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DATASET BRIEF

Global profiling of protein lactylation in *Caenorhabditis elegans*

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Abstract

Lactylation, as a novel posttranslational modification, is essential for studying the functions and regulation of proteins in physiological and pathological processes, as well as for gaining in-depth knowledge on the occurrence and development of many diseases, including tumors. However, few studies have examined the protein lactylation of one whole organism. Thus, we studied the lactylation of global proteins in *Caenorhabditis elegans* to obtain an in vivo lactylome. Using an MS-based platform, we identified 1836 Class I (localization probabilities > 0.75) lactylated sites in 487 proteins. Bioinformatics analysis showed that lactylated proteins were mainly located in the cytoplasm and involved in the tricarboxylic acid cycle (TCA cycle) and other metabolic pathways. Then, we evaluated the conservation of lactylation in different organisms. In total, 41 *C. elegans* proteins were lactylated and homologous to lactylated proteins in humans and rats. Moreover, lactylation on H4K80 was conserved in three species. An additional 238 lactylated proteins were identified in *C. elegans* for the first time. This study establishes the first lactylome database in *C. elegans* and provides a basis for studying the role of lactylation.

KEYWORDS
Caenorhabditis elegans, lactylation, LC-MS/MS, posttranslational modification

1 | INTRODUCTION

Lactate has long been regarded as a waste product of cellular respiratory metabolism under anaerobic conditions, and its biological function has received much attention due to the Warburg effect in tumor cells [1]. In recent years, evidence has emerged that lactate can be used as a major circulating carbohydrate fuel [2], promote tumor

Abbreviations: Kla, lysine lactylation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PSMs, peptide-to-spectrum matches; TCA, tricarboxylic acid; KEGG, Kyoto Encyclopedia of Genes and Genomes; *C. elegans*, *Caenorhabditis elegans*.

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angiogenesis [3–4], and act as a signaling molecule to mediate intracellular or intercellular communication [5]. However, the molecular mechanisms underlying its biological functions remain to be explored in depth.

Posttranslational modification (PTM) is a covalent process that proteins undergo during or after translation [6]. With the development of high-resolution mass spectrometry and modified peptide enrichment techniques, a variety of cellular metabolites have been newly identified as precursors for the acylation of proteins, such as crotonylation, butyrylation, succinylation, and propionylation [7]. In 2019, Zhao et al. identified lactate as a precursor for lactylation and a regulator of the lactylation of proteins [8]. Subsequently, Gaffney DO et al. used mass spectrometry to demonstrate that proteins can be lactylated [9], and these two groups provided new opportunities for studies on the function and mechanism of lactylation. Subsequent studies further found that Class I HDACs (HDAC 1–3) were the most effective lactylated-lysine "erasers" in vitro, and HDAC1 and 3 exerted delactylase activity in cells [10]. In addition, YiaC and CobB regulate lactylated lysines in *Escherichia coli*, in which YiaC acts as a lactyl-CoA (writer) [11]. Hence, these findings provide a solid theoretical basis for elucidating the mechanism by which lactylation is regulated. Previous studies suggested that histone lactylation plays a role in disease pathogenesis. For example, the results of one study showed that H3K18la, H3K23la, and pan histone lactylation were accumulated in the nucleuses of mouse oocytes, fertilized eggs, and preimplantation embryos, but histone lactylation was reduced in hypoxic culture, which in turn impaired preimplantation development in mice [12]. Histone lactylation contributes to tumorigenesis by facilitating YTHDF2 expression [13] and regulates RNA m6A modifications to promote tumor immunosuppression [14]. Lactylation modifications are widespread in the human proteome and are mainly present on nonhistone lysines [15]. For example, lactylation was associated with prostate cancer [16], sepsis [17], hepatocellular carcinoma [18–19], gastric cancer cells [20], human lungs [21], etc. However, the function of lactylation in one whole organism has not been thoroughly studied. *Caenorhabditis elegans* is widely used as a model organism for basic biology research and has been extensively studied in proteomics [22]. Of its nearly 20,000 protein-coding genes, 60–80% are homologous to human genes [23].

