Proteomic Analyses Reveal Divergent Ubiquitylation Site Patterns in Murine Tissues*⑤

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Posttranslational modifications of proteins increase the complexity of the cellular proteome and enable rapid regulation of protein functions in response to environmental changes. Protein ubiquitylation is a central regulatory posttranslational modification that controls numerous biological processes including proteasomal degradation of proteins, DNA damage repair and innate immune responses. Here we combine high-resolution mass spectrometry with single-step immunoenrichment of di-glycine modified peptides for mapping of endogenous putative ubiquitylation sites in murine tissues. We identify more than 20,000 unique ubiquitylation sites on proteins involved in diverse biological processes. Our data reveals that ubiquitylation regulates core signaling pathways common for each of the studied tissues. In addition, we discover that ubiquitylation regulates tissue-specific signaling networks. Many tissue-specific ubiquitylation sites were obtained from brain highlighting the complexity and unique physiology of this organ. We further demonstrate that different di-glycine-lysine-specific monoclonal antibodies exhibit sequence preferences, and that their complementary use increases the depth of ubiquitylation site analysis, thereby providing a more unbiased view of protein ubiquitylation. *Molecular & Cellular Proteomics 11: 10.1074/mcp.M112.017905, 1578–1585, 2012.*

Ubiquitin is a small 76-amino-acid protein that is conjugated to the ε-amino group of lysines in a highly orchestrated enzymatic cascade involving ubiquitin activating (E1), ubiquitin conjugating (E2), and ubiquitin ligase (E3) enzymes (1). Ubiquitylation is involved in the regulation of diverse cellular processes including protein degradation (2, 3, 4), DNA damage repair (5, 6), DNA replication (7), cell surface receptor endocytosis, and innate immune signaling (8, 9). Deregulation of protein ubiquitylation is implicated in the development of cancer and neurodegenerative diseases (10, 11). Inhibitors targeting the ubiquitin proteasome system are used in the treatment of hematologic malignancies such as multiple myeloma (12, 13).

Recent developments in the mass spectrometry (MS)-based proteomics have greatly expedited proteome-wide analysis of posttranslational modifications (PTMs) (14–17). Large-scale mapping of ubiquitylation sites by mass spectrometry is based on the identification of the di-glycine remnant that results from trypsin digestion of ubiquitylated proteins and remains attached to ubiquitylated lysines (18). Recently, two monoclonal antibodies were developed that specifically recognize di-glycine remnant modified peptides enabling their efficient enrichment from complex peptide mixtures (19, 20). These antibodies have been used to identify thousands of endogenous ubiquitylation sites in human cells, and to quantify site-specific changes in ubiquitylation in response to different cellular perturbations (20–22). It should be noted that the di-glycine remnant is not specific for proteins modified by ubiquitin but also proteins modified by NEDD8 or ISG15 generate an identical di-glycine remnant on modified lysines making it impossible to distinguish between these modifications by mass spectrometry. However, expression of NEDD8 in mouse tissues was shown to be developmentally down-regulated (23), and ISG15 expression in bovine tissues is low in the absence of interferon stimulation (24). In cell culture experiments it was shown that a great majority of sites identified using di-glycine-lysine-specific antibodies stems from ubiquitylated peptides (20).

The rates of cell proliferation and protein turnover in mammals vary dramatically between different tissues. Immortalized cell lines, often derived from cancer, are selected for high proliferation rates and fail to represent the complex conditions in tissues. Tissue proteomics can help to gain a more comprehensive understanding of physiological processes in multicellular organisms. Analysis of tissue proteome and PTMs can provide important insights into tissue-specific processes and signaling networks that regulate these processes (25–32). In addition, development of mass spectrometric methods for analysis of PTMs in diseased tissues might lead to the identification of biomarkers.

