including separation of peptides by regular C_{18} reversed-phase liquid chromatography (LC), high-pH reversed-phase LC, strong cation exchange (SCX), and hydrophilic interaction chromatography (HILIC) [13, 20–22]. The deuterium present in some of the labels has been shown to lead to a small shift in retention time for the deuterium-containing peptides, but as long as quantification of the peptides is based on the entire extracted ion peaks of each of the three *m/z* values for a peptide, this has little to no effect on quantitation accuracy [23]. In addition to generic separation strategies, dimethylation has also been used successfully in combination with specific enrichment methods, especially for phosphorylated peptides. These include combinations with immobilized metal ion affinity chromatography (IMAC) [24, 25] and TiO_2 affinity chromatography [26] enrichment as well as the specific immunoprecipitation of peptides containing phosphorylated tyrosine residues [27].

Since most chemical labeling strategies for quantitative proteomics are sensitive to deviations in reaction efficiency and sample handling, efforts have been made toward the automation and robustness of the dimethylation procedure. In 2008, Raijmakers et al. [28] showed that it was possible to perform reductive amination on peptides that were non-covalently bound to C_{18} solid-phase material and that this could be performed online with LC-MS/MS analysis in an automated fashion. As this method placed restrictions on the size and complexity of the sample to be labeled and analyzed, it was later extended to automated labeling combined with SCX separation [29] and even automated labeling combined with online two-dimensional separation (C_{18}-SCX) [30]. A very detailed protocol describing the original in-solution labeling as well as the solid-phase labeling and automated online labeling is available [31].

Although most often performed at the peptide level, stable isotope dimethyl labeling can also be done on intact proteins, for example, to aid the identification of protein N-termini [32, 33]. It is more difficult to force the reaction to completion and, if used for proteomics approaches, it places restraints on the proteases that can be used, as neither trypsin nor Lys-C cleave dimethylated lysine residues [34]. The use of the dimethylation reaction has also been explored for purposes other than quantitative proteomics, such as the improvement of peptide identification by electron transfer dissociation fragmentation [35] and as an aid in the de novo sequencing of peptides and proteins for which no genome sequences are available [35]. Although those applications might be more specialized, the stable isotope dimethylation procedure is now being applied widely for quantitative proteomics, especially for samples where metabolic labeling is not easily achievable, for example, patient material or other tissue samples, as described in more detail later in this review. In this review we will not describe in detail the protocols for stable isotope dimethyl labeling, as these are readily available [31, 36], but we will instead focus on applications and data analysis.

Data analysis

From a bioinformatics perspective, stable isotope dimethylation is a relatively straightforward technique. Quantification is performed at the MS level, by comparing the relative abundances of the differentially labeled peptides, whereas identification is achieved at the MS/MS level, by setting N-terminal and lysine dimethylation as peptide modification. Two main threats frequently affecting stable isotope labeling [37] have been reported to possibly hamper the quantification of dimethylated peptides: retention time shifts between the labeled pairs and overlap of their isotopic clusters. Nevertheless, effective strategies to overcome both issues have been devised since the first studies introducing the procedure.

Retention time shifts can affect heavy deuterated peptides when reversed-phase chromatography is used, causing them to be eluted prior to the corresponding light ones [38]. When labeled pairs are deemed chromatographically resolved, retention time shifts can simply be circumvented by quantifying peptide extracted ion chromatograms (XICs) rather than their intensities in one particular scan [31]. A similar approach was originally described in the seminal publication by Hsu et al. [15], in which all spectra containing both the H4- and D4-labeled isotopologues of a given peptide were combined to produce a composite MS spectrum. The number of combined spectra was dependent on the peptide elution time and on the instrument duty cycle: at an acquisition rate of one scan per second, this typically meant combining 20 to 60 spectra, which was generally sufficient to span the whole peptide elution profile and mathematically equivalent to performing whole XIC area integration. By this strategy, the inaccuracy of quantification caused by differential elution between the deuterated and the nondeuterated peptides, the so-called deuterium effect, was found to be minor. Peptide ratios were then calculated from the relative intensities of the monoisotopic peaks in the composite MS spectrum.

Similar conclusions about a negligible deuterium effect were drawn by Ji and Li [23], who explicitly indicated the importance of summing up intensities over the entire elution profiles, in order to achieve accurate quantification. They were also the first to devise a dedicated strategy to tackle the *overlapping issue* [23], which occurs whenever the mass shift between the peptide pairs is smaller than their isotopic envelope, and was found to become more critical as the mass of the original peptides increases, especially when approaching 3 kDa [19]. The problem can particularly affect dimethyl-labeled peptides without a lysine residue, because they have a mass shift of only 4 Da between their isotopologues, resulting in the overlap of the fifth and consecutive peaks of the lighter peptide on the isotopic distribution of the heavier one. The overlap can clearly hamper quantification accuracy, with a typical upward bias for the heavy labeled peptides. In the proposed strategy, when overlapping occurred, the peptide sequences were submitted to the Web-based tool MS-Isotope, which is embedded in the ProteinProspector package [39] in order to model their theoretical isotopic distributions and to disentangle their relative contributions. In brief, the contribution of the lighter peptide was subtracted from the convoluted isotope pattern in order to obtain the correct peak distribution of the heavier peptide. Correct peptide ratios were then determined by using the deconvoluted monoisotopic peak intensities.

In the early work, quantification of the different peak intensities was performed manually, or by means of simple scripts that allowed batch processing [40]. At a later stage, when the technique started to gain popularity, many more

software tools were adapted to allow the quantification of dimethyl-labeled peptides. Today, the most used tools include open-source software, such as MSQuant [41], MaxQuant [42], PVIEW [43], and the XPRESS algorithm [44] embedded in the Trans-Proteomic Pipeline [45], commercial software, such as Proteome Discoverer (Thermo Fisher Scientific), and commercial software packages, such as Mascot Distiller (http://www.matrixscience.com/distiller.html).

To our knowledge, no systematic study has been presented yet to compare the quantification performance of the different software packages on dimethyl labeling (as has been performed, e.g., for SILAC labeling [46]). For this reason, a few recommendations that can help drive the choice of the most appropriate tool will be given in the remainder of this introduction.

The initial consideration is purely pragmatic: the choice cannot prescind from the instruments and tools already present and commonly used in the laboratory. Proteome Discoverer and MaxQuant, for instance, can only analyze Thermo .raw data files. Similarly, Mascot Distiller should be excluded a priori if Mascot is not the common or favorite database searched for identification. The second consideration is merely economic: commercial packages such as Mascot Distiller are quite expensive, but the cost can be justified when versatility is a prerequisite, in terms of supported instruments and labeling techniques. A third practical consideration regards software usability, which is often directly related to the presence of a good graphical user interface. To this extent, MSQuant has often been preferred to other tools that give a more "black box" feeling and has become the most used tool for dimethyl labeling quantification. The program probably gained its popularity thanks to its interactive feedback during the quantification process, which allows visual inspection of differentially expressed peptides and proper integration over their entire XICs, and because of the possibility to directly interface its output with postprocessing tools such as StatQuant [47] for normalization, outlier detection, and further statistical analysis.

On a more technical note, the choice should also be based on the possibility to deal with the retention time shift and the overlapping issues introduced above. To overcome the deuterium effect, as already mentioned, simple strategies that calculate peptide ratios based on the area under the XIC peaks should be always preferred to more advanced algorithms such as the one implemented in MaxQuant, which compares the intensities of the isotopologues in all scans across the XIC and fits a straight line through the origin of their scatter plot. This algorithm was originally implemented for SILAC data and works very well for high-quality data, but becomes unreliable in the presence of large retention time shifts. In this regard, Mascot Distiller gives the option to choose between the two quantification strategies. The second issue has been almost completely disregarded by quantification software, probably because it is known to affect only very large peaks. To our knowledge, Overlapping Peaks Finder [19] is still the only tool purposely devised for dimethyl labeling. The algorithm can be used in the form of a postprocessing script and can correct quantitative results obtained by quantitation software programs that evaluate peptide ratios based on the XICs of their monoisotopic peaks ions, such as MSQuant and XPRESS.

The last consideration regards peptide identification. For most tools the quantification process is search-results-driven, so only peptides identified by the database search are matched to the precursor signals for quantification. Recent algorithms, though, have enabled a multiplexed strategy for feature-driven quantification, in which feature detection is anticipated and drives database searches by better tailored constraints. When, for instance, MaxQuant is used, the labeling state of paired peptides is determined in advance, so as to allow separate database searches where each methylation state is set as a fixed modification. With a slightly different approach, PVIEW treats "light" methylation as a fixed modification and "intermediate" and "heavy" methylations as modifications that are variable with respect to the "light" one. These customized approaches should allow one to use a smaller search space than the one created by setting "light", "intermediate," and "heavy" methylations as variable modifications (as in Proteome Discoverer), thus reducing the probability to match false-positive identifications.

