Global Lysine Crotonylation Profiling of Mouse Liver

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Lysine crotonylation (Kcr) is a recently discovered post-translational modification (PTM) originally identified in histones.[1] However, p300 has been reported to catalyze histone crotonylation in a medium, provided the original work is properly cited.

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Lysine crotonylation (Kcr) is a relatively new post-translational modification (PTM) originally identified in histones.[1] However, p300 has been reported to catalyze histone crotonylation in a medium, provided the original work is properly cited.

Lysine crotonylation (Kcr) is a recently discovered post-translational modification that potentially regulates multiple biological processes. With an objective to expand the available crotonylation datasets, LC-MS/MS is performed using mouse liver samples under normal physiological conditions to obtain in vivo crotonylome. A label-free strategy is used and 10 034 Class I (localization probabilities > 0.75) crotonylated sites are identified in 2245 proteins. The KcrE, KcrD, and EKcr motifs are significantly enriched in the crotonylated peptides. The identified crotonylated proteins are mostly enzymes and primarily located in the cytoplasm and nucleus. Functional enrichment analysis based on Gene Ontology and Kyoto Encyclopedia of Genes and Genomes shows that the crotonylated proteins are closely related to the purine-containing compound metabolic process, ribose phosphate metabolic process, carbon metabolism pathway, ribosome pathway, and a series of metabolism-associated biological processes. To the best of the authors’ knowledge, this research provides the first report on the mouse liver crotonylome. Furthermore, it offers additional evidence that crotonylation exists in non-histone proteins, and is likely involved in various biological processes. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium with the dataset identifiers PXD019145.

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The ProteomeXchange Consortium (PX) registered the dataset with the datasets identifiers PXD019145 (1 of 5)

Since the donor crotonyl-CoA is sensitive to various stimulations, Kcr is capable of transmitting environmental information to gene expression machinery.[2] Although Kcr is involved in many pathways that regulate diverse cellular functions, and the liver plays a key role in metabolic processes, the liver-associated in vivo Kcr datasets have not yet been reported. Therefore, in this study, we performed LC-MS/MS for global profiling of crotonylation in mouse liver under normal physiological conditions. To this end, we obtained the livers from nine C57BL/6 male mice, pooled them into three biological replicates (sample1, sample2, and sample3, n = 3 per sample), and performed label-free crotonylome analysis. The workflow for the study is presented in Figure 1A and the detailed methods are listed in the Supporting Information.

Raw MS/MS data were subjected to MaxQuant[19] analysis. For the label-free crotonylome, 57 735 (16.02%) out of 360 415 MS/MS spectra were matched to peptides. After excluding those from reversed or potential contaminant proteins, a total of 66 406 peptide-to-spectrum matches (PSMs) in 2945 proteins were identified at a 1% peptide FDR, and 44 383 PSMs (11 408 unique peptides with 11 066 crotonylated sites) in 2657 proteins were crotonylated (Figure 1B). According to the evaluation of the reversed database, the FDR of crotonylated sites was also below 0.75 for 2245 proteins.
1%. A total of 8544 (≈75%) crotonylated peptides (Figure 1C) and 8160 (≈74%) crotonylated sites (Figure 1D) were identified in all the three samples, indicating Kcr was relatively stable among each biological replicate. One spectrum example of crotonylated peptides with a mediocre score is shown in Figure S1, Supporting Information, demonstrating the existence of Kcr.

The characteristics of this label-free crotonylome are shown in Figure S2, Supporting Information. We determined the peptide length distribution (Figure S2A, Supporting Information), spectral count per crotonyl peptide (Figure S2B, Supporting Information), and Andromeda score (Figure S2C, Supporting Information). The number of crotonylated sites in one peptide varied from one to three (Figure S2D, Supporting Information). All identified peptides had near-zero distribution of mass error, and most were <2 ppm (Figure S2E, Supporting Information), suggesting the mass accuracy of the MS data for further analysis. Moreover, using the localization probability of 0 to 1 that assigned each quartile into a class, 99.5% of the Kcr sites could be grouped to Class I, with localization probabilities > 0.75 (Figure S2F, Supporting Information). For further analysis, 10 034 Class I quantifiable crotonylated sites in 2245 proteins that possessed the Kcr sites data in at least two biological replicates were selected. The intensity of each sample was divided by their corresponding median for normalization of the quantified Kcr sites using the Perseus (v.1.6.5.0) software. Next, the scatter plot, and density curve were constructed using log2(intensity), and the Pearson correlation coefficients (Figure S2G, Supporting Information) were estimated, which indicated a high degree of consistency between the biological replicates.

All peptides containing quantified Class I Kcr sites were submitted to MoMo modification motifs (http://meme-suite.org/tools/momo) for motif analysis using the motif-x algorithm, and the five motifs with the highest score were selected. Glutamate (E) and aspartate (D) residues, the two acidic amino acid residues, were identified as the most common residues adjacent to the crotonylated lysine (Figure 2A). Previous studies have shown that KcrE, KcrD, and EKcr motifs also exist conservatively in other species, suggesting that crotonylation recognizes specific amino acid features and might undergo strict regulatory processes.

