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Identification of proteome-wide and functional analysis of lysine crotonylation in multiple organs of the human fetus

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Abstract

Lysine crotonylation (Kcr) is a novel post-translational modification that is important in functional studies. However, our understanding of Kcr in the developing human fetus brain, heart, kidney, liver, and lung remains restricted. In this study, we used high-resolution LC-MS/MS and high-sensitivity immunoaffinity purification to analyze Kcr in the brain, heart, kidney, liver, and lung of 17-week fetus. A total of 24,947 Kcr modification sites were identified in 5,102 proteins, resulting in the most diverse Kcr proteome of fetus organs ever reported. We investigated the universality and specificity of Kcr during the development of several organs in 17-week fetus using bioinformatics analysis. Kcr proteins were found to be closely associated with the synthesis, transcription and translation of genetic material, energy production and metabolic processes. Importantly, the expression of Kcr proteins in each organ was closely related to the organs' developmental functions. Furthermore, several highly modified Kcr proteins may be important targets during fetus organ development. This discovery advances our understanding of fetus organ development and establishes the groundwork for future research into the regulatory mechanisms of crotonylation in fetus organ development.

Keywords Fetus, Multi-organ, Lysine crotonylation, Development, Bioinformatics

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Introduction

The disclosure of epigenetic features at various stages of human fetus development and the provision of a comprehensive understanding of the molecular features at various stages of fetal development have been made possible by the successive development of sophisticated histology techniques and analytical methods in recent years. These developments are extremely significant as they will guide the development of fetus in the future and the survival of newborns. Because there is a limited supply of different fetal organ tissues, the majority of the present knowledge about the molecular characteristics of fetal organ development comes from animal research. A rabbit model of lung development at different periods was used to simulate the molecular characteristics of the human lung organs at the pseudoglandular, tubular, saccular, and alveolar stages. It was discovered that there were notable differences in the expression of certain genes and pathways at different stages of lung development. A rabbit model of lung development at different periods was used to simulate the molecular characteristics of the human lung organs at the pseudoglandular, tubular, saccular, and alveolar stages. It was discovered that there were notable differences in the expression of certain genes and pathways at different stages of lung development [1]. Several studies have shown that lincRNAs have important roles in fetal neurological, cardiovascular, reproductive and pancreatic development, broadening our insights into the gene regulation of lincRNAs during fetal development [2]. In addition, the latest report is a multi-omics mapping of human embryonic dewlap and joint development using state-of-the-art spatial transcriptomics, adding to our understanding of cellular lipid determination in human skeletal development [3]. It is clear that tissue development is significantly influenced by histological investigations. The alteration of crotonylation in many organs during fetal development, however, has not been thoroughly studied and warrants more research.

Protein post-translational modifications (PTMs) are processes that chemically modify amino acid side chains after protein synthesis, including phosphorylation, acetylation, ubiquitination, crotonylation, methylation, and glycosylation [4–6]. The complexity of the proteome has become more apparent with the identification of these PTMs. Among these, lysine crotonylation (Kcr) modification is a novel acylation modification that was just found in 2011 by the University of Chicago team led by Prof. Yingming Zhao. It was initially described in the prestigious journal Cell and garnered a lot of attention. The alteration, which is mostly present on histones in transcriptionally active chromatin areas and entails the addition of a crotonyl group to lysine residues in proteins, is intimately linked to the control of reproduction. Protein lysine residues, which are often located on histones in transcriptionally active chromatin areas and are intimately associated with reproductive control, are modified by the addition of a crotonyl group [7]. Crotonylation plays an important role in a variety of biological processes, including regulation of metabolic enzyme activity, control of DNA-protein interactions, and protein-protein or protein-nucleic acid interactions during replication and transcription [8, 9]. Kcr is linked to a number of physiological and pathological processes, including fetal development [10], brain hippocampal neuron activation [11], cardiac hypertrophy [12], hepatocellular carcinoma metastasis and recurrence [13], non-small cell lung cancer cell apoptosis [14], and inflammation and fibrosis in diabetic nephropathy [15]. Members of this group's research have demonstrated that during human fetal lung development, multiple metabolic pathways gradually come into play as the lung matures and metabolic function is refined [16]. Furthermore, research has showed that non-histone Kcr is down-regulated in gastric, liver, and kidney cancers, but up-regulated in lung, pancreatic, esophageal, thyroid, and colon cancers [17]. It is clear that non-histone Kcr plays an important role in several organs. The 17th week of pregnancy is a vital phase in fetal organ development, during which main organs such as the brain, heart, lungs, kidneys, and liver have formed and began to function. At 17 weeks, the development and function of these organs have reached a relatively mature stage, providing an excellent foundation for investigating the role of crotonylation modification in organ function. We can gain insight into the role of Kcr in normal development and provide a foundation for understanding its abnormal changes in related diseases by studying the crotonylation modifications in the brain, heart, kidney, liver, and lung organs of 17-week-old fetuses. This, in turn, can provide potential biomarkers and targets for early disease diagnosis and treatment.