In this study, we combined high-resolution mass spectrometry with immunoenrichment of di-glycine modified peptides to investigate endogenous ubiquitylation sites in murine tissues. We identified more than 20,000 ubiquitylation sites from five different murine tissues and report the largest ubiquitylation dataset obtained from mammalian tissues to date. Furthermore, we compared the performance of the two monoclonal di-glycine-lysine-specific antibodies available for enrichment of ubiquitylated peptides, and reveal their relative preferences for different amino acids flanking ubiquitylation sites.

EXPERIMENTAL PROCEDURES

Tissues—Mouse tissues were dissected from C57BL/6 mice, rinsed with PBS and frozen immediately in liquid nitrogen. The frozen tissue samples were cryogrinded using a Retsch MM 400 ball mill and lysed in modified radioimmunoprecipitation assay buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% sodium deoxycholate) supplemented with protease inhibitors (Complete protease inhibitor mixture tablets, Roche Diagnostics) and 5 mM N-ethylmaleimide (Sigma). Lysates were sonicated and cleared by centrifugation at $16000 \times g$.

MS Sample Preparation—Twenty milligrams of protein from tissue lysates were precipitated in acetone and subsequently re-dissolved in denaturation buffer (6 M urea, 2 M thiourea in 10 mM HEPES pH 8.0). Cysteines were reduced with 1 mM dithiothreitol and alkylated with 5.5 mM chloroacetamide (33). Proteins were digested with endoproteinase Lys-C (Wako Chemicals) and sequencing grade modified trypsin (Sigma) after four-fold dilution in water. Protease digestion was stopped by addition of trifluoroacetic acid and precipitates were removed by centrifugation. Peptides were purified using reversed-phase Sep-Pak C18 cartridges (Waters, Milford, MA), re-dissolved in immunoprecipitation buffer (10 mM sodium phosphate, 50 mM sodium chloride in 50 mM 3-(*N*-morpholino)propanesulfonic acid pH 7.2). Precipitates were removed by centrifugation. Subsequently the samples were split in two equal parts; one part was used to enrich modified peptides with 100 μg of purified di-glycine-lysine-specific GX41 monoclonal antibody (Lucerna). One hundred microliters of protein G-conjugated Sepharose (Invitrogen, Carlsbad, CA) was used to recover antibody-peptide complexes. In parallel, peptides were immunoprecipitated using 40 μl of di-glycine-lysine antibody resin provided in the Ubiquitin Remnant Motif Kit (Cell Signaling Technology, Danvers, MA). The Ubiquitin Remnant Motif Kit affinity resin is provided without any further details about the amount of antibody and the matrix used to couple it. Peptides were incubated with the antibodies for 4 h at 4 °C on a rotation wheel. The beads were washed three times in ice-cold immunoprecipitation buffer followed by three washes in water. Immunoenriched peptides were eluted with 0.15% trifluoroacetic acid in H_2O, fractionated in six fractions using micro-column-based strong-cation exchange chromatography (SCX) and desalted on reversed phase C18 StageTips as described previously (34, 35). The average enrichment of di-glycine-lysine in the peptide fractions was 35% (supplemental Fig. S1*A*). Interestingly, we observe only a small percentage of peptides with multiple di-glycine-lysines. Di-glycine-lysine containing peptides have a higher molecular weight and a lower hydropathicity compared with the unmodified peptides in the enriched peptide fractions (supplemental Fig. S1*B*).

MS Analysis—Peptide fractions were analyzed on a hybrid linear ion-trap Orbitrap (LTQ-Orbitrap Velos, Thermo Scientific) or quadrupole Orbitrap (Q-Exactive, Thermo Scientific) mass spectrometer equipped with a nanoflow HPLC system (Thermo Scientific) as described (36–38). Peptide samples were loaded onto C18 reversed

phase columns (15 cm length, 75 μm inner diameter) and eluted with a linear gradient from 8 to 40% acetonitrile containing 0.5% acetic acid in 3–4 h. The mass spectrometer was operated in data dependent mode, automatically switching between MS and MS2 acquisition. Survey full scan MS spectra (*m/z* 300–1200) were acquired in the Orbitrap. The 10 most intense ions were sequentially isolated and fragmented by higher-energy C-trap dissociation (HCD) (39). An ion selection threshold of 5000 counts was used. Peptides with unassigned charge states, as well as with charge state less than +3 were excluded from fragmentation. Fragment spectra were acquired in the Orbitrap mass analyzer. A lock mass ion from ambient air (*m/z* 445.120025) was used for internal calibration of measurements in the Orbitrap on LTQ-Orbitrap Velos mass spectrometers (40).