Applications of stable isotope dimethyl labeling in proteomics

As mentioned already, stable isotope dimethyl labeling has been incorporated into many types of experiments, albeit mostly for the purpose of relative quantification of protein expression. We have broadly separated these applications into global quantitative expression proteomics, quantitative analysis of posttranslational modifications (PTMs), and protein interaction studies. As more than 70 applications have been reported since 2003, we have made a selection and we highlight a few studies in more detail, but also provide a more comprehensive list of published work using stable isotope dimethyl labeling.

Stable isotope dimethyl labeling for quantitative expression proteomics

The most widely used application of stable isotope dimethyl labeling in proteomics is as a quantification tool to determine globally the relative levels of protein expression between cellular or tissue states. A general strategy in an expression proteomics experiment using stable isotope dimethyl labeling can be illustrated by experiments performed by Munoz et al. [48] to compare the proteomes of human embryonic stem cells (hSECs), human induced pluripotent stem cells (hiPSCs), and the fibroblast cells from which the latter originated. In order to

characterize the differences and similarities in the protein content of hESC and hiPSC, an in-depth quantitative proteome profiling experiment was executed using two different hiPSC lines, their two parental fibroblast cell lines, and one hESC line. Skin fibroblast (i.e., IMR90) and foreskin fibroblasts (4Skin) were reprogrammed into hiPSCs. A schematic of the experimental workflow is shown in Fig. 2a. Cells were lysed and peptides were digested. hiPSC peptides were labeled with light isotopes, hESC peptides were labeled with intermediate-mass isotopes, and parental fibroblast cell peptides were labeled with heavy isotopes. Labeled peptides were mixed in a 1:1:1 ratio based on total peptide amount determined by running an aliquot of the labeled samples in a regular LC-MS experiment and comparing the overall peptide signal intensity, fractionated by SCX, and analyzed by LC-MS/MS. Independent biological replicates were performed, whereby the light and intermediate (hiPSC and hESC) isotope labels were swapped. Peptides appeared as a triplet in the mass spectra. Because the light, intermediate-mass, and heavy peptides are nearly chemically identical, except for their mass difference [31], the ratio of peak intensities directly yields the ratio of the proteins in hiPSCs, hESCs, and fibroblasts, respectively.

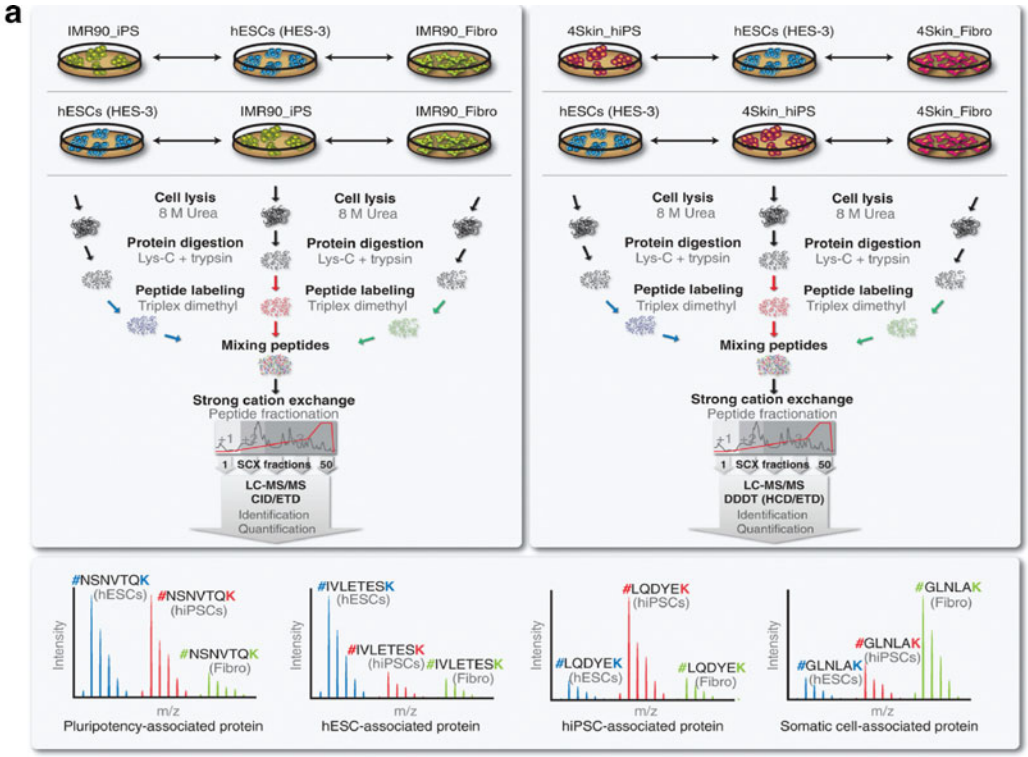
Combining all the data from the two experiments and two replicates, more than 10,000 proteins were identified, of which 7,000 proteins could be quantified. The comparative analysis focused on a smaller set of 2,683 proteins quantified in all experiments and replicates. The data revealed that protein expression of 98.7 % of quantified proteins was alike in the hESC and hiPSC proteomes, whereby only a small group of 58 proteins was found to be differentially expressed (Fig. 2b, c). These quantitative results indicate the reprogramming process remodeled the proteomes of both fibroblast cell lines to a proteome profile, which closely resembles the hESC proteome, making at the proteome level hiPSCs nearly indistinguishable from hESCs. In a comparable study, Phanstiel et al. [49] analyzed molecular differences between hESCs and hiPSCs at the proteome level using iTRAQ isobaric tagging for quantification. Both studies came to the identical conclusion that at the proteome level hiPSCs are nearly indistinguishable from hESCs. Moreover, from a technical point of view, it was also interesting to see that the depth of proteome coverage and the accuracy and precision in protein quantification were alike, implying that stable isotope dimethyl labeling and iTRAQ can achieve similar levels of depth and accuracy in quantification.

The broad potential and suitability of stable isotope dimethyl labeling for global quantitative expression proteomics has been demonstrated by several groups on samples from a variety of cells, tissues, and body fluids. To illustrate this, point we give an overview of recent studies in Table 1. Clearly the method is suitable for a wide range of samples, allowing deep coverage of the proteome and allowing adaptation to minute amounts of sample.

We finish this section by highlighting an interesting study using dimethyl labeling in population proteomics, highlighting that the method can also be used to probe large amounts of samples, as represented when proteomes need to be probed differentially across and within populations to define and better understand protein variation. With the massive number of samples from several populations to be analyzed, dimethyl labeling was used as it is cost-effective. Parker et al. [50] reported the differential proteomes of honeybees from geographically different bee farms with the aim of determining the diversity of protein expression in commercial honeybee populations and of identifying the mechanisms used by the bees to adapt to different ecological conditions. They conducted a large-sale quantitative proteomics analysis of the midgut proteome from adult nurse bees from eight different locations. Between four and 11 colonies from each location were sampled, each colony was sampled in triplicate, and each replicate comprised midguts from five nurse bees. They applied a dimethyl triplex labeling and a random block experimental design to derive relative expression profiles for all proteins across all colonies studied. One block was a triplex analysis of bees from three different colonies. In total 58 blocks were analyzed. Overall, 578 proteins were quantified, from which 170 proteins showed differential expression in midguts correlating with distinct populations. Their dimethyl-labeling-based quantitative proteomics study revealed that bees indeed adapt to different climates. For example, the major energy-producing pathways of mitochondria were consistently higher in bees in colder climates, whereas upregulation of some proteins involved in metabolism was observed in bees originating from warmer climates [50].