In this study, we investigated the Kcr modification of five organs, namely the brain, heart, liver, kidney, and lung, during 17-week fetus development, as well as the biological functions involved. Multiple organs' commonality and specificity characteristics during fetus development were found. It is believed that Kcr modification can provide useful guidance in future fetus development.

Methods

Sample collections

In this study, a case of 17-week fetus abortion was collected. Crotonylation modification studies were performed on five organ tissues - brain, heart, lung, liver and kidney - from a fetus terminated at 17 weeks. The organ tissues were collected and saline washed three times in a sterile, ultra-clean bench to get rid of extra blood and other contaminants. The tissues were sliced into blocks smaller than 0.6 cm^3 using sterile tissue shears, then

placed in a cryopreservation tube and kept at -80 °C for later use. The source of the tissues used in this study complied fully with Shenzhen People's Hospital's institutional ethics and legal guidelines on the voluntary termination of pregnancy specimens. The Ethics Committee of Shenzhen People's Hospital gave its approval for all tissue acquisitions (LL-KY-2023080-01). Additionally, an informed consent document was signed by the donor patients.

Protein extraction

The tissues of each organ were removed from – 80 °C and placed separately in a pre-cooled mortar. Liquid nitrogen was then injected to completely ground the tissues to powder. The tissues were then ground into powder and put into 15 ml centrifuge tubes that had been chilled beforehand. To completely lyse the cell, each sample was put on ice and four times the volume of the lysis buffer (8 M urea, 1% protease inhibitor, 3 μM TSA, and 50 mM NAM) was added for ultrasonic homogenization. Centrifugation at 12,000 g for 10 min at 4 °C was used to gather the protein supernatant. Lastly, the BCA method was used to determine the protein concentration.

Trypsin digestion

The isolated proteins were gradually mixed with 20% TCA, vortexed, then precipitated for two hours at 4 °C. After centrifuging the precipitate for five minutes at 4,500 g, it was rinsed two or three times with cooled acetone. Centrifugation was used to remove the supernatant, which was then diluted by adding 200 mM TEAB. Sonication was used to break up the precipitate, and trypsin was added in a 1:50 ratio to digest it overnight. Next, 5 mM dithiothreitol (DTT) was added, and the proteins were reduced for 30 min at 56 °C. Alkylation with 11 mM iodoacetamide (IAA) was performed for 15 min at room temperature while being shielded from light to finish the digestion process.

Fractionation and enrichment of Kcr peptides

The Kcr peptides of each sample were fractionated using a NanoElute ultra-high performance liquid phase system. Briefly, in the presence of mobile phase A (0.1% formic acid and 2% acetonitrile) and mobile phase B (0.1% formic acid and 100% acetonitrile), the peptides were divided into eight fractions by setting different liquid phase gradients and finally dried by vacuum centrifugation.