In this study, we used adult *C. elegans* as study subjects with three biological replicates (Lac 09, Lac13, and Lac17), and the experiment was based on LC-MS/MS. We identified 1836 Class I (localization probabilities > 0.75) lactylated sites out of 487 proteins (Table S1) and explored the preference of lactylation modification in secondary structure and subcellular localization. Bioinformatics analysis showed that lactylated proteins were mainly involved in the tricarboxylic acid cycle (TCA cycle) and other metabolic pathways. To this end, we compared with the reported human [18–21] and rat [24] lactylation datasets. This paper establishes the first atlas of lactylation in *C. elegans* and expands the lactylome database, providing more valuable evidence for in-depth studies of lactylation. The MS data have been deposited to the ProteomeXchange Consortium with the dataset identifier PXD041277 (Data Reviewer link).

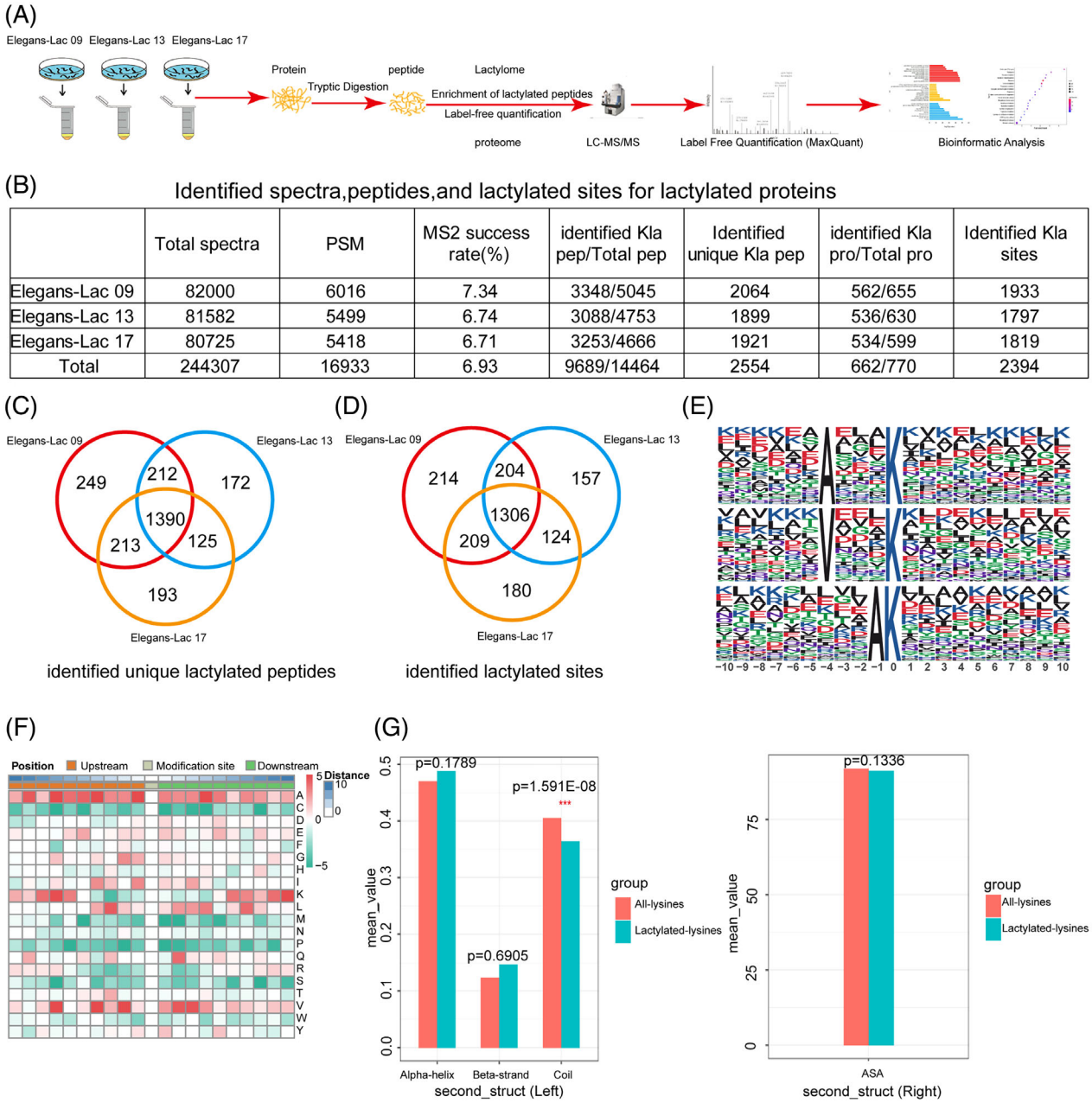
2 | RESULTS

2.1 | Global profiling of the lactylome in *C. elegans*

We performed bottom-up lactylome analysis in *C. elegans* obtained from three biological replicates (Lac09, Lac13, and Lac17) under normal physiological conditions. The workflow for the study is presented in Figure 1A, and the detailed methods are listed in the Supporting Information. Mass spectrometry (MS) raw files were analyzed using MaxQuant version 1.6.15.0 [25]. For the lactylome, a total of 16,933 (6.93%) of 244,307 MS/MS spectra were matched to peptides. As potential contaminant or reversed proteins were removed, 14,464 peptide-to-spectrum matches (PSMs) in 770 proteins were adjusted to a 1% peptide false discovery rate (FDR), and 9,689 PSMs (2,554 unique peptides with 2,394 lactylated sites) in 662 proteins were lactylated (Figure 1B). A total of 1390 (≈54%) unique lactylated peptides (Figure 1C and Table S2) and 1306 (≈55%) lactylated sites (Figure 1D and Table S2) were identified in all three samples, suggesting that lactylation is relatively stable across biological replicates. The 2394 lactylated sites were assigned to a category at each quartile based on the lactylated site localization probabilities calculated by MaxQuant using the PTM scoring algorithm [25–26]. Among them, a total of 2377 (99.3%) lactylated sites were Class I (localization probability > 0.75), 5 (0.2%) were Class II ($0.5 <$ localization probability ≤ 0.75), 12 (0.5%) were Class III ($0.25 <$ localization probability ≤ 0.5), and there were no class IV sites ($0.25 \geq$ localization probability) (Figure S1A). To validate the MS data, we constructed homemade pipelines [27]. For the lactylome data, we determined the peptide length distribution (Figure S1B), and the Pearson correlation coefficients indicated that the reproducibility of the data among the three biological replicates was reasonable (Figure S1C). All identified peptides were checked for mass errors, and the distribution was