Received: 8 November 2021 | Revised: 12 January 2022 | Accepted: 13 January 2022

DOI: 10.1002/jms.4812

RESEARCH ARTICLE

Journal of MASS SPECTROMETRY WILEY

Clickable report tags for identification of modified peptides by mass spectrometry

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Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: SFB1309; Fonds der Chemischen Industrie

Abstract

The identification and quantification of modified peptides are critical for the functional characterization of post-translational protein modifications (PTMs) to elucidate their biological function. Nowadays, quantitative mass spectrometry coupled with various bioinformatic pipelines has been successfully used for the determination of a wide range of PTMs. However, direct characterization of low abundant protein PTMs in bottom-up proteomic workflow remains challenging. Here, we present the synthesis and evaluation of tandem mass spectrometry tags (TMT) which are introduced via click-chemistry into peptides bearing alkyne handles. The fragmentation properties of the two mass tags were validated and used for screening in a model system and analysis of AMPylated proteins. The presented tags provide a valuable tool for diagnostic peak generation to increase confidence in the identification of modified peptides and potentially for direct peptide-PTM quantification from various experimental conditions.

KEYWORDS
AMPylation, chemical proteomics, MS-tags, protein post-translational modifications, reporter ions

1 | INTRODUCTION

Post-translational protein modifications play a critical role in many cellular functions.[1] This creates numerous PTM proteins or so-called proteoforms, which largely exceed the number of encoded genes and generates an extraordinary diversity of protein properties.[2] However, techniques to confidently quantify and identify the site of modification are missing. This is, in particular, a challenging issue for low abundant and unstable PTMs such as AMPylation.[3–8] Although the number of available linkers for enrichment complemented by various chemical proteomic approaches is quite large, there is a vacancy of the linker that would yield a reporter ion upon MS/MS fragmentation and thus improve the site identification rates.[9–11] So far, isobaric labeling has been mainly used for protein quantification in bottom-up proteomics.[12] The large-scale employment of mass spectrometry-based proteomics has taken off. The isobaric strategies have allowed for the multiplication of the sample's measurement to minimize the measurement time while providing precise quantification of proteins prepared under different conditions. In parallel, chemical proteomic strategies have been utilized for the identification of PTM proteins using small compound PTM analogs containing an alkyne handle that allows the downstream enrichment of the modified proteins.[13–18] It remained challenging to quantify and compare PTM stoichiometry between conditions because modified and unmodified peptides displayed

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different ionization properties. Furthermore, different total protein amounts, as well as shifted retention times during LC separation, contribute to the abovementioned problems.[19,20] Here, we report the synthesis of two MS-tags conjugated to alkyne-modified proteins via click chemistry, which produce a reporter ion upon fragmentation in proof-of-concept experiments. The reporter ions are then used to improve the identification of the modified peptides by the search algorithms.

2 | RESULTS

Based on the structure of commercially available TMT-tag and the recently reported sulfoxide-containing MS-tag, we have designed and synthesized two novel MS-tags.[21,22] The presented 2,6-dimethylpiperidine-based (DMP) and sulfoxide-containing (SOX) tags contain azido group for bioorthogonal Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) with alkyne-modified peptides or proteins. Thus, the two new MS-tags enable selective labeling of modified peptides for MS analysis, in contrast to the original TMT-tag reagent bearing an *N*-hydroxysuccinimide ester (NHS) group to react with all available primary amines within the protein sample (Figure 1).[18]