Dimethyl labeling for the analysis of protein posttranslational modifications

Next to the measurement of global protein expression profiles, MS has also become a tool of choice for the analysis of protein PTMs. It can unravel PTMs without prior knowledge and it is typically much more comprehensive, specific, and quantitative than antibody-based methods. Recent parallel developments in MS and chromatography, including new techniques for enriching peptides bearing a PTM, and concomitant improvements in bioinformatics software have facilitated the global analysis of PTMs, making it possible to pinpoint them with single amino acid resolution [51–53]. However, identification of PTM sites by MS can be hindered by the low abundance of the modified peptides, especially in the case of signaling proteins, which are also often modified at substoichiometric levels. Luckily, this problem can largely be addressed by modification-specific enrichments. However, for functional annotation, the goal of large-scale PTM studies is not just to catalog many PTM sites but more importantly to determine how the identified PTMs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4Skin_hiPS
4Skin_Fibro
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Lys-C + trypsin
Peptide labeling
Triplex dimethyl
Mixing peptides
Strong cation exchange
Peptide fractionation
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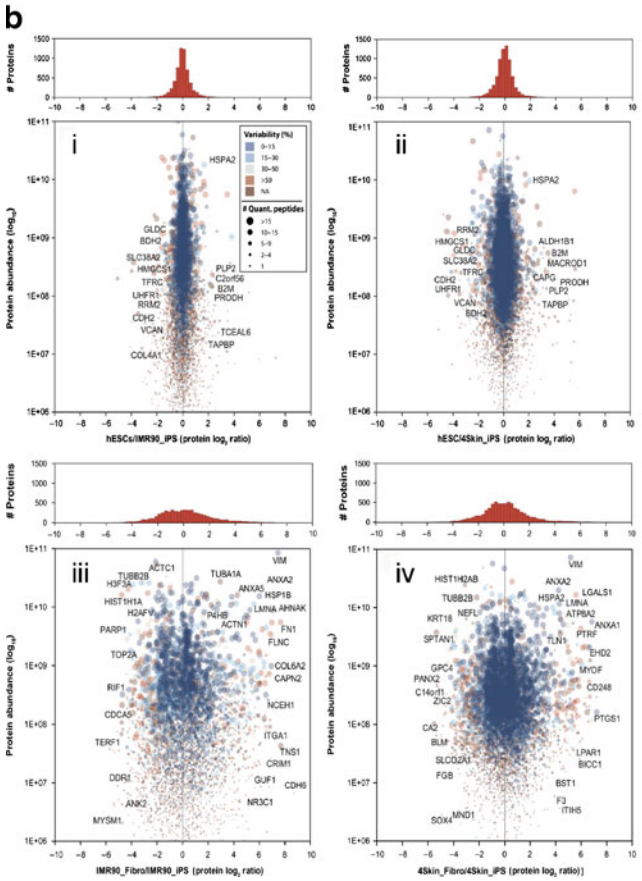
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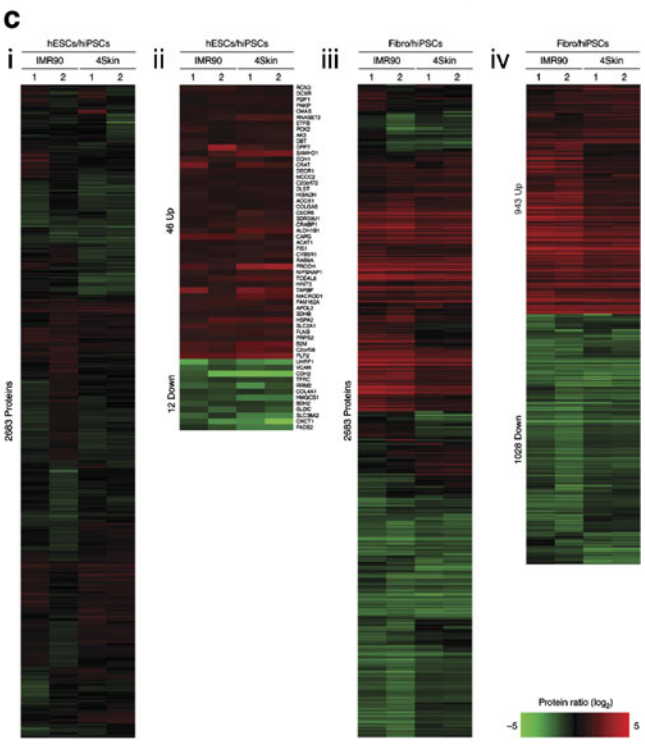
Proteins
Protein abundance (log10)
hESCs/IMR90_iPS (protein log2 ratio)
hESC/4Skin_iPS (protein log2 ratio)
IMR90_Fibro/IMR90_iPS (protein log2 ratio)
4Skin_Fibro/4Skin_iPS (protein log2 ratio)
Variability (%)
Quant. peptides


c
hESCs/hiPSCs
Fibro/hiPSCs
IMR90
4Skin
2683 Proteins
46 Up
12 Down
943 Up
1028 Down
Protein ratio (log2)

◀ **Fig. 2** Principle of a quantitative proteomics experiment and data analysis based on stable isotope dimethyl labeling, exemplified by quantitative proteome profiling of human induced pluripotent stem cells (*hiPSCs*) and human embryonic stem cells (*hESCs*). **a** Illustrative experimental design in a quantitative proteomics experiment based on stable isotope dimethyl labeling, as used for a quantitative proteome profiling of hiPSCs and hESCs. Quantitative proteomics experiments using two different hiPSC lines were conducted, whereby in the first experiment (*top left*), IMR90_iPS were compared with hESCs (HES-3) and with the parental fibroblast cell line, IMR90_Fibro. In the second experiment (*top right*), 4Skin_iPS were compared with hESCs (HES-3) and the parental fibroblast cell line, 4Skin_Fibro. Cells were lysed, and proteins were extracted and subsequently digested with Lys-C and trypsin. Peptides were labeled using triplex dimethyl chemistry, equally mixed, and prefractionated by using strong cation exchange (*SCX*). Peptides originating from hESCs were labeled with a light isotope (*blue*) and peptides originating from hiPSCs and its parental fibroblast cell line were labeled with intermediate-mass (*red*) and heavy (*green*), isotopes respectively. Two biological replicates were performed for each experiment, where the labels were swapped between hiPSCs and hESCs. SCX fractions were analyzed by high-resolution liquid chromatography–tandem mass spectrometry (*LC-MS/MS*). The peak intensities of the identified peptides were proportional to their abundance. **b** Global quantitative proteomic comparisons of hESCs, two hiPSCs, and their two precursor fibroblast cell lines. The absolute protein abundance ($\log_{10}$ scale) is plotted against the relative protein ratios ($\log_2$ scale) for the hESCs/IMR90_iPS (*i*), hESCs/4Skin_iPS (*ii*), IMR90_Fibro/IMR90_iPS (*iii*), and 4Skin_Fibro/4Skin_iPS (*iv*) comparisons. The number of peptides used for the quantification and the calculated variability or relative standard deviation of the peptide ratios are represented in the plots by the spot size and color scale, respectively. In the plots for hESCs and hiPSCs, most of the proteins were quantified with close to zerofold change, which indicates that hESC and hiPSC proteomes are very similar. By contrast, the plots for hiPSCs and their precursor fibroblast cells reveal large differences in their proteomes. The histograms of the frequencies show the density of proteins in each analysis using a bin size of 0.25 ($\log_2$). **c** Proteome differences between hESCs, hiPSCs, and their parental fibroblasts. In total, 2,683 proteins were consistently quantified in all four data sets, i.e., two replicates (label swaps) of the IMR90 and 4Skin experiments. Relative protein abundances are represented as heatmaps for hESC/hiPSC (*i*) and fibroblast/hiPSC (*iii*) comparisons. With use of significance analysis of microarrays, 58 proteins (2.2 %) were found significantly regulated between hESCs/hiPSCs (*ii*) and 1,927 (73.4 %) were found significantly regulated between fibroblasts/hiPSCs (*iv*). *Red* and *green* indicate upregulated and downregulated events, respectively. *CID* collision-induced dissociation, *ETD* electron transfer dissociation, *DDDT* data-dependent decision tree, *HCD* high-energy collisional dissociation (All data from [48])

change after a particular cell signaling pathway is activated or during disease development.

Stable isotope dimethyl labeling in combination with targeted enrichment provides a nice platform for the quantitative analysis of protein PTMs, and is still cost-efficient when several milligrams of starting material is required to enable the detection of the low-abundance modified peptides.

Dimethyl labeling application in quantitative phosphoproteomics

Phosphorylation is one of the most important and most well studied PTMs as it plays a critical role in the regulation of several cellular processes, including cell cycle, growth, apoptosis, and differentiation. Several approaches have been developed to facilitate the enrichment of low-abundance phosphopeptides, for example, phosphotyrosine immunoprecipitation, TiO_2 affinity chromatography, and IMAC [52, 54]. Dimethyl labeling is well suited to large-scale quantitative phosphoproteomics as it can be easily incorporated with any kind of phosphoprotein or phosphopeptide enrichment approach even when starting with milligrams of material (Table 2). As an illustrative example, we describe below how dimethyl labeling was incorporated in our laboratory with two important enrichment techniques in quantitative phosphoproteomics studies. We give examples for the targeted analysis of tyrosine phosphorylation using phosphotyrosine immunoprecipitation and the more global analysis of protein phosphorylation using SCX in combination with TiO_2 affinity chromatography to profile differential phosphorylation events in stem cells following stimulation leading to differentiation [27, 55].

Examining the self-renewal process in human embryonic stem cells

Human embryonic stem cells (hESCs) exhibit two exceptional properties, which are the ability to self-renew and the ability to differentiate into all cell types of the human body [56]. We were interested in how hESCs self-renew and maintain their undifferentiated state. Self-renewal of hESCs is regulated by several factors; one of them is fibroblast growth factor (FGF) 2. FGFs execute their biological actions by activating cell surface FGF receptors, which are members of the receptor tyrosine kinase family, resulting in activation of further downstream signaling pathways, including the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3-K) pathways. To understand the role of FGF signaling in hESCs, we performed two large-scale, targeted, quantitative phosphoproteomics experiments to investigate phosphorylation events following FGF-2 stimulation [27, 55].