Peptide Identification and Computational Analysis—Raw data files were analyzed using MaxQuant (development version 1.2.6.20) (41). Parent ion and MS2 spectra were searched against a database containing 84,790 murine protein sequences obtained from the UniProtKB released in February 2012 using Andromeda search engine (42). Spectra were searched with a mass tolerance of 6 ppm in MS mode, 20 ppm in HCD MS2 mode, strict trypsin specificity and allowing up to three missed cleavage sites. Cysteine carbamidomethylation was searched as a fixed modification, whereas N-terminal protein acetylation, methionine oxidation, n-ethylmaleimide modification of cysteines, and di-glycine-lysine were searched as variable modifications. Di-glycine modified lysines were required to be located internally in the peptide sequence. Site localization probabilities were determined by MaxQuant using the PTM scoring algorithm as described previously (41, 43). The data set was filtered based on posterior error probability (PEP) to arrive at a false discovery rate of below 1% estimated using a target-decoy approach (44). Raw data will be made available on request. Statistical analysis was performed using the R software environment. Ubiquitylation sequence pattern analysis was performed using iceLogo (45). Gene Ontology biological process term enrichment analysis was performed using the DAVID bioinformatics resource (46). Properties of the enriched peptides were determined using the ProtParam tool from the Biopython software package (47).

RESULTS

To map endogenous ubiquitylation sites in murine tissues, we extracted brain, heart, kidney, liver, and skeletal muscle from 10-week-old male C57BL/6 mice. The corresponding tissues were pooled and lysed together to minimize inter-individual differences. Equal amounts ($\sim$20 mg) of proteins from each tissue were digested using trypsin and peptides containing di-glycine remnants were immunoenriched using two different di-glycine-specific monoclonal antibodies (19, 20). The peptides were further fractionated using micro-column-based strong-cation exchange chromatography and analyzed by liquid chromatography connected to a hybrid linear ion-trap Orbitrap (LTQ-Orbitrap Velos, Thermo Scientific) or quadrupole Orbitrap (Q-Exactive, Thermo Scientific) mass spectrometer, as described previously (36–38). Raw data were analyzed using MaxQuant and filtered to obtain a false discovery rate (FDR)[1] below 1% (41) (Fig. 1*A*).