After dissolving the digested Kcr peptides in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0), the pan- Kcr antibody beads were added, and the mixture was slowly incubated at 4 °C for the entire night. To get rid of non-specific Kcr peptides, the beads were then rinsed twice with water after being rinsed four times with NETN buffer. The peptides were then desalted for LC-MS/MS analysis after being eluted with 0.1% trifluoroacetic acid.

LC-MS/MS analysis

A NanoElute Ultra High Performance Liquid System was used to extract the Kcr peptides from each sample. The Kcr peptides were dissolved in 0.1% formic acid and then put onto an analytical column that was in reverse phase. In the presence of mobile phase A (0.1% formic acid and 2% acetonitrile) and B (0.1% formic acid and 100% acetonitrile), various liquid phase gradients were established, and the flow rate was kept constant at 450 nL/min. A nanospray ion source was used to separate the peptides, and a Q Exactive Plus mass spectrometer was used for MS/MS analysis. The electrospray voltage was 2,000 V and the scanning range was 100-1,700 m/z. Data was acquired using PASEF mode, and 10 MS/MS scans were conducted in succession after one MS scan, with a dynamic exclusion time of 30 s. The automatic gain was controlled at 5×10^4, and a fixed first mass of 100 m/z was used for generating the MS/MS profiles.

Database search

Maxquant 1.6.6.0 was used for database searches. The reverse decoy database and the Homo sapiens-9,606 (20,366 sequences) protein database were used for MS/MS profile analysis. The following were a few of the setup parameters: The protein was identified, PSM was identified, and PSM was identified below 1% FDR. The cleavage was performed using trypsin/P; the number of missed cleavage sites was 4; the minimum peptide length was set at 7; the maximum peptide modification was set at 5; the mass error tolerance for fragmentation ions was set at 20 ppm; the fixed modification was aminomethylation on Cys; the variable modifications were crotonylation on lysine, oxidation on Met, and acetylation on the protein's N-terminus.

Bioinformatics analysis

The UniProt-GOA database was used to perform GO annotation and classify the annotation results into three categories: molecular structures, cellular components, and biological processes.

Wolfpsort subcellular localization software was used to predict the subcellular location of Kcr proteins.

Venn diagrams were used to screened for co-expressed Kcr proteins, MoMo software was used to analyze motifs of Kcr modification sites, and the R package was used to create motif and heat maps.

The DAVID website was used to examine the GO and KEGG functional enrichment of variously expressed proteins, and the R program was used to create bubble and heat maps.

Results

Identification of Kcr proteins and modification sites

We collected a case of flow products and analyzed Kcr modifications on brain, heart, kidney, liver and lung. The majority of the peptides were dispersed between 7 and 20 amino acids, according to mass spectrometry-based assay quality control, which is in line with the general pattern of trypsin digestion and HCD rupture mode (Fig. 1a). Furthermore, mass errors for the great majority of the detected peptides were within 10 ppm (Fig. 1b). These outcomes demonstrate the accuracy of our crotonylation data. Heterogeneity among the five samples was discovered by correlation analysis, and the findings are displayed as a heat map (Fig. 1c).

The results revealed that 5,102 proteins and 24,947 Kcr modification sites were discovered (Fig. 1d). Kcr protein sites identified in each organ are shown in Fig. 1e and f. In brain tissue, 2,635 proteins and 8,827 Kcr modification sites were identified, with 2,484 proteins having low modification sites (1 to 10 Kcr modification sites one protein), 125 proteins having medium modification sites (10–20 Kcr modification sites one protein), and 26 proteins having high modification sites (more than 20 Kcr modification sites one protein). In heart tissue, 2,756 proteins and 10,913 Kcr modification sites were found, with 2,566 low modification sites, 149 middle modification sites, and 41 high modification sites. In kidney tissues, 2,545 proteins and 8,542 Kcr modification sites were discovered, with 2,404 being low modification sites, 115 middle modification sites, and 26 high modification sites. There were 1,898 proteins and 5,773 Kcr modification sites found in liver tissue, with 1,813 being low modification sites, 75 being medium modification sites, and 10 being high modification sites. In lung tissue, 3,035 proteins and 10,483 Kcr modification sites were identified, with 2,867 low, 129 medium, and 39 high.