Since FGF-2 signaling is initiated via receptor tyrosine kinases, we initially performed an analysis of the early tyrosine phosphorylation events following FGF-2 stimulation. We treated hESCs with FGF-2 for 0, 5, and 15 min. As tyrosine phosphorylation is less frequent in cells compared with serine and threonine phosphorylation, 6 mg lysate from each time point was digested and dimethyl-labeled, whereby the non-stimulated hESCs (0 min) were labeled with light dimethyl labels, the 5-min-stimulated hESCs were labeled with intermediate-mass labels, and the 15-min-stimulated hESCs were labeled with heavy labels. The differentially labeled samples were mixed 1:1:1 based on the total peptide amount determined by running an aliquot of the labeled samples in a regular LC-MS experiment and comparing the overall peptide signal intensity and the signal intensity of the samples

Table 1 Selected applications of stable isotope dimethyl labeling in quantitative expression proteomics

Sample types	Dimethyl labels	Analysis	Quantification tool	Reference
Human carcinoma cells	$2xCH_3$, CHD_2	SCX, LC-MALDI-MS	Ratios calculated manually from monoisotopic signal intensities	[22]
Bovine photoreceptor cells	$2xCH_3$, $2xCHD_2$	LC-MS	MSQuant	[89]
Human osteoprogenitor cells	$2xCH_3$, $2xCHD_2$ (protein labeling)	LC-MS	XInteract (XPRESS) (part of the Trans-Proteomic Pipeline)	[90]
Human uroepithelial cells	$2xCH_3$, $2xCHD_2$	SCX, LC-MS	Ratios manually calculated from peak heights	[91]
Human carcinoma tissue	$2xCH_3$, $2xCHD_2$	LC-MS	In-house adapted version of MSQuant	[92]
Honeybee abdominal tissue	$2xCH_3$, $2xCHD_2$, $2x^{13}CD_3$	LC-MS	MSQuant	[93]
Rat liver microsomes	$2xCH_3$, $2xCHD_2$	LC-MS	In-house program PQ-SIDL (http://peptide.ce.ncu.edu.tw)	[94]
Human urine	$2xCH_3$, $2xCHD_2$	LC-MS	In-house software	[95]
Bovine milk	$2xCH_3$, $2xCHD_2$	LC-MS	MaxQuant	[96]
Arabidopsis plastoglobules	$2xCH_3$, $2xCHD_2$	LC-MS	Ratios manually calculated from peak areas	[97]
Potatoes	$2xCH_3$, $2xCHD_2$, $2x^{13}CD_3$	SCX, LC-MS	In-house software	[98]
Pseudomonas putida S12	$2xCH_3$, $2xCHD_2$	LC-MS	MSQuant	[99]
Clostridium phytofermentas	$2xCH_3$, $2xCHD_2$	LC-MS	Ratios calculated manually from peak areas	[100]

CH_3 labeled using CH_2O and $NaBH_3CN$, *CHD_2* labeled using CD_2O and $NaBH_3CN$, *$^{13}CD_3$* labeled using $^{13}CD_2O$ and $NaBD_3CN$, *SCX* strong cation exchange, LC liquid chromatography, *MALDI* matrix-assisted laser desorption/ionization, *MS* mass spectrometry

enriched for tyrosine-phosphorylated peptides by phosphotyrosine immunoprecipitation. The enriched peptides were then analyzed by LC-MS/MS (Fig. 3). We identified and quantifies 300 unique tyrosine phosphorylation sites. As expected, an increase in tyrosine phosphorylation of all FGF receptors and their canonical downstream effectors (e.g., MAPK and PI3-K) was identified. Interestingly, a large number of proteins not directly involved in the canonical FGF pathway, especially Src substrates, were observed with increased phosphorylation upon FGF-2 stimulation, although only relatively small increases were observed for their Src family kinases. Moreover, cluster analysis revealed that the identified Src substrates had the same tyrosine phosphorylation profiles in response to FGF-2 as identified in all FGF receptors and their downstream targets. Our finding suggested for the first time the importance of Src kinase signaling in maintaining the undifferentiated hSEC phenotype, which could be confirmed by our Src kinase inhibition assays [57]. From a technical point of view, it is interesting to note that in these experiments we labeled 6 mg of material; however, we only analyzes nanogram amounts of material acquired following phosphotyrosine immunoprecipitation, discarding the rest to waste. Similar experiments have been reported by Wolf-Yadlin et al. [58], who used iTRAQ labeling for the quantitative readout.

Table 2 Selected applications of stable isotope dimethyl labeling in quantitative phosphoproteomics

Sample types	Dimethyl labels	Analysis	Quantification tool	Reference
MCF-7 cells	$2xCH_3$, $2xCHD_2$	IMAC-HILIC, LC-MS	MASCOT Distiller (used average peak area of the first 3 or 4 isotopic peaks across the elution profile)	[101]
Human lymphobastoid cells	$2xCH_3$, $2x^{13}CHD_2$	TiO_2, LC-MS	Ratios calculated manually from relative peak intensities	[102]
Human lymphoid cells	$2xCH_3$, $2xCHD_2$, $2x^{13}CD_3$	SCX, online TiO_2, LC-MS	In-house dimethyl-adapted version of MSQuant	[103]
Mouse brain tissue	2xCH3, $2xCD_3$	SCX, online IMAC, LC-MS	Ratios calculated manually from peak areas	[104]
Zebra fish embryos	$2xCH_3$, $2xCHD_2$	SCX, online TiO_2, LC-MS	In-house dimethyl-adapted version of MSQuant	[26]
Rat uteri	$2xCH_3$, $2xCHD_2$	IMAC, MALDI, LC-MS	Ratios calculated manually from relative peak heights	[24]
Arabidopsis	$2xCH_3$, $2xCHD_2$	Zr^{4+}-IMAC, LC-MS	Ratios calculated manually from relative intensities	[105]

CH_3 labeled using CH_2O and $NaBH_3CN$, *CHD_2* labeled using CD_2O and $NaBH_3CN$, *$^{13}CHD_2$* labeled using $^{13}CD_2O$ and $NaBH_3CN$, *CD_3* labeled using CD_2O and $NaBD_3CN$, *$^{13}CD_3$* labeled using $^{13}CD_2O$ and $NaBD_3CN$, *IMAC* immobilized metal ion affinity chromatography, *HILIC* hydrophilic interaction chromatography, *LC* liquid chromatography, *MS* mass spectrometry, *TiO_2* TiO_2 affinity chromatography, *SCX* strong cation exchange, *MALDI* matrix-assisted laser desorption/ionization

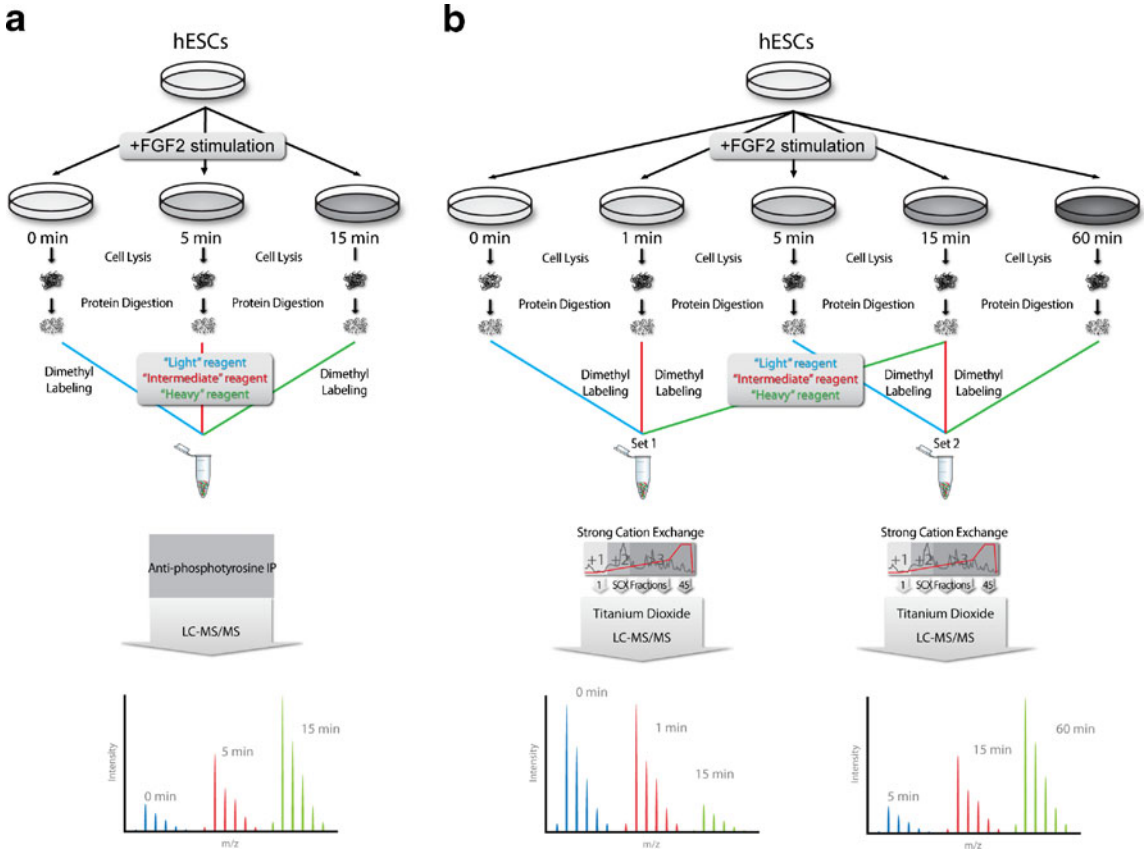


Fig. 3 Overview of the experimental design used in a quantitative phosphoproteomics study monitoring signaling responses in fibroblast growth factor 2 (*FGF2*)-stimulated hESCs cells. FGF2-stimulated cells were harvested at different time points following stimulation and lysed, after which the extracted proteins were digested. The resulting peptides were stable-isotope-labeled using triplex stable isotope dimethyl labeling. **a** Targeted phosphotyrosine profiling. The peptides extracted from hESCs stimulated for 0, 5, and 15 min were mixed in a 1:1:1 ratio and phosphotyrosine immunoprecipitation (*IP*) was performed to enrich the samples for phosphotyrosine peptides prior to LC-MS/MS. **b** Global phosphopeptide profiling. The peptides extracted from hESCs stimulated for 0, 1, 5, 15, and 60 min were mixed as depicted to create two sets of samples. SCX and TiO_2 affinity chromatography were used to enrich the samples for phosphopeptides prior to LC-MS/MS. The two experiments combined provided global differential-phosphorylation-related signaling events during the early time course following FGF2 stimulation. (Adapted from [55])