close to zero and mostly below 2 ppm (Figure S1D), confirming that the mass accuracy of the MS data was acceptable. A spectral count of each lactylated peptide (Figure S1E) and Andromeda score (Figure S1F) were obtained. For the proteomics data, we assessed the peptide length, spectral count per peptide, Andromeda score, and mass error (Figure S2). Based on the homemade pipeline, all samples passed quality control and were used for subsequent bioinformatics analysis.

2.2 | Analysis of lactylated site patterns

To define the possible Kla motifs in lactylated proteins, the characteristic sequences of modified sites and their enrichment statistics were obtained by MoMo [28], and the following conservative patterns around lactylated peptides were obtained: xxxxxxAxxx_K_xxxxxxxxxx, xxxxxxVxxx_K_xxxxxxxxxx, and xxxxxxxxxA_K_xxxxxxxxxx (where x indicates a random amino acid residue) (Figure 1E). These motifs differ from those identified in human gastric cancer cells [20] and human lungs [21]. Heatmaps were created for the amino acid sequences



	Total spectra	PSM	MS2 success rate(%)	identified Kla pep/Total pep	Identified unique Kla pep	identified Kla pro/Total pro	Identified Kla sites
Elegans-Lac 09	82000	6016	7.34	3348/5045	2064	562/655	1933
Elegans-Lac 13	81582	5499	6.74	3088/4753	1899	536/630	1797
Elegans-Lac 17	80725	5418	6.71	3253/4666	1921	534/599	1819
Total	244307	16933	6.93	9689/14464	2554	662/770	2394

FIGURE 1 Identification and secondary structure of lactylated proteins in *Caenorhabditis elegans*. (A) A bottom-up lactylome and proteomics workflow for the *C. elegans*. (B) Identified spectra, peptides, and lactylated sites for lactylated proteins. (C) Venn diagram of identified unique lactylated peptides in the three biological replicates. (D) Venn diagram of identified lactylated sites in the three biological replicates. (E) The three types of conserved motif sites of lysine. (F) Motif analysis of lactylated sites. (G) Distribution of lactylated lysines and all lysines in the alpha-helix, beta-strand, and disordered coil (left), as well as protein surface accessibility (right).

surrounding lactylation sites; red indicates high frequency, and green indicates low frequency (Figure 1F). To further explore the preference of lactylation on secondary structure and surface accessibility, the NetSurfP algorithm [29] was used to predict the secondary structure and surface accessibility for lactylated sites and all the lysine residues of lactylated proteins (Figure 1G). The analysis revealed that lysine residues on α-helices ($p = 0.1789$) and β-folds ($p = 0.6905$) were more likely to undergo lactylation; in contrast, lysine residues on coils (p = 1.591E-08) were less likely to undergo lactylation (Figure 1G, left). Furthermore, the surface accessibility of lactylated sites and all lysine residues of lactylated proteins showed no significant differences ($p = 0.1336$) (Figure 1G, right).