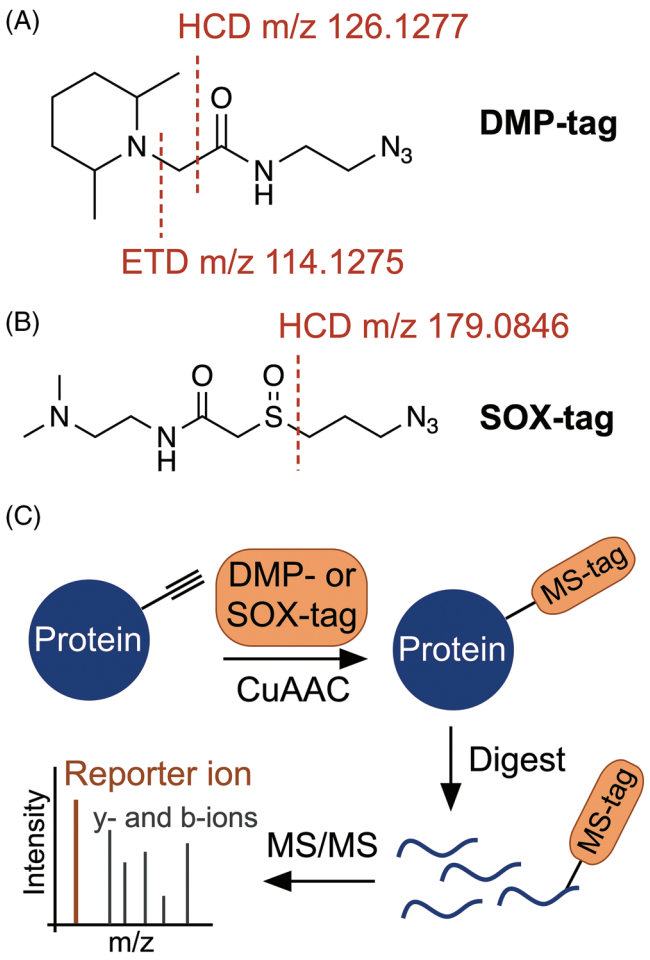

FIGURE 1 (A,B) Structure of the DMP- and SOX-tag with characteristic reporter ion masses. (C) Overall strategy to identify modified peptides in complex samples using the DMP- and SOX-tag

The study has been initiated by the synthesis of two new MS-tags (Figure 2). First, the DMP-tag containing 2,6-dimethylpiperidine was prepared from the carboxylic acid derivative **1** by HATU catalyzed amide coupling with 2-azidoethylamine, yielding after 3 days the desired DMP-tag at a 63% yield. Next, the synthesis of the sulfoxide-based tag was carried out in a total of five steps. In brief, the synthesis starts with nucleophilic substitution of ethyl bromoacetate with 3-mercaptopropanol, followed by activation of the hydroxyl group by tosylation (**2**) and subsequent conversion to azide **3** to equip the linker of the MS-tag with moiety suitable for click chemistry. Even though hydrolysis of the ester was a side reaction during nucleophilic substitution, obtained acid **3** was used in 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and hydroxybenzotriazole (HOBt) catalyzed amide coupling giving compound **4**, which was oxidized by mCPBA to the final SOX-tag. Although all the steps provided moderate to good yields, the final oxidation showed a somewhat lower yield of 17% caused by side reactions and instability during purification. Both reagents DMP- and SOX-tags show good stability when stored as ready-to-use solutions in DMSO at −20°C.

To explore in detail the fragmentation properties of the DMP- and SOX-tags, we have established a model protein-PTM system in which the free thiol of the cysteine residue C58 of bovine serum albumin (BSA) was modified with the cysteine reactive probe IA-alkyne, which contains a terminal alkyne (Figure 3). The alkyne decorated BSA resembles the protein PTM isolated from cells treated with an alkyne-containing probe, which is common in chemical proteomic workflows that aim to map protein PTMs.[15,18,23–25] The alkyne-modified BSA was further decorated with either DMP- or SOX-tag using the CuAAC. Next, the BSA was proteolytically cleaved by chymotrypsin, and the resulting peptide mixtures were desalted on