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[1] The abbreviations used are: FDR, false discovery rate; GO, gene ontology; HCD, higher-energy C-trap dissociation; IPI, International Protein Index; PEP, posterior error probability; PTM, posttranslational modification; SCX, strong-cation exchange chromatography; UPS, ubiquitin proteasome system.
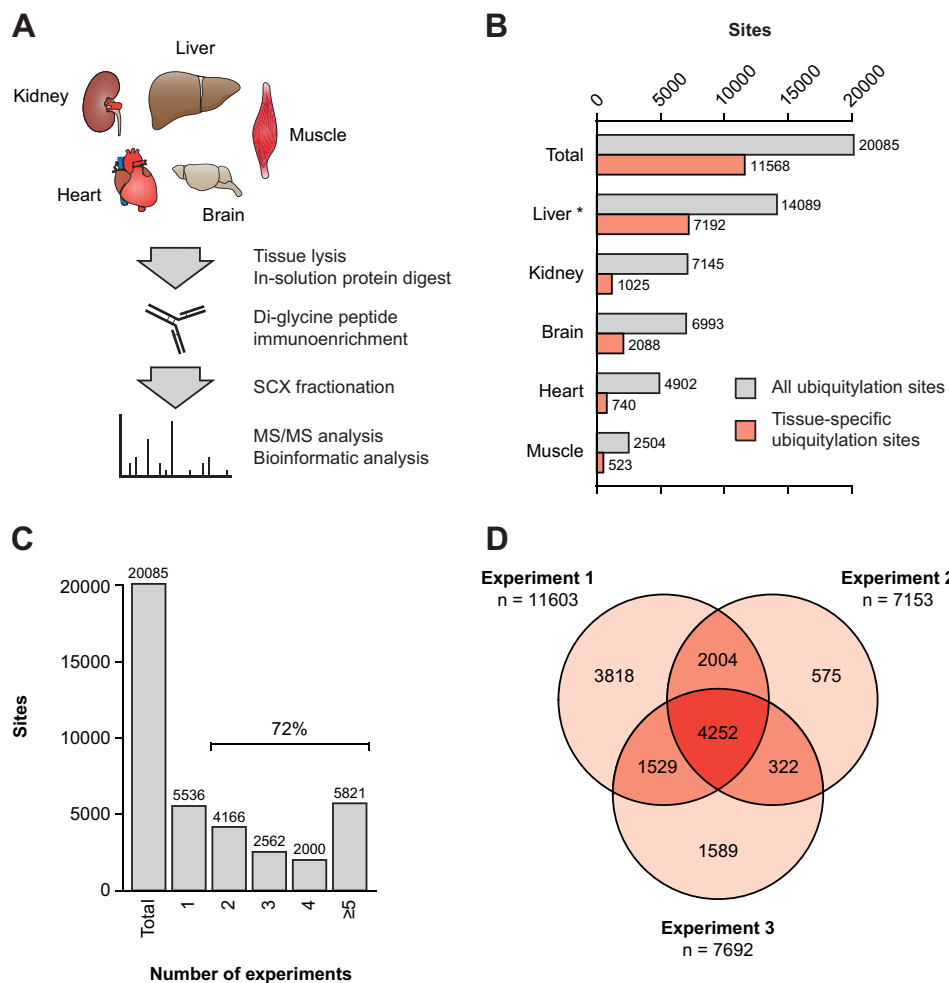
F<small>IG</small>. 1. **Identification of endogenous ubiquitylation sites in murine tissues by mass spectrometry.** *A*, Frozen tissues were cryogrinded and proteins were solubilized in modified radioimmunoprecipitation assay buffer. Proteins were digested using trypsin, and di-glycine modified peptides were enriched using two different di-glycine-lysine-specific monoclonal antibodies. Enriched peptides were fractionated using strong-cation exchange chromatography and analyzed by mass spectrometry. *B*, The bar chart illustrates the total number of ubiquitylation sites and the number of tissue-specific ubiquitylation sites identified in each tissue. The number of ubiquitylation sites identified from all tissues (except liver) is combined from two independent replicate experiments. The number of ubiquitylation sites identified from liver tissue is combined from three independent replicate experiments (indicated by an asterisk). *C*, Number of experiments in which individual sites were found. Over 70% of the reported sites were found in two or more experiments. *D*, Venn diagram illustrating the experimental overlap between the three replicate experiments performed from liver tissue.

Using the described workflow and two different di-glycine-specific antibodies, we identified more than 20,000 unique endogenous ubiquitylation sites that were mapped to a single lysine with localization probability >0.9 (Fig. 1*B* and supplemental Table S1). The largest number of sites was identified from liver (14,089 sites). In contrast, only 2504 sites were identified from skeletal muscle although the same amount of protein lysate was used in these experiments. About 58% of the reported ubiquitylation sites were identified only in one of the studied tissues, with liver contributing the largest fraction of unique sites (Fig. 1*B*). Notably, a relatively large number of tissue-specific ubiquitylation sites were found in brain. To generate a high quality data set, we performed at least two replicate experiments using both di-glycine-specific antibod-

ies for each tissue. Over 70% of the reported sites were identified in more than one experiment (Fig. 1*C*). In case of liver, we performed three replicate experiments and identified over 7000 di-glycine-lysine sites in each experiment with site overlap ranging from 67–92% (Fig. 1*D*).