2.3 | Comprehensive characterization of the lactylome and proteome of *C. elegans*

We selected 1836 Class I (localization probabilities > 0.75) lactylated sites in 487 proteins that were detected in at least two biological replicates (Table S1) and identified a total of 2416 proteins in the

proteome (Table S3) for subsequent bioinformatic analysis. The detailed methods are listed in the Supporting Information. The proportion of lactylated proteins among 2416 proteins was determined (Figure S4A). The Venn diagram showed that 13 lactylated proteins were difficult to detect by conventional proteomics methods. However, the lactylated enrichment technique led to the discovery of proteins that were not detected in the proteomics dataset. To clarify the biological roles of proteins in *C. elegans* under normal physiological conditions, we determined their subcellular localization, cellular component, molecular function, and associated biological processes. To illustrate the subcellular distribution of the lactylated proteins in *C. elegans*, we annotated all of the lactylated proteins containing Class I (localization probabilities > 0.75) lactylated sites using UniProt protein annotations. Subcellular localization investigation demonstrated that the lactylated proteins were mainly located in the cytoplasm (33.2%), the secretion (14.8%), and the mitochondrion (10.1%), followed by the nucleus (8.6%) (Figure 2A), which is different from subcellular localization of lactylated proteins in other species and tissues [19–21, 30–33]. The results demonstrated that these lactylated proteins exhibited different subcellular distribution patterns among different species and cell types. The subcellular localization distribution in the proteome was compared, and the lactylated proteins were relatively overrepresented in the cytoplasm (33.2 vs. 21.9%), the secretion (14.8 vs. 3.6%), and the mitochondrion (10.1 vs. 6.6%) but underrepresented in the nucleus (8.6 vs. 18.1%) (Figure 2B).

GO enrichment analysis of the cellular component revealed that lactylated proteins were mainly enriched in the cytosol and cytosolic ribosome (Figure 2C, middle, Table S4). GO-based analysis of molecular function revealed that lactylated proteins were mainly enriched in terms including the structural constituent of the ribosome (Figure 2C, upper, Table S4). Lactylated proteins were enriched in a variety of biological processes, including peptide biosynthesis processes and translation processes (Figure 2C, lower, Table S4), which were related to protein synthesis and metabolism. The GO enrichment analysis of proteins in the proteome showed that for the biological process (BP) category (Figure 2D and Table S5), proteins were significantly enriched in the reproductive processes, the sexual reproduction processes, and the multicellular organismal reproductive processes. Both lactylome and proteomics data revealed significant enrichment in various metabolic pathways, including the citrate cycle (TCA cycle), proteasome, ribosome, carbon metabolism, phagosome, biosynthesis of amino acids, oxidative phosphorylation, mTOR signaling pathway, biosynthesis of cofactors, and longevity regulating pathway-worm (Figure 2E, 2F, Table S6, S7). Separately, the lactylome data also showed enrichment in energy metabolic pathways such as pyruvate metabolism (Figure S3A), glycolysis/gluconeogenesis (Figure S3B), propanoate metabolism (Figure S3C), glyoxylate and dicarboxylate metabolism (Figure S3D), glutathione metabolism (Figure S3E), and 2-Oxocarboxylic acid metabolism (Figure S3F). These results indicated that lactylated proteins focused on the energy metabolic pathways and revealed that most key enzymes involved in these pathways were lactylated [9]. To further clarify the biological regulation and functions of lactylated proteins in *C. elegans*, we classified these proteins based on Eukaryotic Orthologous Group (KOG) annotation. The lactylated proteins could be divided into 23 KOG functional categories. The top three KOG terms obtained from the analysis of lactylated proteins were energy production and conversion [C]; translation, ribosomal structure and biogenesis [J]; and posttranslational modification, protein turnover, and chaperones [O] (Figure 3A and Table S8). These results are identical to those obtained in other species [19, 30]. Based on the proteomics data, the top three KOG terms were translation, ribosomal structure and biogenesis [J], posttranslational modification, protein turnover, chaperones [O], and signal transduction mechanisms [T] (Figure S4B and Table S9). PPI networks among KEGG pathways with significant enrichment of the lactylome and proteome were performed via the STRING website (https://string-db.org/) and Cytoscape (V.3.7.2) software (Figure 3B).