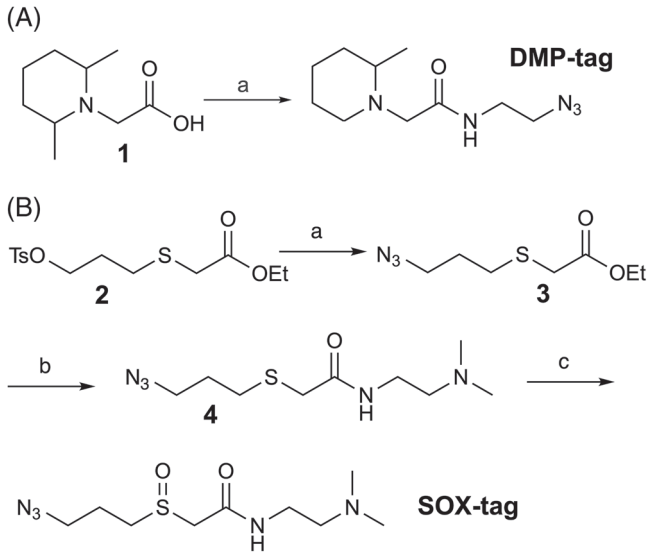

FIGURE 2 (A) Synthetic approach to DMP-tag (a) 2-azidoethan-1-amine, EDC, HOBt, DIPEA, DMF, r.t., 72 h, 63%. (B) Synthesis of the SOX-tag. (a) NaN_3, EtOH, 95°C, 18 h, 60%. (b) N^1,N^1-dimethylethane-1,2-diamine, EDC, HOBt, DIPEA, DCM, r.t., 18 h, 55%. (c) mCPBA, H_2O, r.t., 1.5 h, 17%

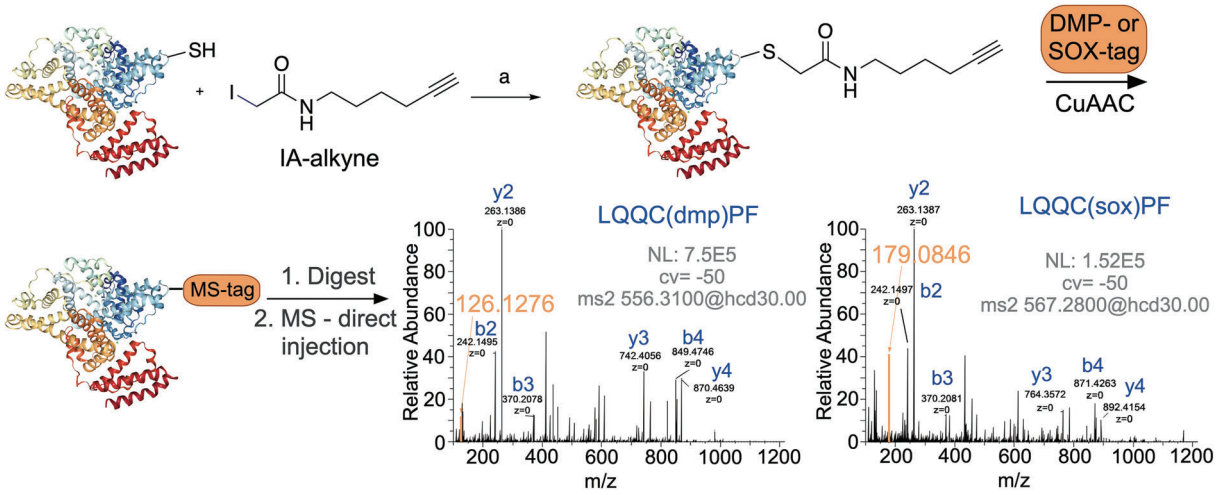

FIGURE 3 Proof-of-concept experiments with the BSA model system and the DMP- and SOX-tags

C18 columns and analyzed by direct injection into the Orbitrap Eclipse Tribrid mass spectrometer with high-field asymmetric waveform ion mobility spectrometry (FAIMS) introduced between the ion source and the Eclipse mass spectrometer.[26] We have started with an analysis of the DMP-tag modified BSA and the resulting DMP-tagged peptide LQQC (dmp)PF and its missed cleavage peptide LQQC (dmp) DEHVKLVNELTEF where dmp refers to the attached DMP-tag. Stepwise optimization of the FAIMs compensation voltage (CV) provided suitable conditions at −50 CV to acquire the MS1 spectra and select the target ions for the MS^2 experiment. To identify the suitable condition for the generation of the MS^2 spectra, with particular focus on the intensity of the reporter ion at 126.1277 m/z, resulting from the fragmentation of the DMP-tag, the higher-energy C-trap dissociation (HCD) energy has been gradually increased. Optimization has revealed that the most effective cleavage occurs at 30 V. A complementary set of experiments has been performed with the electron-transfer dissociation (ETD) fragmentation technique, showing a somewhat lower intensity of the corresponding reporter ion at 114.1275 m/z (Figure S1). In parallel, the fragmentation properties of the SOX-tag were assessed to show the anticipated reporter ion at 179.0846 m/z using HCD fragmentation. However, it surprisingly produced a complementary reporter ion at 131.1178 m/z as a major fragment upon ETD fragmentation (Figure S2). For both the DMP-tag and SOX-tag, better fragmentation was observed for species with higher charge peptide precursor ions. Measurement of the negative control, the BSA peptides, which were not modified with the DMP-tag or SOX-tag, but only with IA-alkyne, confirmed the specificity of all reporter ions (Figure S3).