To uncover biological processes associated with ubiquitylated proteins, and to compare ubiquitylation profiles between the examined tissues, we performed Gene Ontology (GO) biological process term enrichment analysis followed by hierarchical clustering (Fig. 2*A* and supplemental Table S2). Our analysis revealed an enrichment of similar terms in the case of skeletal muscle and heart. In muscle tissues, proteins involved in muscle contraction such as myosin family proteins and proteins involved in metabolic processes are ubiquity-
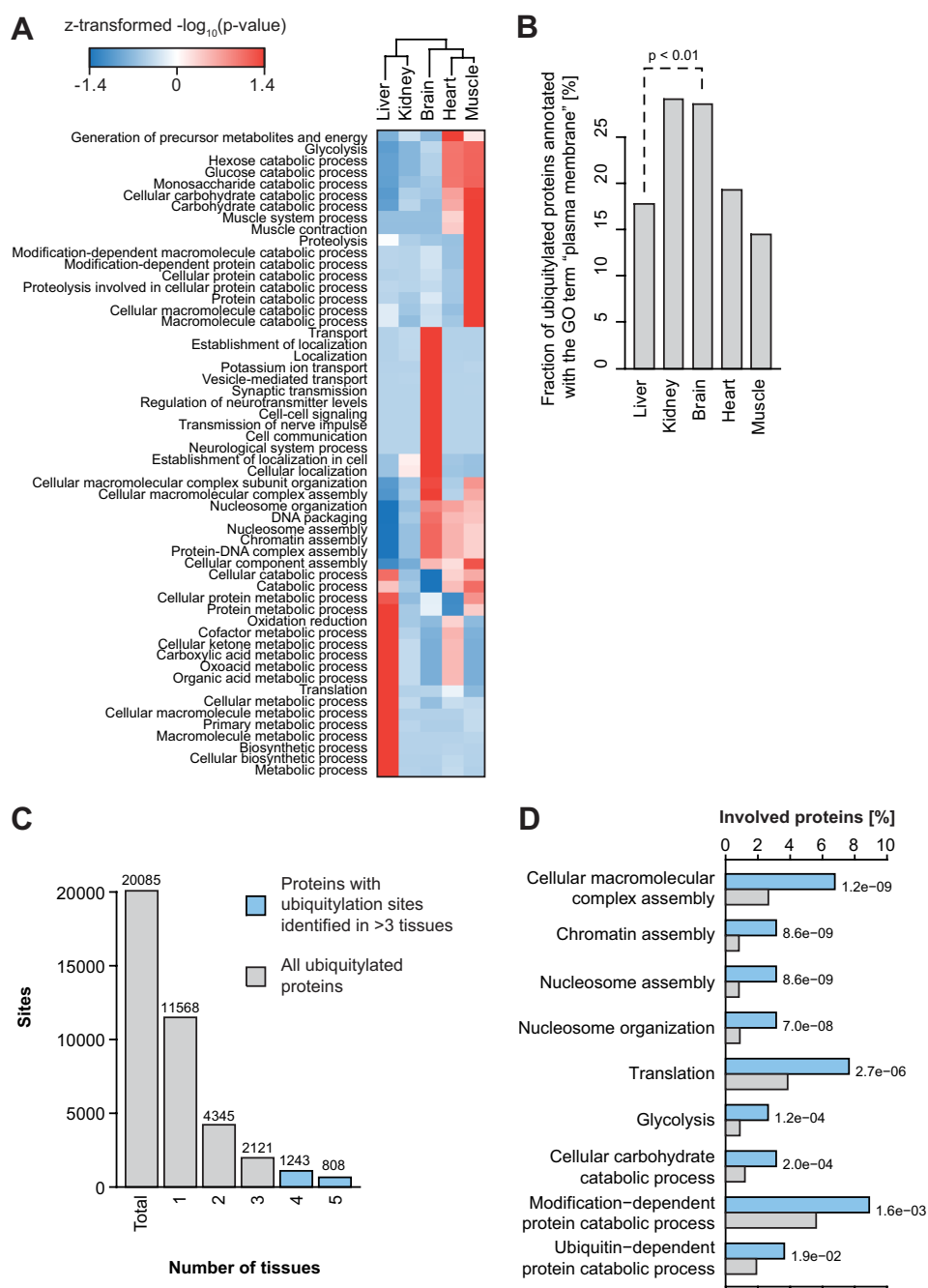
FIG. 2. **Ubiquitylated proteins participate in tissue-specific biological processes.** *A*, The enrichment of GO biological process terms among ubiquitylated proteins in different tissues was analyzed and illustrated as heat map. Only sites that were found in two or more replicate experiments were used for the analysis. The complete results of the analysis can be found in supplemental Table S2. *B*, The bar chart illustrates the fraction of ubiquitylation sites on plasma membrane proteins in different tissues. All proteins annotated with the GO cellular compartment term "plasma membrane" were considered as plasma membrane proteins. Fisher exact test was applied to determine the significance of the observed differences. *C*, The bar chart illustrates the number of ubiquitylation sites detected in one, two, three, four, or five tissues. Sites identified in at least four out of five tissues are indicated with light blue bars. *D*, GO biological process term enrichment analysis of proteins with ubiquitylation sites that were identified in at least four of the studied tissues.