2.4 | Interaction network and wide conservation analysis of lactylated proteins

The majority of key TCA cycle enzymes were lactylated in protein-protein interaction (PPI) networks. Fifteen enzymes with 57 Kla sites were identified (Figure 3B), including cytoplasmic aconitate hydratase (aco-1), putative aconitate hydratase (aco-2), isocitrate dehydrogenase [NAD] subunit alpha-1 (idha-1), putative citrate synthase (cts-1), dihydrolipoyl dehydrogenase (dld-1), malate dehydrogenase, mitochondrial (mdh-2), malate dehydrogenase, cytoplasmic (mdh-1), succinate-CoA ligase [ADP/GDP-forming] subunit alpha (sucl-1), succinate-CoA ligase [GDP-forming] subunit beta (sucg-1), succinate dehydrogenase [ubiquinone] iron-sulfur subunit (sdhb-1), succinate–CoA ligase [ADP-forming] subunit beta (suca-1), fumarate hydratase (fum-1), 2-oxoglutarate dehydrogenase (ogdh-1), succinate dehydrogenase [ubiquinone] flavoprotein subunit (sdha-1), and dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex (dlat-1). Notably, mdh-2 was a critical regulatory enzyme in the TCA cycle and had the highest number of Kla sites (n = 9). To clarify the core lactylated proteins involved in the TCA cycle, the betweenness centrality (BC) of each lactylated protein was calculated in the protein-protein interaction (PPI) network (Figure S4C and Table S10). Mitochondrial malate dehydrogenase (mdh-2) is an important enzyme in the TCA cycle among the top 15 lactylated proteins with high BC values [34]. To investigate the conservation of lactylation in different organisms, we completed a BLAST analysis of our lactylated *C. elegans* protein sequences with the recently reported human hepatocellular carcinoma [18–19], human gastric cancer cells [20], human lungs [21], and rats [24] lactylomes. The results showed that 41 proteins were lactylated and homologous in the three species (Figure 3C and Table S11). Moreover, only H4K80la was conserved in the three species (Figure 3D and Table S12). The identified overlapping sites in different lactylomes suggested that lactylation is conserved in different organisms, and this modification may play an immense role in basic biological processes. To determine the biological functions of homologous