Having characterized the fragmentation properties of the DMP- and SOX-tag in our model system, we have continued to test the possibility of using these MS-tags for the identification of modified peptides on the whole proteome level. For this, HeLa cells lysate was treated with IA-alkyne and further reacted with the DMP- or SOX-tag using click chemistry. Subsequently, the labeled proteome was acetone precipitated, trypsin digested and measured by LC-MS/MS using the 2 h gradient with alternating FAIMS CV voltages between −50 and −70 V. The MS^2 has been acquired in the orbitrap, m/z range was adjusted to span from 110 to 1100 m/z and HCD fragmentation set to 30 V.[27] Next, MaxQuant searched the resulting spectra for the peptides modified with cysteine reactive probe and labeled with the DMP- or SOX-tag. The reporter ions were set up as diagnostic peaks. From the single run, MaxQuant identified an overall 10 802 peptides in the DMP-tag sample (Table S1). Among the total number of peptides, 3578 were modified with more than 99% of all MS^2 spectra containing the corresponding diagnostic peak of the DMP-tag (Figure 4A). In comparison, MSFragger search has found on average 18 673 peptides and 4783 modified peptides. SOX-label showed somewhat lower numbers with MaxQuant finding in total 14 601 peptides and 1992 modified peptides again with more than 99% containing the SOX-tag reporter ions, and again higher numbers resulted from the MSFragger search—17 380 peptides and 2916 modified peptides showing the efficiency of the offset search (Table S2). The average site identification probability for both tags with high-resolution MS^2 is over 99%. For comparison, the samples have been measured using the low-resolution ion trap MS^2 acquisition as well (Figure S4). This led to a significant increase in the total number of identified peptides but lower number of DMP-modified peptides (2325) and SOX-modified peptides (1550; both calculated with MaxQuant). This observation is in line with previously reported improvement of modified peptide identification rates by the high-resolution MS^2 spectra and demonstrates the feasibility of our approach compatible with a wide range of MS measurement setups.[28] Moreover, the identified DMP-modified peptides using the low-resolution MS^2 might contain interfering reporter ions at 126.0913 m/z from acetylated lysine.[29] Together, the application of the DMP- and SOX-tag with IA-alkyne in the proof-of-principle experiments show the high efficiency of the reporter ion release, which opens a way for relative quantification of modified peptides between various experimental conditions when used with isotopically labeled tags. However, the absolute quantification of modified peptides would need to be determined for each