Next, to obtain an overview of crotonylation in mice, the proteins containing quantified Class I Kcr sites were subjected to Ingenuity Pathway Analysis (QIAGEN Bioinformatics). It was observed that most of the crotonylated proteins were located in the cytoplasm and nucleus. However, proteins in the plasma membrane, extracellular space, and other locations also had Kcr modification, demonstrating that Kcr broadly exists among different types of proteins (Figure 2B). Furthermore, protein type classification revealed that many of the annotated crotonylated proteins were enzymes; however, transporters, peptidases,
transcription regulators, along with various other types of proteins were also found to be crotonylated, thereby providing additional evidence that Kcr exists among different protein types and may participate in several biological processes (Figure 2C).

To better understand the biological function of crotonylated proteins in mouse liver, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed for all proteins containing quantified Class I Kcr sites using ClusterProfiler (v3.12.0), R package. GO biological process analysis showed that the crotonylated proteins were enriched in the purine-containing compound metabolic process, ribose phosphate metabolic process, small molecule catabolic process, and a series of other metabolic processes (Figure 3A). Additionally, KEGG pathway analysis found that carbon metabolism and ribosome pathways were especially prominent for crotonylated proteins. Proteasome, peroxisome, and other metabolic pathways were also related to crotonylation (Figure 3B). Similarly, we found that ribosome, proteasome, and several metabolic pathways frequently appeared in other crotonylation-related studies with different species,[12,16,22] suggesting that crotonylation-regulated pathways are relatively conserved.

To assess the content of crotonylated peptides in this label-free dataset from multiple dimensions, we then performed four
additional database searches. First, we replaced crotonylation (K) with acetylation (K), a common acylation modification, as a variable modification using MaxQuant, which identified only 5633 peptides in 634 proteins as acetylated (Table S2, Supporting Information), supporting that the primary searches successfully identified crotonylation instead of acetylation. Next, we used MaxQuant and simultaneously set crotonylation (K), acetylation (K), propionylation (K), and butyrylation (K) as variable modifications. Among the 84 785 identified peptides in 3096 proteins, 44 847 peptides in 2704 proteins were recorded as crotonylated, which was much higher than the counts for the other three acylations (Table S3, Supporting Information). Then, we performed open searches using pFIND3 (EVA.3.0.11, 48.81% PSMs were crotonylated, results in Table S4, Supporting Information) [23] and MSFragger (v12.2. 33.01% PSMs were crotonylated, results in Figure S3 and Table S5, Supporting Information) [24] respectively. All the above four analysis results revealed that Kcr is the most frequent PTM in this dataset. The absolute count of the crotonylated peptides from all the search results confirmed that the crotonylated peptides existed in the liver crotonylome datasets; however, by some measures, it represents less than 50% of the total peptide content, indicating that the presence of Kcr should not be forced upon proteomics datasets with the assumption that all peptides are crotonylated.

Furthermore, three label-free raw files (without enrichment for modified peptides; unpublished) of the mouse under physiological conditions were used as a negative control, and the open search results showed a dominant Δ Da delta mass peak, but unapparent natural PTMs (Figure S4, Supporting Information). The relatively low crotonylation without enrichment indicated that the real abundance of Kcr is not very high in the liver and that the anti-crotonyl antibody is an effective tool to observe these events.

Compared to the whole proteome, peptides with PTMs have a low relative abundance. Special considerations in sample handling, data acquisition, and post-acquisition processing might constrain reproducibility, quantitative efficacy, throughput, and depth for large-scale PTMs analysis by MS. [25] In this study, we performed antibody-based enrichment of crotonylated peptides, which helped to increase the sensitivity of the MS analysis for identification and quantification. However, antibody-based enrichment prevents the acquisition of direct information on relative abundances (crotonylated peptides/all peptides in the whole proteome), because crotonylated peptides and non-crotonylated peptides were separated into different sample pools, [26] and only the pool containing enriched crotonylated peptides was detected by MS. In other words, the antibody enrichment strategy is effective and useful for "enrichment," but might not be relied upon for absolute quantification. Improvement for MS analysis of PTMs is in progress, covering all aspects of the MS process. [25–27] In order to be rigorous, we applied different database search strategies, and used raw files without enrichment for modified peptides as a negative control in this study.

To our knowledge, this study reports the first crotonylome of mouse liver. Using the label-free strategy and conventional closed search of MaxQuant, we identified a total of 10 034 Class I crotonylated sites in 2245 proteins from livers of nine mice. It is also evident that crotonylation exists in non-histone proteins and might participate in multiple biological processes, offering fundamental information for future crotonylation studies.
Conflict of Interest

The authors declare no conflict of interest.

Keywords

crotonylation, dataset, label-free, liver, mouse

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