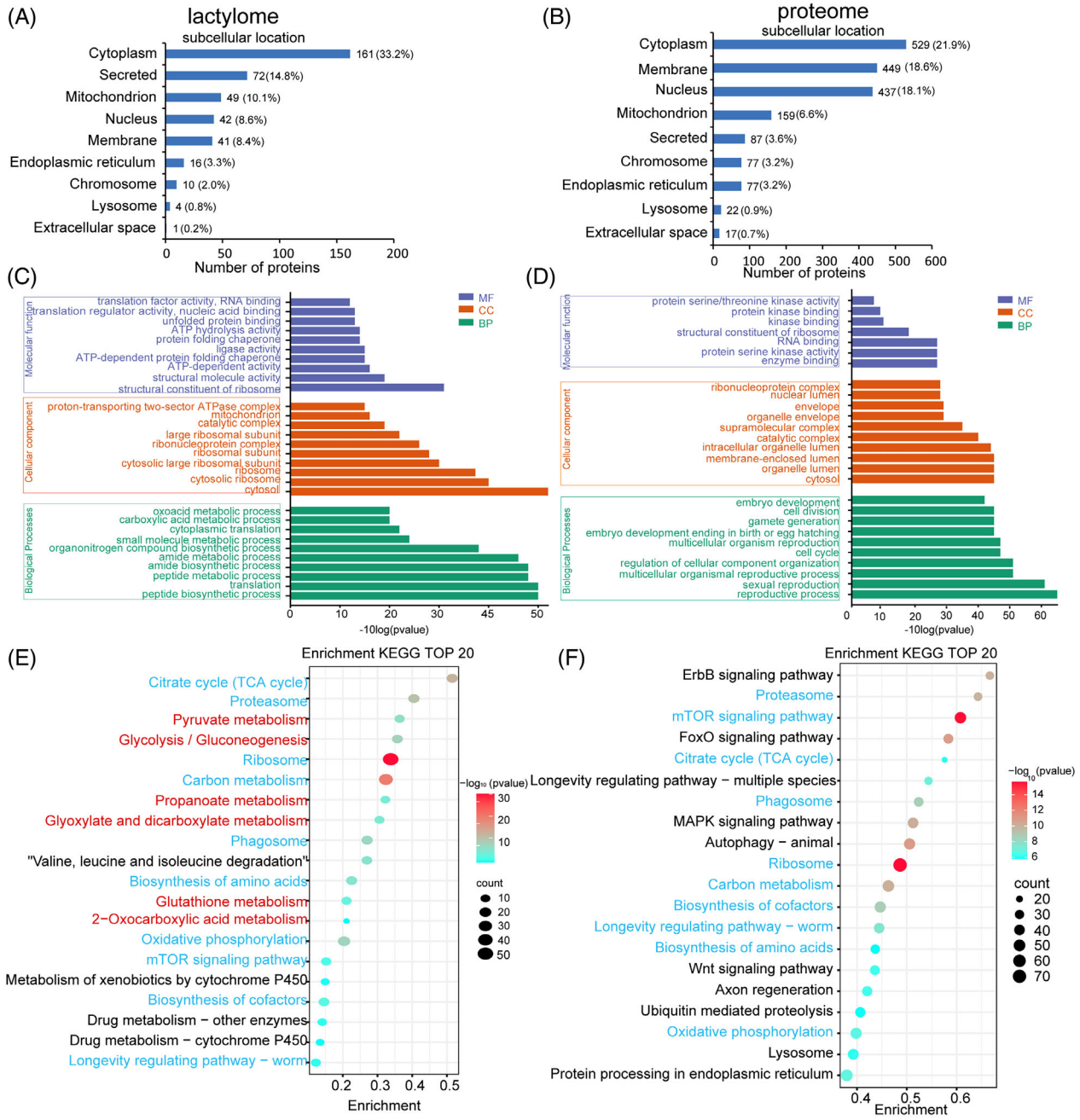


FIGURE 2 Functional classification and enrichment analysis of the lactylome and proteome. (A) UniProt protein annotations for subcellular localization analysis of lactylated proteins with Class I (localization probabilities > 0.75) lactylated sites in the lactylome. (B) Subcellular localization analysis of identified proteins in the proteome. (C) GO analysis of lactylated proteins with Class I (localization probabilities > 0.75) lactylated sites in the lactylome. (D) GO analysis of identified proteins in the proteome. (E) KEGG enrichment analysis of lactylated proteins with Class I (localization probabilities > 0.75) lactylated sites in the lactylome. (F) KEGG enrichment analysis of identified proteins in the proteome.

proteins in the three species, we performed a KEGG enrichment analysis. Consequently, a large number of conserved lactylated proteins are related to carbon metabolism, butanoate metabolism, pyruvate metabolism, and valine, leucine and isoleucine degradation (Figure S5B, C, and D Table S13, S14, and S15). An additional 238 lactylated proteins were identified in *C. elegans* for the first time (Figure 3C and Table S16). Then, we analyzed the lactylation of proteins related to the TCA cycle in three species (Figure 3E).

3 | DISCUSSION AND CONCLUSION

The first lactylation atlas of global proteins of *C. elegans* was reported in this study. We identified 1836 class I (localization probabilities > 0.75) lactylated sites from 487 proteins in the lactylome and a total of 2416 proteins in the proteome for further analysis. In the pattern analysis of lactylated sites (Figure 1G), lactylated sites were more likely to occur on α-helices ($p = 0.1789$) and β folds ($p = 0.6905$) and less likely to