Compared with dimethyl labeling, this increases the cost of the experiment tremendously; moreover, the iTRAQ label may have a negative effect on the efficiency of the phosphotyrosine immunoprecipitation. In a comparison between iTRAQ and dimethyl labeling, Boersema et al. [27] reported quantitatively identical results following EGF stimulation in HeLa cells, indicating that both methods can perform equally well.

To further understand the role of FGF-2 on hESCs, we extended our tyrosine phosphorylation profiling by performing a more global phosphoproteomics study under identical biological conditions aiming to include now also the vast majority of phosphorylations in cells that are on serine and threonine residues, albeit at the expense of detecting many tyrosine phosphorylations [55]. We performed a multidimensional strategy combining SCX chromatography to reduce sample complexity and by TiO_2 affinity chromatography off-line to enrich the samples for phosphopeptides, and dimethyl labeling for quantification. The hESCs were stimulated with FGF-2 for 0, 1, 5, 15, and 60 min. Then, 1.5 mg lysate from each time point was digested and dimethyl-labeled, whereby for the first group, the nonstimulated hESCs (0 min) were labeled with light dimethyl labels, the 5-min stimulated hESCs were labeled with intermediate-mass labels, and the 15-min-stimulated hESCs were labeled with heavy labels. For the second group, the 1-min stimulated hESCs were labeled with light dimethyl labels, the 15-min-stimulated hESCs were labeled with intermediate-mass labels, and the 60-min-stimulated hESCs were labeled with heavy labels. Samples from each group were mixed in a 1:1:1 ratio and fractionated by SCX. The SCX fractions abundant in phosphopeptides were further enriched for phosphopeptides with TiO_2 affinity chromatography off-line and analyzed by LC-MS/MS (Fig. 3). A total of 1,653 phosphopeptides corresponding to 810 proteins were quantified. In agreement with our study mentioned above, a rapid increase in phosphorylation was observed in all FGF receptors as well as downstream targets of the MAPK,

PI3K, and Src family members. Complementing our previous analysis, we observed in this study that FGF-2 also regulated several other downstream signaling pathways, including Wnt and actin/cytoskeletal pathways. The observed changes in phosphorylation emphasized that FGF-2 may directly regulate the pluripotency transcription factors to maintain the undifferentiated phenotype [55].

Technically similar applications of dimethyl labeling combined with a wide range of phosphoenrichment techniques are listed in Table 2. To end this section, we highlight our recent study of a novel phosphopeptide enrichment approach based on a combination of low-pH SCX and Ti^{4+}-IMAC. We have shown that the Ti^{4+} method can boost the number of phosphopeptides identified in dimethyl-labeling-based quantitative proteomics. We identified more than 9,000 unique phosphorylation sites from a single experiment consisting of only 400 μg triple-dimethyl-labeled lysate [25].

Overall, all these studies combined reveal that stable isotope dimethyl labeling provides a versatile platform for quantitative phosphoproteomics studies as it can be incorporated in every workflow chosen to perform enrichment of phosphopeptides and phosphoproteins and can be applied to every kind of cell, tissue, or organelle.

Dimethyl labeling applications in quantitative glycoproteomics

Glycosylation is one of the most frequent PTMs occurring often on cell-surface and extracellular proteins. The two main forms are O-linked oligosaccharides bound to serine or threonine residues and N-linked glycans attached to asparagine residues. Changes in the glycosylation profile have been reported to be associated with several diseases; therefore, quantitative glycoproteomics is rapidly gaining interest. Several enrichment strategies have been developed for the study of glycosylated proteins. The two major approaches are lectin affinity and hydrazide chemistry, which are mostly applied for the analysis of N-linked glycoproteins [59, 60]. Alternative enrichment methods include HILIC and TiO_2 affinity chromatography for glycoproteins and peptides containing sialic acids [61, 62]. Stable isotope labeling combined with different glycoprotein enrichment approaches has been utilized in several quantitative glycoproteomics studies [59, 60]. Dimethyl labeling is well suited for quantitative glycoproteomics study of both N-linked and O-linked glycosylation as it can be combined with all the above-mentioned enrichment strategie,s as described in more detail next.

Quantitatitive N-linked glycoproteomics profiling in cardiac remodeling

Parker et al. [63] described a quantitative glycoproteomics strategy to profile the myocardial N-glycoproteome during cardiac modeling after ischemia/reperfusion injury in a rat model. Their initial profiling used iTRAQ for quantification with 100 μg starting material. The starting material was sufficient for only one enrichment step and only 80 glycopeptides could be quantified with altered abundance. To increase the coverage of the glycopeptides, they performed dimethyl labeling for quantification so the amounts of starting material could be increased and therefore complementary glycopeptide enrichment methods based on different chemical properties of the attached glycans could be performed. Membrane proteins (2 mg) from control heart and those from hearts subjected to ischemia/reperfusion injury were digested and dimethyl-labeled. The labeled peptides were mixed and subjected to three different enrichment methods, TiO_2 affinity chromatography for sialic acid containing glycopeptides, ZIC-HILIC for neutral glycopeptides, and hydrazide capture. A total of 590 glycosylation sites were quantified and 123 glycopeptides were identified with abundance changes. Of these, 46 glycopeptides were in agreement with those obtained by the iTRAQ approach, whereas dimethyl labeling added another 62 glycopeptides with altered abundance. These glycosylation sites mapped to proteins from the three phases of cardiac remodeling ranging from extracellular matrix proteins and cell-surface glycoproteins to proteins associated with sarcolemma and electrochemical signaling and solute transport [63].

Dimethyl labeling can also be combined with lectin affinity chromatography enrichment. Wei et al. [64] established an analytical platform that can enrich, identify, and quantify glycoproteins in complex plasma samples for biomarker discovery in prion disease. Blood from mice inoculated with prion protein was collected 108, 158, and 198 days after inoculation. Plasma samples from seven control and seven infected mice from each time point were pooled and glycoproteins were enriched by lectin affinity chromatography. Glycoproteins were digested, differentially dimethyl labeled, and analyzed by two-dimensional reversed-phase–reversed-phase LC-MS/MS. Dimethyl labeling revealed around 100 proteins with altered expression in the plasma collected from prion-inoculated animals. As a consequence of the data, they proposed serum amyloid P-component protein as a potential biomarker for the progression of prion disease [64].

Probing the dynamics of O-linked N-acetylglucosamine glycosylation in the brain

O-linked *N*-acetylglucosamine (O-GlcNAc) is an intracellular, reversible form of O-glycosylation that shares many features with phosphorylation. Khidekel et al. [65] have reported a new quantitative proteomics strategy for monitoring the dynamics of O-GlcNAc glycosylation, for which the method is termed quantitative isotopic and chemoenzymatic tagging (QUIC-Tag). The method combines selective,

chemoenzymatic tagging of O-GlcNAc proteins, which allows selective biotinylation and avidin chromatography purification of the proteins using dimethyl labeling for quantification (Fig. 4). The application of the QUIC-Tag method was shown in the study of O-GlcNAc glycosylation in neurons and brain where the modification is abundant. The QUIC-Tag method was used to probe global glycosylation changes as a response to extracellular stimuli. Interestingly, O-GlcNAc modification was identified in several proteins essential for synaptic function in neurons, suggesting a role of the modification in mediating the communication between neurons. Their data also showed for the first time that besides glucose concentration, extracellular stimuli can induce and regulate O-GlcNAc glycosylation in the brain [65].

Dimethyl labeling for identification of proteolytic products and natural N- and C-termini

Endogenous proteolysis is a major, irreversible PTM that can modify protein activity, function, localization, and life span. Proteolysis generates proteins with neo-N- and/or neo-C-termini not originally present in the initially translated polypeptides. Proteases are one of the largest enzyme classes in mammalian organisms. However, for half of the proteases, substrates are relatively unknown and for the other half annotation of the substrate degradome is incomplete.