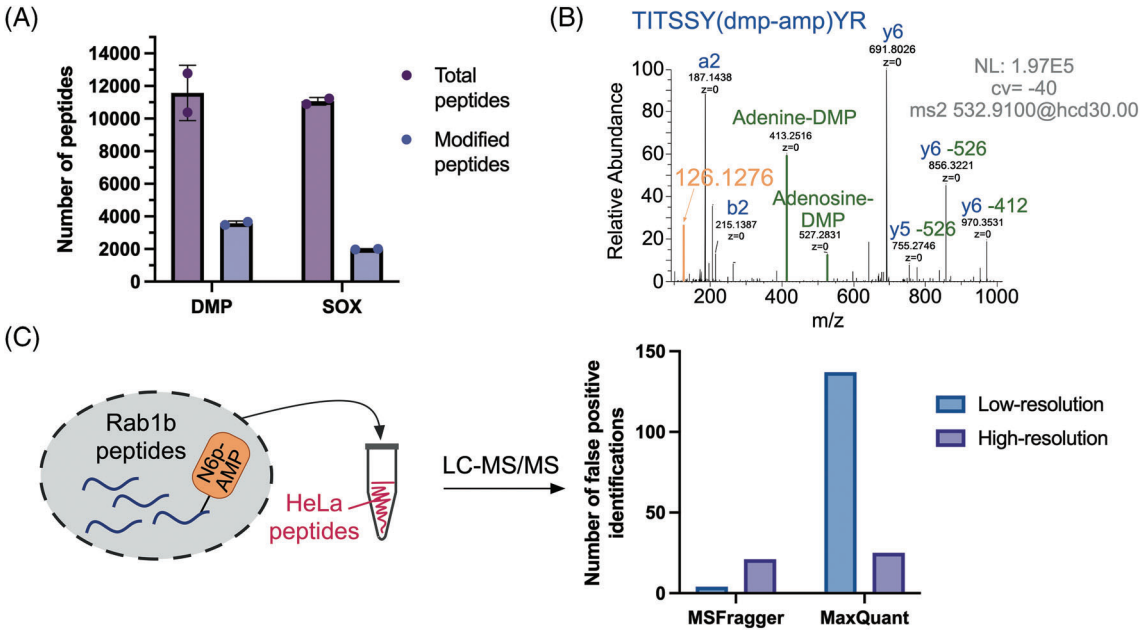

FIGURE 4 Analysis of cysteine DMP- and SOX-labeling on whole proteome level and Rab1b AMPylation with DMP-tag. (A) Total peptides and modified peptides found by MaxQuant in the DMP- and SOX-tag labeled cysteines using high-resolution MS^2 acquired in orbitrap. (B) Fragmentation properties of DMP-labeled AMPylated peptide from Rab1b. (C) Comparison of the MaxQuant and MSFragger search fidelity using the spiked in unnatural N^6-propargyl AMPylated peptide from Rab1b

application because of its dependence on the used chemical proteomic probe, its metabolic incorporation rate or reactivity and CuAAC efficiency.

Encouraged by the results, we focused on our better performing DMP-tag to study protein AMPylation.[30] First, in an in vitro reaction of the well-described pair of AMP-transferase DrrA and its substrate Rab1b with ATP or N^6-propargyl ATP the AMPylated Rab1b was prepared and characterized by intact protein MS (Figure S5 and S6).[31,32] Both, wt and N^6-propargyl modified proteins were then coupled with DMP-tag, reduced, alkylated and trypsinized. The resulting peptide mixture was analyzed by direct injection into the mass spectrometer. Of note, Rab1b is modified on Y77 with the tryptic peptide TITSSYYR, which makes the site identification in particular challenging because of six possible modification sites. The FAIMS compensation voltage optimization allowed us to select and enhance the intensity of the desired peptides for MS^2 experiments. The unmodified Rab1b peptide served as a control. Interestingly, the unmodified peptides were found only as double-charged peptides, whereas the AMPylated peptide was predominantly triple charged. The following fragmentation experiments corroborate previous reports and add additional insight on fragmentation properties.[3,4,33] The fragmentation of the control AMPylated TITSSYYR peptide by HCD provided all characteristic ions and neutral losses. These were also paralleled in the analysis of the N^6-propargyl AMP modified peptide. The measurement was repeated with the attached DMP-tag to explore the possibility to modulate fragmentation properties and improve the site identification rate of this unstable PTM. However, the DMP-AMP-peptide exhibits the same fragmentation properties, but as expected, it has yielded an additional reporter ion at 126.1276 m/z with HCD and a low intensity 114.1275 m/z reporter ion when ETD was used (Figures 4B and S7). The artificial Rab1b DMP-AMP-peptides were spiked in the HeLa whole proteome tryptic digest and analyzed via LC-MS/MS. Indeed, it was possible to identify the desired DMP-AMP-peptide from the Rab1b by MaxQuant and MSFragger. Of note, the score was improved when the neutral losses were defined, but it led to the incorrect localization of the modification on the peptide (Table S3). This could be due to the fact that the modified Rab1b peptide contains six potentially modified sites out of eight amino acids in total. The MS^2 acquired in the ion trap resulted in false-positive identifications in both MaxQuant and MSFragger. The MaxQuant search was set up to search for unnatural modified peptides (with N^6-propargyl AMP) but identified 137 modified peptides instead of one from Rab1b (Figure 4C). In comparison, the MSFragger search showed greater stringency by finding only four modified peptides, which were inherently incorrect but did not find the Rab1b peptide (Figure 4C). The high-resolution MS^2 acquired in the orbitrap has led to improvement of the MaxQuant search and in MSFragger to correct assignment of the modified Rab1b peptide (Figure 4C).