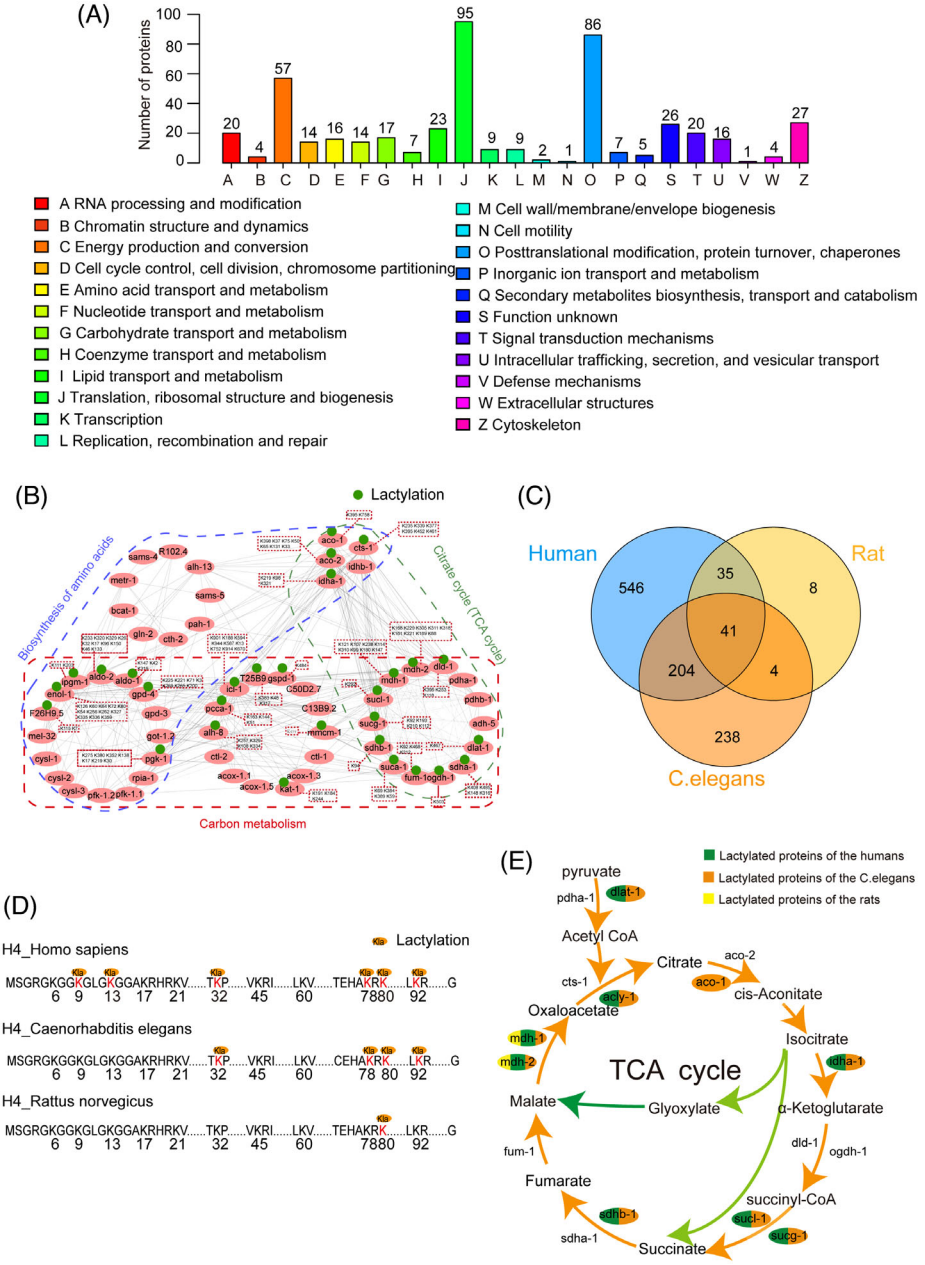


FIGURE 3 Global profiling of lactylated proteins in *C. elegans* and other research. (A) Eukaryotic orthologous group (KOG) analysis of lactylated proteins with Class I (localization probabilities > 0.75) lactylated sites. (B) Identified proteins in the proteome and lactylated proteins with Class I (localization probabilities > 0.75) lactylated sites in the lactylome are involved in the citrate cycle (TCA cycle), carbon metabolism, and biosynthesis of amino acids. The proteins identified in the proteome that are highlighted in pink circles and green dots were determined to be modified by lactylation in this study, and the red box indicates the lactylation sites. (C) Venn diagram showing the overlap of lactylation proteins identified in *C. elegans*, human lungs, human gastric cancer cells, human hepatocellular carcinoma, and rats. (D) H4 lysine lactylation sites in *C. elegans*, humans (human lungs, human gastric cancer cells, human hepatocellular carcinoma), and rats. Lysines subjected to modifications are indicated by red. The numbers indicate modified lysine positions on H4 proteins. (E) Schematic diagram of lactylated proteins related to the tricarboxylic acid (TCA) cycle in three species.