The group of Overall [66–71] has developed two of the most powerful quantitative proteomics approaches for labeling and isolating N- or C-terminal peptides, referred to as terminal amine isotopic labeling of substrates (TAILS) and C-terminal amine-based isotope labeling of substrates (C-TAILS). The approaches are designed for comparison of protease-treated and control untreated proteomes as well as for comparison of proteolytic processing in different samples, as shown in Fig. 5a. The two methods combine an enrichment step for protein termini with stable isotope labeling, mostly with dimethyl labeling for quantification (1) to assign specific cleavage events to the protease of interest and (2) to distinguish the protease-induced proteolytic products from background proteolytic products in a control sample.

TAILS was developed to enrich, identify, and quantify both natural and proteolysis-generated N-termini. As a critical step, at the protein level, dimethyl labeling is incorporated to

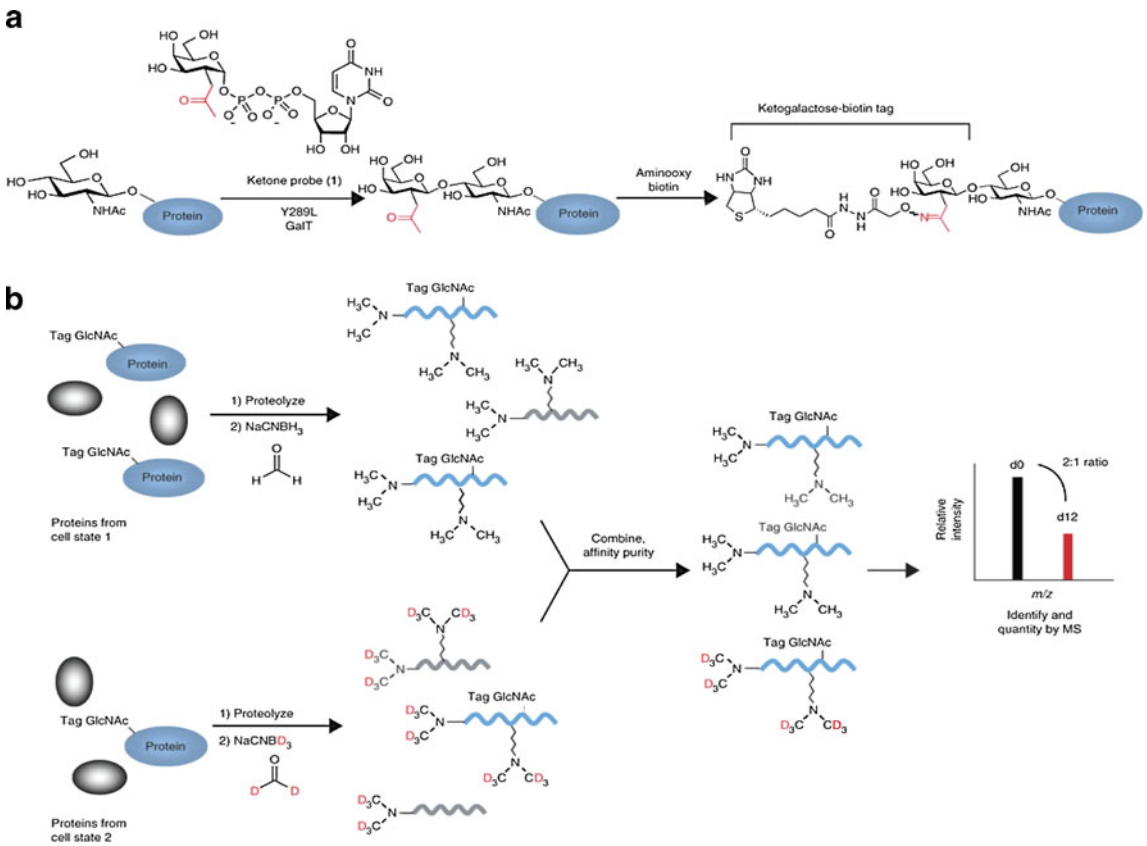
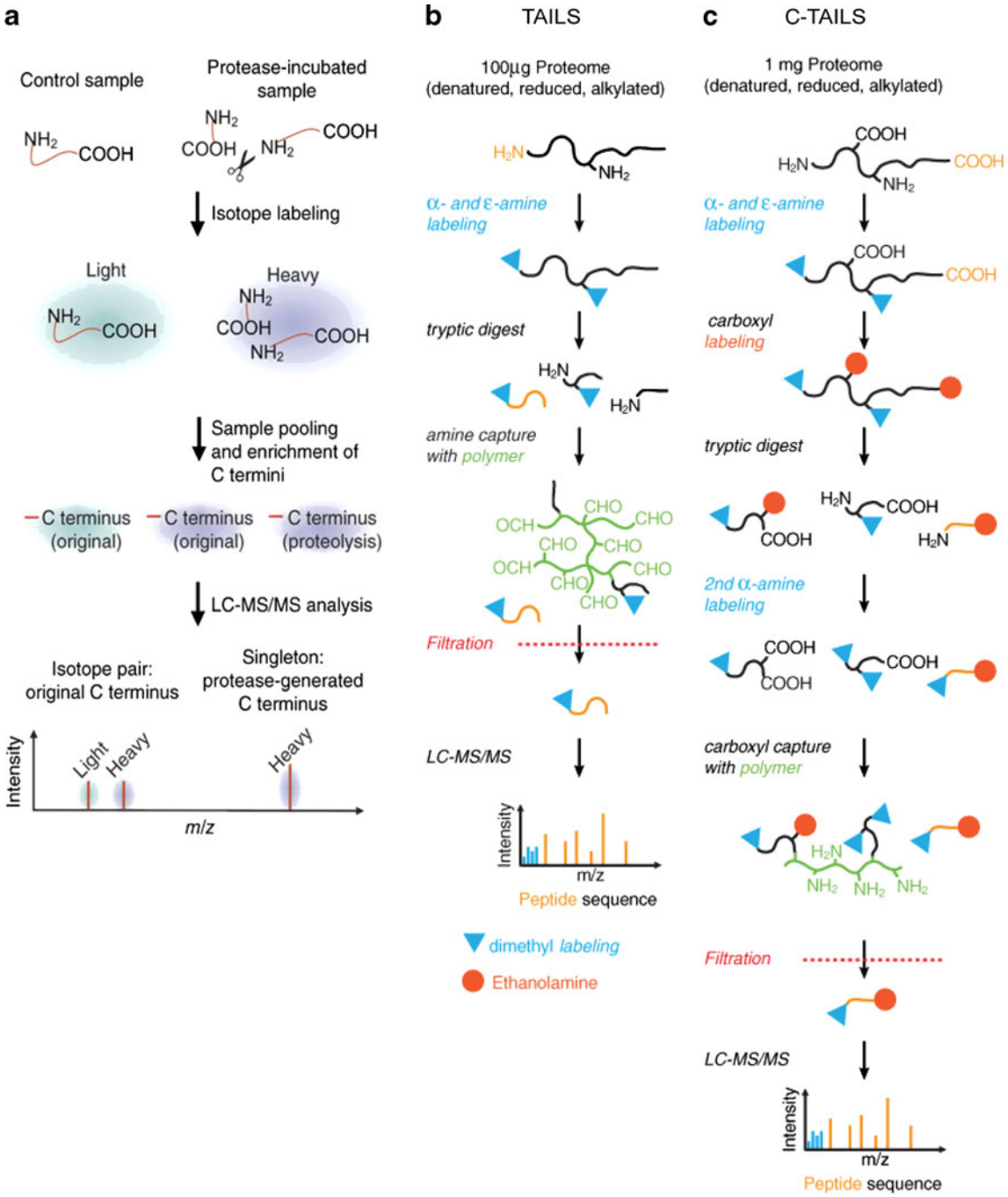


Fig. 4 Overview of the QUIC-Tag approach including stable isotope dimethyl labeling. **a** O-linked *N*-acetylglucosamine (O-GlcNAc)-glycosylated proteins are chemoenzymatically tagged with a ketogalactose sugar, which allows selective biotinylation of the proteins. **b** O-GlcNAc proteins from two different cell states are selectively tagged, digested, and differentially labeled by stable isotope dimethyl labeling. The mixtures are subsequently combined, and O-GlcNAc peptides are selectively enriched by avidin chromatography prior to quantification by liquid chromatography–mass spectrometry (*MS*). (Adapted from [65])

modify natural and proteolysis-generated N-terminal and lysine amines, thus protecting the modified amines from trypsin and simultaneously labeling the proteins. As lysine dimethylation prevents trypsin cleavage of lysine, the resulting generation of longer peptides by trypsin aids the identification of the relatively short N-terminal peptides. After tryptic digestion, negative enrichment was performed by using an amine–reactive, aldehyde-derivatized polymer to capture internal trypsin-generated N-termini in order to separate them from protected N-termini (Fig. 5b). Quantification of the heavy and light ratios of the peptides together with stringent bioinformatics search criteria enables discrimination of protease-cleaved neo-N-terminal peptides from the background proteolysis products; therefore, substrate repertoire of a specific protease can be identified and their accurate cleavage sites can be assigned [66–68].

TAILS has been demonstrated in the study of broad-specificity matrix metalloproteinase 2 cleavage sites and substrates in mouse fibroblast secretomes. TAILS identified more than 200 cleavage sites from more than 100 substrates.