lated. Brain showed a very distinct pattern with enrichment of brain-specific terms such as "synaptic transmission" and "regulation of neurotransmitter levels." In agreement with this, comparison of ubiquitylation sites identified in brain with sites identified in other tissues revealed that many ubiquitylation sites in brain are present on proteins residing in the plasma membrane such as neurotransmitter receptors and ion channels (Fig. 2*B*).
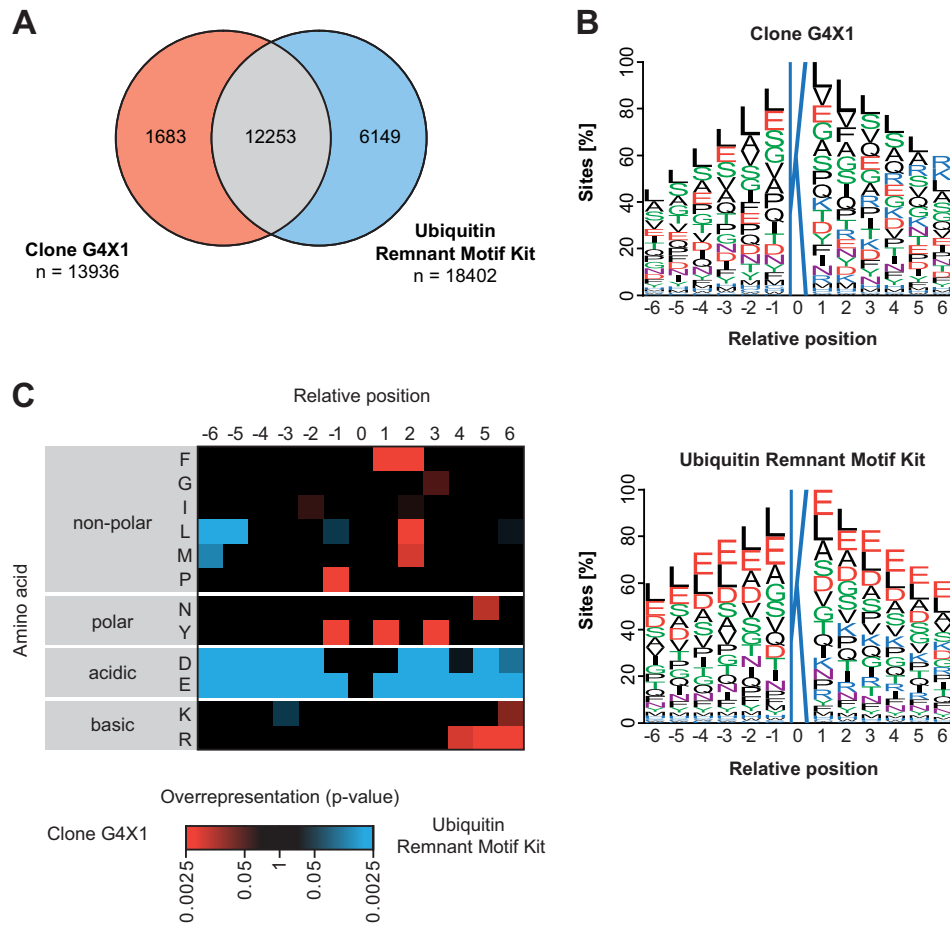
FIG. 3. **Sequence preferences of di-glycine-specific monoclonal antibodies.** *A*, Number of ubiquitylation sites identified with the two different antibodies. The red and blue area illustrates the ubiquitylation sites identified only with the monoclonal antibody clone GX41 or the Ubiquitin Remnant Motif Kit. *B*, Sequence motif analysis of ubiquitylation sites identified exclusively with the monoclonal antibody clone GX41 or the Ubiquitin Remnant Motif Kit, respectively. *C*, Amino acid preferences exhibited by the different di-glycine-lysine antibodies. The relative frequencies of amino acids flanking ubiquitylation sites identified with the two different antibodies were compared using IceLogo. Amino acids that were found overrepresented near di-glycine-lysines identified with the monoclonal antibody clone GX41 are colored in *red*. Amino acids overrepresented in proximity to ubiquitylation sites identified with the Ubiquitin Remnant Motif Kit are shown in *blue*. Only amino acids with significant overrepresentation are shown.