Our and others' previous attempts to search for AMPylated peptides in whole proteome tryptic digest proved to be challenging. In particular, Pieles et al have synthesized two adenosine analogs containing ^{15}N and ^{13}C stable isotopes, which were used for metabolic labeling of AMPylated proteins. Although it was possible to identify reporter ion clusters of labeled adenosines in in vitro activity assays, the search of the labeled peptides on a whole proteome has shown a rather low efficiency.[4] Therefore, in our study, we have decided to

use a commercial DMP-specific antibody to enrich the DMP-modified peptides.[34] In principle, the main advantage compared to other approaches is the possibility of enriching and selectively eluting only the modified peptides without the necessity to use additional chemical or enzymatic cleavage of the linker used for the enrichment. The possibility of enriching DMP-modified peptides was first tested on a model system with an IA-alkyne probe coupled with the DMP-tag. The anti-DMP antibody was applied on the peptide level and resulted in a two-fold increase of the DMP-modified peptides in the sample comparison to DMP-modified peptides without enrichment (Figure 5 and Table S4). The same approach was then applied to pro-N6pA treated cell lysates, which resulted in the labeling of DMP-AMP-modified peptides. Even though we have identified numerous AMPylated peptides, there was no overlap with previously found AMPylated proteins using complementary methods.[15,33] We hypothesize that this is mainly caused by challenging bioinformatic analysis, which has to deal with a complex mixture of ions after fragmentations. Although harnessing the potential of reporter ions and neutral losses presence might be a great advantage in future.

In summary, we have designed and synthesized two clickable MS-tags based on DMP- and SOX-moieties, which were evaluated using the single digested modified BSA protein and on whole proteome level with cysteine reactive IA-alkyne. Further on, we have applied the DMP-tag in the analysis of protein AMPylation and attempted enrichment of the AMPylated peptides using the DMP-specific antibody. This study extends the repertoire of available MS-linkers, opens the possibility to further develop isotopically labeled derivatives of DMP- and SOX-tags for quantification of PTM-peptides obtained from different cell types or stress conditions. Moreover, we have generated a high-quality MS spectra resource for optimization of the PTM search algorithms, which is freely available to the community.

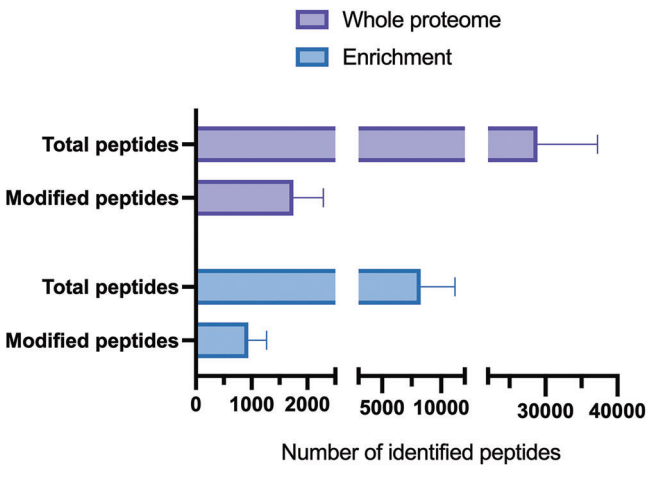

FIGURE 5 DMP-IA-alkyne-modified peptides identification rate in the whole proteome and after the enrichment using the DMP-specific antibody

ACKNOWLEDGMENTS

This research was supported by the Liebig fellowship from Fonds der Chemischen Industrie to P.K. and T.B., LMUexcellent Junior Fund to P.K. and Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – SFB 1309.

AUTHOR CONTRIBUTION

P.K. conceived the study, assisted with MS measurements, and wrote the manuscript. D.M. carried out the synthesis of the tags and participated in MS measurements. A.T. performed MS samples preparation and MS measurements. T.B. carried out the affinity enrichment of AMP-DMP modified peptides. M.-K.W. overexpressed the DrrA and Rab1b and performed in vitro AMPylation. All authors have revised the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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How to cite this article: Makarov D, Telek A, Becker T, von Wrisberg M-K, Schneider S, Kielkowski P. Clickable report tags for identification of modified peptides by mass spectrometry. *J Mass Spectrom*. 2022;57(3):e4812. doi:10.1002/jms.4812