Subcellular localization analysis and GO classification of co-expressed Kcr proteins

We used Venn analysis to investigate the correlation and heterogeneity of Kcr proteins in the five organs and discovered that 1,154 proteins were expressed in all five organs, with 311 proteins specifically expressed in the heart, 457 proteins in the brain, 386 proteins in the lungs, 147 proteins in the liver, and 106 proteins in the kidney (Fig. 2a). The proteins that were co-expressed in the five organs were classified using subcellular localization (Fig. 2b) and GO (Fig. 2c). As demonstrated, the greatest component was cytoplasm (43.12%), followed by the nucleus (23.81%) and mitochondria (13.16%). It was projected that some co-expressed Kcr proteins would be dispersed in the extracellular (7.27%), plasma membrane (3.90%), and cytoplasm (4.85%). The results of GO classification revealed that cellular process (22.81%) was the largest category in the classification of biological processes, followed by biological regulation (17.41%) and metabolic process (16.27%). The largest group of cellular components was organelle (15.42%), followed by cytosol (10.17%) and membrane (9.95%). In molecular function classification, binding (45.58%) was the most common category, followed by catalytic activity (19.58%) and molecular function regulator activity (7.06%).

Functional enrichment analysis of co-expressed Kcr proteins

To further study the biological functional significance of the co-expressed Kcr proteins, GO and KEGG enrichment analysis were carried out on the proteins from all five organs. The findings of the GO analysis are displayed in Fig. 3a-c. In terms of biological processes, co-expressed Kcr proteins were considerably enriched in purine-containing compound metabolic process, RNA splicing, purine nucleotide metabolic process, and genetic material synthesis (Fig. 3a). Significantly associated with cell-substrate junction, focal adhesion and vesicle lumen cellular components (Fig. 3b). In terms of MF, it was significantly correlated with cadherin binding, actin binding, and ATP hydrolysis activity functions (Fig. 3c). Based on KEGG pathway enrichment analysis, our visualization of the top 30 significantly enriched pathways revealed that co-expressed Kcr proteins were significantly enriched in Pathways of neurodegeneration - multiple diseases, Amyotrophic lateral sclerosis, and Alzheimer's disease (Fig. 3d). Most importantly, the terms "transcription" and "translation" were confirmed. Furthermore, we divided the KEGG enrichment results into six categories: metabolism, human biseases, organismal systems, cellular processes, genetic information processing, and environmental information processing, and the results are given in the form of hierarchical network diagram (Fig. 4).

PPI analysis of co-expressed Kcr proteins

PPI analysis of co-expressed Kcr proteins was performed using the string database, and the interacting proteins were categorized into five clusters by different algorithms: translation and ribosome, mitochondrial and ribonucleoside, catabolic process, splicing and telomere regulation, and it was found that there was a correlation between the co-expressed Kcr proteins (Fig. 5a). Translation and ribosome functions have the strongest interactions with Kcr proteins.

Motif analysis of co-expressed Kcr proteins

To establish the Kcr proteomic profile during fetus development. We analyzed the Motif features of the crotonylation sites on five organ co-expressed Kcr proteins. Heatmaps reflect peptide sequences consisting of 10