TAILS has also been used to study mouse inflammatory bronchoalveolar fluid and revealed the substrates of the poorly defined breast cancer protease matrix metalloproteinase 11 [66]. Applications of TAILS have also been shown by Tholen et al. [72], who were interested in the contribution of the endolysosomal cysteine endoprotease, cathepsin L (Cstl) to extracellular proteolysis of mouse embryonic fibroblasts. TAILS was used to determine changes in the cleavage pattern of secreted proteins between wild-type and Cstl knockout mouse embryonic fibroblasts and to identify the Cstl-dependent cleavage sites. In total, TAILS identified more than 1,500 protein termini, with this number being reduced by more than 300 by Cstl deficiency.

A similar strategy was applied to study the protein C-terminome [69, 70]. C-TAILS is currently one of the few methods that allow proteome-wide enrichment of C-terminal peptides. C-TAILS was developed to enrich, identify, and quantify both natural and proteolysis-generated C-termini. As an additional step to N-terminal TAILS, endogenous and protease-generated neo-C-terminal groups together with aspartate and glutamate side chain carboxyl groups have to be chemically protected at the protein level. Dimethyl labeling is incorporated to label and protect amines at the protein and peptide level. Trypsin digestion yields the natural protein C-terminal peptides and neo-C-terminal peptides, now with protected carboxyl groups. Original C-terminal and neo-C-terminal peptides which lack free carboxyl groups are isolated by negative enrichment [69, 70] (Fig. 5b). A nice application of C-TAILS was reported by Schilling et al. [69], who identified hundreds of C-terminal peptides in the *Escherichia coli* proteome.

◀ **Fig. 5** Overview of N-terminal amine isotopic labeling of substrates (*TAILS*) and C-terminal terminal amine isotopic labeling of substrates (*C-TAILS*) approaches including stable isotope dimethyl labeling. **a** Overview of the TAILS and C-TAILS approaches that can be used to identify protease-generated neotermini. The workflow depicts protease-generated C-termini as an example. A proteome sample is incubated with the targeted protease, which generates neo-C-terminal protein. After proteolysis, the protease is inactivated and the samples are denatured and reduced. To distinguish induced proteolysis from background proteolysis, control and protease-treated samples are differentially labeled using dimethylation with light and heavy isotopes, respectively. After trypsin digestion and labeling, both samples are mixed, and C-terminal peptides are isolated. In LC-MS/MS analysis of background proteolysis, C-termini are present equal roughly equal amounts in both samples and thus appear as heavy-isotope- and light-isotope-labeled peptides, whereas the targeted protease-generated C-termini appear exclusively in the heavy labeled form. **b** and **c** Representation of N- and C-terminal peptide enrichment strategies used in TAILS and C-TAILS. To enrich N-terminal peptides, protein amine groups are protected via dimethyl labeling (represented by *blue triangles*). After trypsin digestion, internal tryptic peptides are coupled to a polymer via their free N-terminus. The original protein N-terminal peptides remain unbound and after separation by ultrafiltration are analyzed by LC-MS/MS. To enrich C-terminal peptides, protein amine groups are protected and simultaneously dimethyl-labeled (represented by *blue triangles*). Then, carboxyl groups are chemically protected (represented by *red circles*). After trypsin digestion, peptide N-termini are again protected and simultaneously dimethyl-labeled (represented by *blue triangles*). N-terminal and internal tryptic peptides are coupled to a polymer via their free C-terminus. The original protein C-terminal peptides remain unbound, and after separation by ultrafiltration are analyzed by LC-MS/MS. (Adapted from [69, 71])

Stable isotope dimethyl labeling applications in interaction proteomics

Rather than individual proteins themselves, protein complexes are the functional units of the cell. Indeed, signal transduction and cellular pathways require protein–protein interactions (PPIs) and the assemblies of proteins into large protein complexes. Therefore, mapping of PPIs and complex components is essential to understand the regulatory mechanisms underlying biological processes. High-throughput yeast two-hybrid screens [73] and affinity purification followed by MS [74] are two major techniques used to study PPIs. A major challenge in using affinity purification for interaction studies is that it is difficult to control/diminish false-positive interactions coming from the complex cellular background, regardless of the bait protein or protocol used. It has been shown that quantitative proteomics can assist in overcoming this problem by performing pull-down and control pull-down experiments in parallel and using incorporation of different stable isotopes to distinguish background proteins (equally abundant in both experiments) from specific interaction partners (absent in the control or at least twofold more abundant in the pull-down). Among the different quantitative proteomics strategies demonstrated in PPI studies, dimethyl labeling has proven to be quite successful in the mapping of PPIs as well as protein–nucleic acid and protein–drug interactions [75–80].

One of the most useful applications of quantitative affinity purification–MS has been the global elucidation of protein interaction networks (PINs) present in a cell or organism [81]. Although, dimethyl labeling is still a relative newcomer in the field, it has been demonstrated as a tool to construct PINs in methicillin-resistant *Staphylococcus aureus* (MRSA-252) [77]. A total of 406 MRSA proteins were cloned and expressed as glutathione *S*-transferase (GST)-fusion proteins and used as bait in high-throughput pull-down experiments, with each pull-down having an internal negative pull-down represented by the GST control. Dimethyl labeling was incorporated into each pull-down pair to distinguish specific interactors from the nonspecific background by quantifying the amount of interactor bound to the GST-fused bait protein relative to the amount in the control GST-alone pull-down. Interactors with at least a twofold enrichment were considered bona fide. In total, 13,219 pairwise interactions involving 608 MRSA proteins were reconstructed into an MRSA PIN, which can serve as an important basis for investigation of *S. aureus* biology and potential functions for previously uncharacterized proteins. Several proteins were identified as central hubs in the PIN, indicating that they may be essential for

network integrity and stability, and thus essential and critical for MRSA viability and growth. The authors also suggested that some of the highly connected proteins can become prospective antimicrobial drug targets. Incorporating dimethyl labeling can be used to quantitatively assess the validity of the interaction and thus also pinpoint false-positive interactions, which is extremely difficult for the classic yeast two-hybrid screen approach.

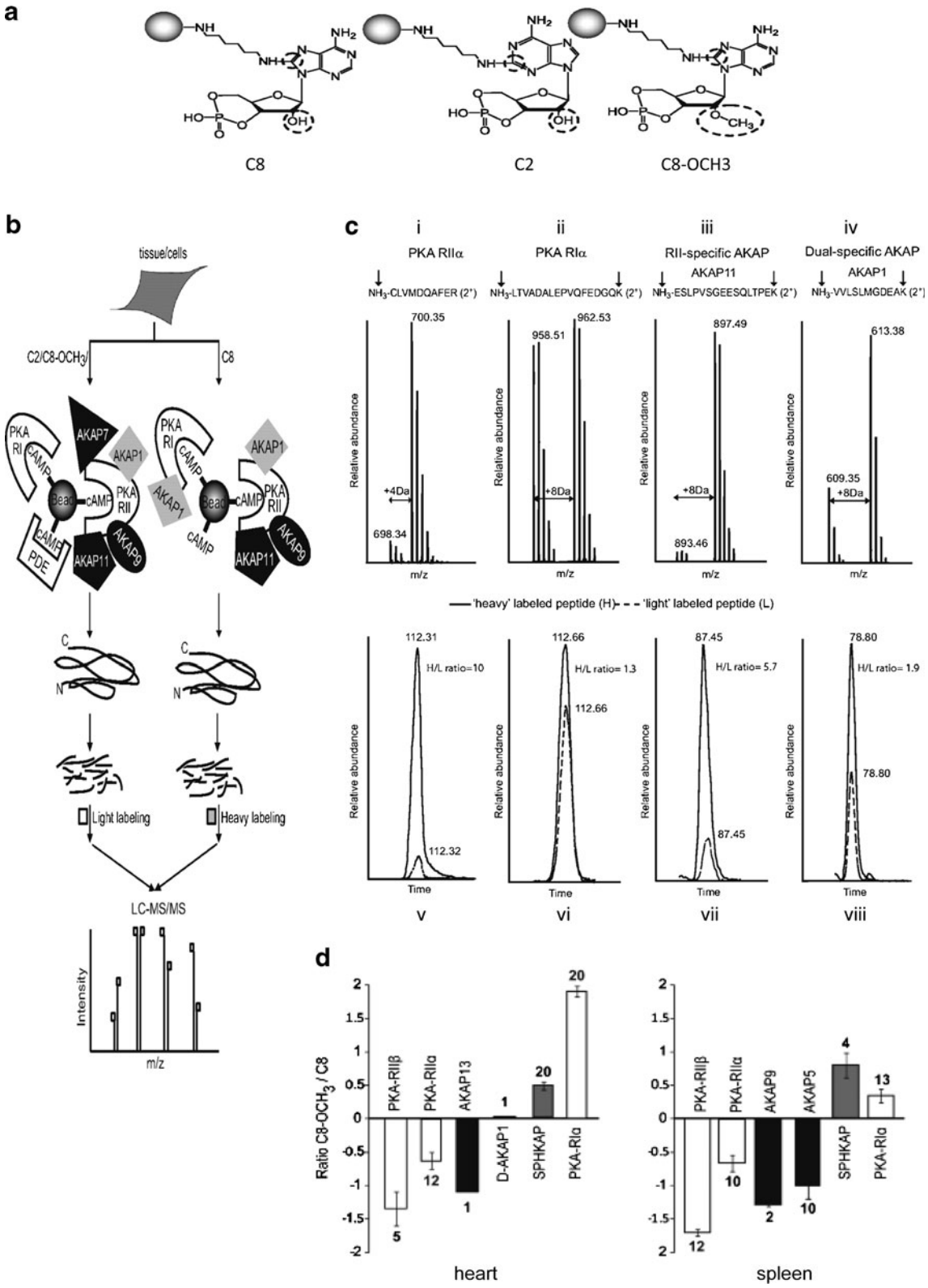
The dimethyl labeling approach outlined above to study PINs can be used to study any protein interaction as long as a bait and a proper control are available. Cheng et al. [76] developed what they termed a quantitative nanoproteomics approach for charting protein complexes (QnanoPX) using gold nanoparticles as a probe for affinity purification. The approach was applied to globally map the transcriptional activation complex of estrogen response element (ERE) in MCF-7 cells. ERE is regulated by the estrogen receptor. When activated by 17β-estradiol (E2), the estrogen receptor is translocated to the nucleus, where it binds to ERE, and recruits other proteins to the complex to subsequently promote transcription of the target genes. To identify ER–ERE complex components, gold nanoparticle probes were functionalized with consensus sequences of 13-bp ERE (positive probes) and control negative probes were functionalized without ERE. The pull-downs by the negative and the positive probes were differentially labeled to distinguish genuine ER–ERE complex components from nonspecific binding proteins. The same strategy was then extended to investigate E2-induced changes in protein expression after 24 h E2 treatment [76]. A pull-down in cells without E2 treatment was used as a control. Among the proteins affected by E2 treatment, they detected several proteins involved in transcriptional regulation by ER, for instance, c-Myc, suggesting it may play a significant role in E2-mediated transcription, which was further substantiated by western blot data. Both of these studies show powerful applications of dimethyl labeling to unravel biological pathways by tackling the generic problems of nonspecific binding and false positives in protein interaction studies.