In contrast to tissue-specific sites, a distinct subset of ubiquitylation sites was commonly identified in multiple tissues (in at least four out of the five tissues analyzed) (Fig. 2*C*). To understand the cellular role of these sites and proteins, we performed GO biological process term enrichment analysis (Fig. 2*D*). Proteins with ubiquitylation sites that were identified in the majority of the tissues participate in ubiquitin-mediated proteasomal degradation, nucleosome organization, metabolic processes and translation. We identified all known sites on ubiquitin itself as well as sites on the ubiquitin-like modifiers NEDD8, SUMO2, and SUMO3. Interestingly, many components of the ubiquitylation machinery, such as E1, E2, and E3 enzymes and de-ubiquitylases, are extensively modified. In addition, many metabolic enzymes, heat-shock proteins, and ribosomal proteins are ubiquitylated in all examined tissues.

Ubiquitylation of histones has been implicated in the regulation of gene transcription and the response to DNA damage

(5, 48). We find ubiquitylation of all core histones in each tissue and many of the identified ubiquitylated lysines are known to be modified by other PTMs, including acetylation and methylation.

Recent proteomic studies have utilized two different monoclonal antibodies for the enrichment of ubiquitylated peptides from human cell lines (20, 21). These studies reported differences in the amino acid sequences flanking the ubiquitylation sites. However, it remained unclear whether these differences were attributable to sequence preferences of the antibodies, or to differences in sample processing workflow. To understand the basis of these results, we compared the two monoclonal antibodies side-by-side. We divided each peptide sample from the five tissues in two equal parts and performed the enrichment of di-glycine modified peptides separately with each antibody. Although the absolute numbers of ubiquitylation sites identified with the two antibodies are not directly