occur on coils ($p = 1.591E-08$) than all lysine residues, which was the same pattern previously found in other species [20]. To characterize the nature of lactylation in *C. elegans*, we used the motif-x algorithm to analyze the sequence pattern of peptides with Class I (localization probabilities > 0.75) lactylated sites [28]. For the sequence motif surrounding Kla residues, we found that alanine (A) and valine (V) residues were the most significant residues in each lactylation motif (Figure 1E). Regarding subcellular localization (Figure 2A), the largest number of lactylated proteins were mainly found in the cytoplasm. The function, metabolism, and interaction of proteins are closely related to their subcellular localization. We then used the Gene Ontology (GO) database [35] to confirm that lactylated proteins were also mainly

located in the cytoplasm (45%) (Figure S5A). In eukaryotes, lactate metabolism mainly occurs in the mitochondria of the cytoplasm. Therefore, it is not surprising that lactate substrate proteins are mainly located in the cytoplasm. The KOG functional annotation (Figure 3A) and KEGG pathway enrichment analysis (Figure 2E) showed that lactylated proteins were significantly enriched in metabolic-related pathways, including the carbon metabolism pathway and the TCA cycle. These findings correspond with previous lactylation-related studies in rice, T.gondii, and Trypanosoma brucei [30–32], suggesting that lactylation-regulated pathways are relatively conserved and lactylation plays an active role in these essential metabolism pathways in *C. elegans*. Therefore, we focused on the association between lactylation and energy metabolism. The majority of key TCA cycle enzymes were lactylated in protein-protein interaction (PPI) networks (Figure 3B), and similar findings were obtained with lactylated proteins in human HEK293 cell lines [9].

Then, we analyzed the lactylation of proteins related to the TCA cycle in the three species (Figure 3E). Interestingly, mdh-1 and mdh-2 are conserved lactylated proteins in three species. Lactylations on dlat-1, acly-1, idha-1, sucg-1, sucl-1, and sdhb-1 in humans were also presented in *C. elegans*. However, lactylation on aco-1 was only identified in *C. elegans*. To determine the location of the lactylated sites of mdh-1 and mdh-2 in the three species, sequence alignment analysis was performed, showing that K110la, K118la, K205la, and K318la of MDH-1, as well as K157la, K239la, K296la, K301la, K307la, and K324la of MDH-2 in humans, were also present in rats. Moreover, eight lactylated sites (K121, K107, K238, K114, K310, K99, K180, K147) in mdh-1 and nine lactylated sites (K168, K229, K305, K311, K318, K161, K221, K189, K88) in mdh-2 were only identified in *C. elegans* (Figure S5E), which might be essential for connecting the citric acid cycle, gluconeogenesis, and glyoxylate shunt [34]. However, the biological significance of these different modification sites should be further investigated.

In conclusion, our work expands the lactylome database and provides a valuable resource and in-depth knowledge on the mechanism of lactylation; however, the following issues need to be resolved in future studies. First, although affinity enrichment techniques can greatly increase the number of lactylated proteins identified, false positives for modification sites still occur. Therefore, future studies should consider that nonmodified synthetic peptides from the *C. elegans* proteome are used as negative lactylated controls. This approach can significantly reduce the false-positive rate of lactylation modification identification, revealing the true modification sites and target proteins. Second, the mechanism underlying how Kla affects metabolic-related pathways in *C. elegans* was not investigated here. Future studies, including genetic manipulation and mutagenesis experiments, are needed to verify the effects of Kla on protein functions.

4 | MATERIALS AND METHODS

The *C. elegans* used were wild-type N2, and trypsin was used for digestion. To enrich the lactylated peptides, the peptides were dissolved in lactated resin (antibody resin No. (PTM-1404) from PTM Bio, Hangzhou Jingjie Biotechnology Co., Ltd.). The peptides were separated by a UHPLC system and then injected into an NSI ion source for ionization before being analyzed by Orbitrap Exploris™480 mass spectrometry, and the MS/MS data were analyzed using the MaxQuant software package (v1.6.15.0) (Supplementary Materials for details).

4.1 | MS Data Reviewer link

Proteomexchange identifier: PXD041277

URL: https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD041277

AUTHOR CONTRIBUTIONS

J.-T. Yang and J.-F. Liu directed and designed the experiments. T. Ding analyzed the data and wrote the manuscript. Y.-H. Yang, Q.-C. Wang, Y. Wu, R. Han, X.-T. Zhang and J. Kong analyzed the data. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The MS data have been deposited to the ProteomeXchange Consortium with the dataset identifier PXD041277 (Data Reviewer link). MS Data Reviewer link Proteomexchange identifier: PXD041277 URL: https://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD041277.

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SUPPORTING INFORMATION

Additional supporting information may be found online https://doi.org/10.1002/pmic.202300185 in the Supporting Information section at the end of the article.

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