Stable isotope dimethyl labeling can also be incorporated in the interaction study of small immobilized molecules with proteins from a lysate to screen for drug targets and off-target binding, often referred to as chemical proteomics [82]. We were interested in probing the interactome of cyclic GMP specific phosphodiesterase 5 (PDE5) inhibitors that resemble sildenafil and vardenafil. A quantitative chemical proteomics approach was performed to investigate whether proteins other than the clinically known target PDE5 could also interact with these drug analogues [78]. We found that only three proteins displayed selective affinity for the PDE5 inhibitor, of which two were isoforms of PDE5, and only one new binder was observed, namely, prenyl-binding protein (PrBP). Follow-up experiments confirmed the specific interaction between the PDE5 inhibitor analogues and prenyl-binding protein, albeit in the low micromolar range, much weaker than the interaction between the drug and PDE5, which is in lower nanomolar range [78]. This approach was also used to assess the relative binding of chemically slightly different PDE5 inhibitors. Their selectivity profiles were determined by differences in abundances of the proteins retrieved by PDE5 inhibitor pull-downs. Our data indicated that slight chemical modifications of the analogues can bias the inhibitor selectivity from PDE5 toward other interacting proteins [80].

Over the past few years we have put significant effort into establishing a cyclic AMP (cAMP)-based quantitative chemical proteomics strategy that combines cAMP-based affinity chromatography with dimethyl labeling and MS to study cAMP signaling pathway [36, 75, 83]. cAMP regulates cellular functions primarily by activating cAMP-dependent protein kinase (PKA), which itself is compartmentalized by A-kinase anchoring proteins (AKAPs) to ensure specificity of cAMP signaling events in space and time [83, 84]. The affinities of PKA for binding to AKAPs differ significantly for the two main classes of PKA-R isoforms, PKA-RI and PKA-RII; therefore, the AKAP family can be roughly divided into three groups on the basis of which the PKA-R subunits bind. Our first attempt combined cAMP-based chemical proteomics with

Fig. 6 Overview of a chemical proteomics approach to unravel cyclic AMP (*cAMP*)-dependent protein kinase (*PKA*) isoform–A-kinase anchoring protein (*AKAP*) specificity in combination with stable isotope dimethyl labeling. **a** Chemical structures of the three immobilized cAMP analogues coupled to agarose beads. **b** Experimental strategy to probe selective enrichment of PKA isoforms and their interacting partners using stable isotope dimethyl labeling. Following the parallel cAMP pull-down using C8 and C2 beads (and also C8-OCH_3 and control EtOH beads), proteins were digested in solution and peptides originating from the pull-down using the C8 beads were labeled with a heavy isotope, whereas those originating from C2 beads (and also C8-OCH_3 and EtOH beads) were labeled with a light isotope in dimethyl labeling. Each set of heavy-isotope- and light-isotope-labeled samples was mixed in a 1:1 ratio and then analyzed by LC-MS/MS for protein identification and quantification. **c** The actual mass spectra of peptide pairs from PKA-R isoforms observed following differential pull-downs using C8 and C8-OCH_3 together with the mass spectra from identified AKAPs. Each detected peptide exists as a pair with a typical 4-Da mass difference (2-Da*m/z* difference) for doubly charged peptides that have no lysine residues, whereas an 8-Da mass difference (4-Da*m/z* difference) is observed for doubly charged peptides with one lysine residue (*plots i–iv*). Plots *v–viii* show extracted ion chromatograms of the peptides. The heavy-isotope-labeled peptides (peptides which are enriched by C8) are represented by a *solid line* and light-isotope-labeled peptides (peptides which are enriched by C8-OCH_3) are represented by a *dashed line*. Each individual peptide pair is used for the assessment of differential binding affinities. *d* Our cAMP-based approach reveals that SPHKAP is a PKA-RI-specific AKAP. Note that in this experiment, peptides from C8-OCH_3 pull-down were labeled with heavy isotopes and peptides from C8 pull-down were labeled with light isotopes. The 2 log C8-OCH3/C8 ratios obtained for each PKA isoform and all pulled-down AKAPs are shown in pull-down in rat heart and pull-down in rat spleen. SPHKAP follows the PKA-RI ratio, indicating its preference for PKA-RI. *PDE* phosphodiesterase. (Adapted from [75, 79])

dimethyl labeling to study and compare the properties of three differently immobilized cAMP analogues, termed C2, C8, and C8-OCH_3 (Fig. 6a). These agarose beads were used for enrichment, isolation, and detection of PKA and AKAPs directly from a crude lysate of cells or tissue (Fig. 6b). We found that both PKA-R isoforms were captured equally well by the



C8 and C2 beads as the dimethyl ratios were close to 1. More interestingly, for the PKA-RI isoform, the C8 to C8-OCH_3 ratio was close to 1, but for PKA-RII this ratio was on average 5, indicating that C8 beads have substantially higher affinity for PKA-RII than C8-OCH_3 beads (Fig. 6c). These PKA-RI/PKA-RII ratios are also reflected in the observed ratios for the AKAPs binding to the R subunits (Fig. 6c). The data indicated that by combining dimethyl labeling with C8 and C8-OCH_3 pull-downs, the differences in PKA-R isoform affinity of the two analogues can be used as a discriminating factor to determine PKA-R/AKAP binding specificity. When applying this strategy to rat heart and spleen tissue, we deduced the specificity of SPHKAP, a novel AKAP. This revealed its unique preference for PKA-RI (Fig. 6d; C8-OCH_3/C8 ratios follow those of PKA-RI), which was later confirmed by in vitro data. These data led to the identification of the first PKA-RI-specific AKAP ever found in mammals [79, 85].

General conclusions

Presently, there are many alternative ways to perform quantitative proteomics. The most robust forms require the use of stable isotope labeling. These stable isotopes can be incorporated either metabolically using popular methods such as ^{15}N labeling [86] or SILAC [7, 8] or chemically using, for instance, ICAT [10], iTRAQ/tandem mass tags [11, 12], or ^{18}O labeling [87, 88]. To this toolbox, stable isotope dimethyl labeling has recently been added. Although it is clear that all these different methods have their strengths and weaknesses, the applications described in this review clearly show that stable isotope dimethyl labeling provides a very versatile method. The weakness of, but not unique to, stable isotope dimethyl labeling is that it cannot be easily multiplexed beyond four channels. Moreover, as quantification is based on MS spectra, and not on MS/MS fragments as in iTRAQ/tandem mass tags, overlapping ion signals originating from other peptides with similar masses can hamper quantification. In summary, the strengths of stable isotope dimethyl labeling are that its low cost and high efficiency allow application on both large (milligram) and also small (submicrogram) amounts of protein input. In addition, it can be used in both off-line and online methods and it can achieve equal depth in protein quantitation as the alternative methods mentioned above. Another advantage of this chemical labeling strategy is that it can be applied to any sample, including those originating from cells, tissue, body fluids, and whole organisms. Therefore, we think the future of stable isotope dimethyl labeling in quantitative proteomics is bright.

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