comparable because of differences in the antibody formulations (for details see Experimental procedures section), we find a large overlap of the identified sites indicating that both antibodies recognize di-glycine in a wide range of sequence contexts (Fig. 3*A*). Direct comparison of the ubiquitylation sites identified with the different antibodies revealed that each antibody enriched a distinct subset of ubiquitylation sites (Fig. 3*B*, 3*C,* and supplemental Fig. S1*C*). The monoclonal antibody clone GX41 (19) showed a significant preference for nonpolar amino acids including leucine and isoleucine as well as for tyrosine in positions next to the modified lysine. The rabbit monoclonal antibody available in the Ubiquitin Remnant Motif Kit (20) showed a preference for the acidic amino acids aspartic acid and glutamic acid surrounding di-glycine modified lysines (Fig. 3*C*). These data indicate that at least one of the available antibodies preferentially recognizes di-glycine modified lysines in specific sequence contexts.

DISCUSSION

In this study we demonstrate the enrichment of endogenous ubiquitylated peptides from murine tissue lysates and their subsequent identification by mass spectrometry. The presented data set contains more than 20,000 endogenous murine ubiquitylation sites and by far exceeds the number of previously known ubiquitylation sites in this important model organism.

Interestingly, our analysis reveals differences in the number of ubiquitylation sites identified in each of the studied tissues. The large number of sites identified from liver tissue might reflect the high rate of protein synthesis and degradation in hepatocytes. This is in accordance with previous reports indicating that hepatocytes contain high proteasome concentrations (3, 49). In contrast, the low number of sites identified in skeletal muscle lysates can possibly be explained by the high abundance of structural proteins in this tissue.

In addition to liver, we identified numerous unique ubiquitylation sites from brain tissue. Many of these sites are located on brain-specific plasma membrane proteins including neurotransmitter receptors and ion-channels, which is in agreement with studies demonstrating the high complexity of the brain proteome (25, 29, 50). Interestingly, GO biological process term analysis of proteins ubiquitylated in different tissues demonstrated that brain and kidney contained a large fraction of ubiquitylation sites on proteins annotated with the term "plasma membrane." The frequent occurrence of ubiquitylation on plasma membrane proteins might reflect the role of ubiquitylation in controlling the function of cell surface receptors in brain and kidney.

Our analysis suggests that in addition to tissue-specific expression of proteins, tissue-specific post-translational modifications of proteins by ubiquitylation may further increase the complexity of the proteome and contribute to the distinct phenotype of cells in multicellular organisms. However, given the vast abundance differences of proteins in different tissues, it is difficult to distinguish tissue-specific ubiquitylation from tissue-specific protein expression.

Notably, a subset of proteins is ubiquitylated in all studied tissues. These proteins include E1, E2 and E3 enzymes and de-ubiquitylases for which it remains unclear whether ubiquitylation is a site-specific regulatory process or occurs as bystander effect because of their involvement in the ubiquitylation machinery. In contrast, the site-specific ubiquitylation of histones is a well-known regulatory mechanism of gene expression and DNA damage response and our results highlight the relevance of these modifications in a physiological context (48).

We show that both monoclonal di-glycine-specific antibodies efficiently enrich di-glycine modified peptides from complex peptide mixtures and find a large overlap between the ubiquitylation sites identified with the two antibodies. However, direct comparison of ubiquitylation sites exclusively identified with one of the antibodies revealed relative preferences for specific amino acids flanking ubiquitylation sites. It remains difficult to define a valid ubiquitylation motif using these apparently biased enrichment strategies. Our results indicate that both antibodies are suited for efficient enrichment of di-glycine modified peptides in routine applications. Complementary use of both antibodies can in certain cases increase the depth of ubiquitylation site analysis and may help to obtain a more unbiased view of this modification.

Importantly, the presented workflow can be adapted to study changes of protein ubiquitylation in murine models of neurodegenerative and other diseases. Furthermore, the method is not limited to murine tissues, but can be applied to analyze ubiquitylation in tissues and primary cells from diverse organisms.

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