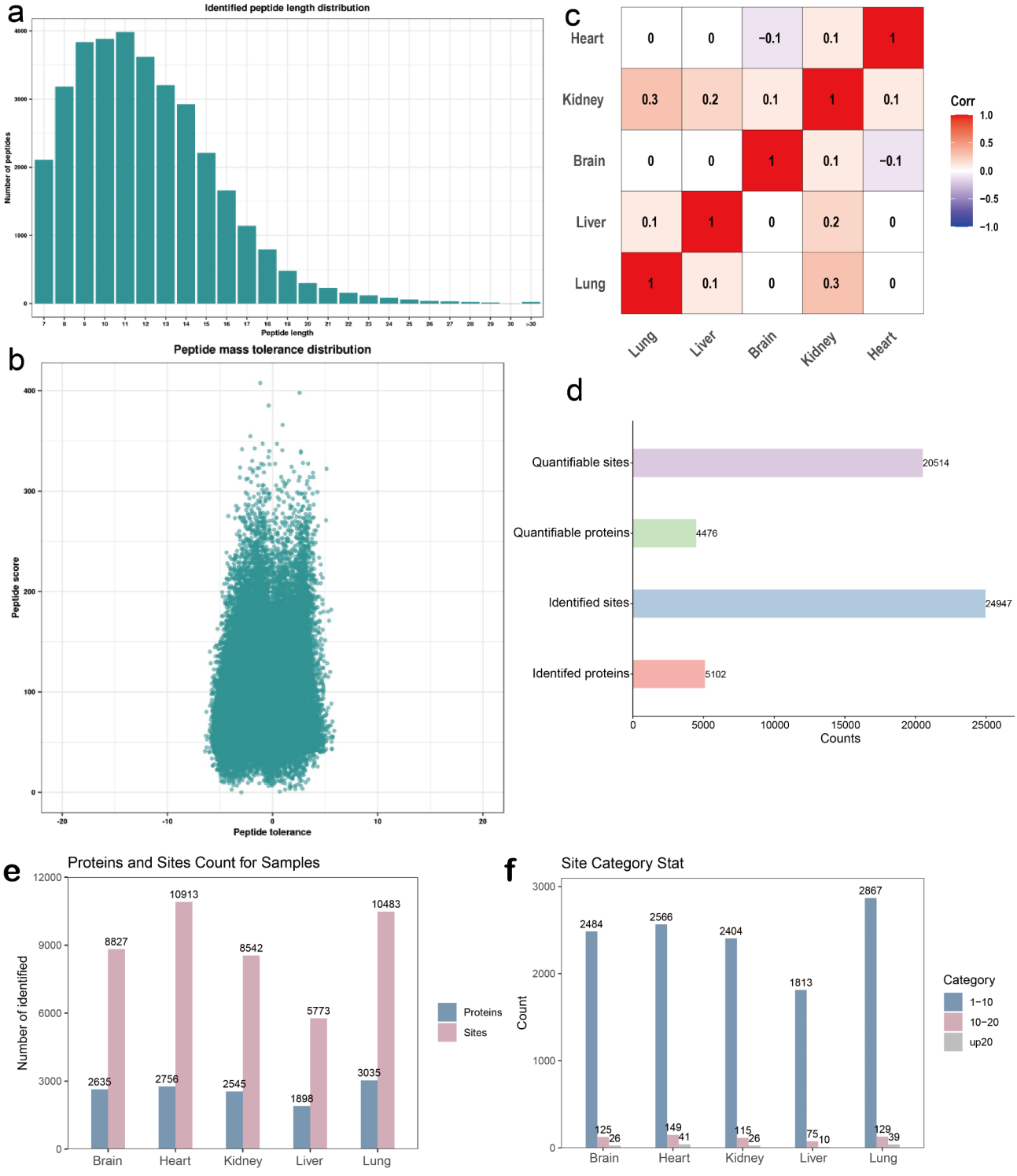


Fig. 1 Lysine crotonylation modifications of brain, heart, kidney, liver, and lung organs in 17-week fetus. (**a**) The length distribution of all identified lysine crotonylation peptides. (**b**) Mass accuracy distribution of all identified Kcr peptides. (**c**) Correlation heat map analysis of each organ sample. (**d**) Basic information statistics of mass spectrometry data results of lysine crotonylation assay for each organ. (**e**) Number of detected Kcr proteins and sites in each organ. (**f**) Crotonylation modification sites from each organ are distributed in a single protein

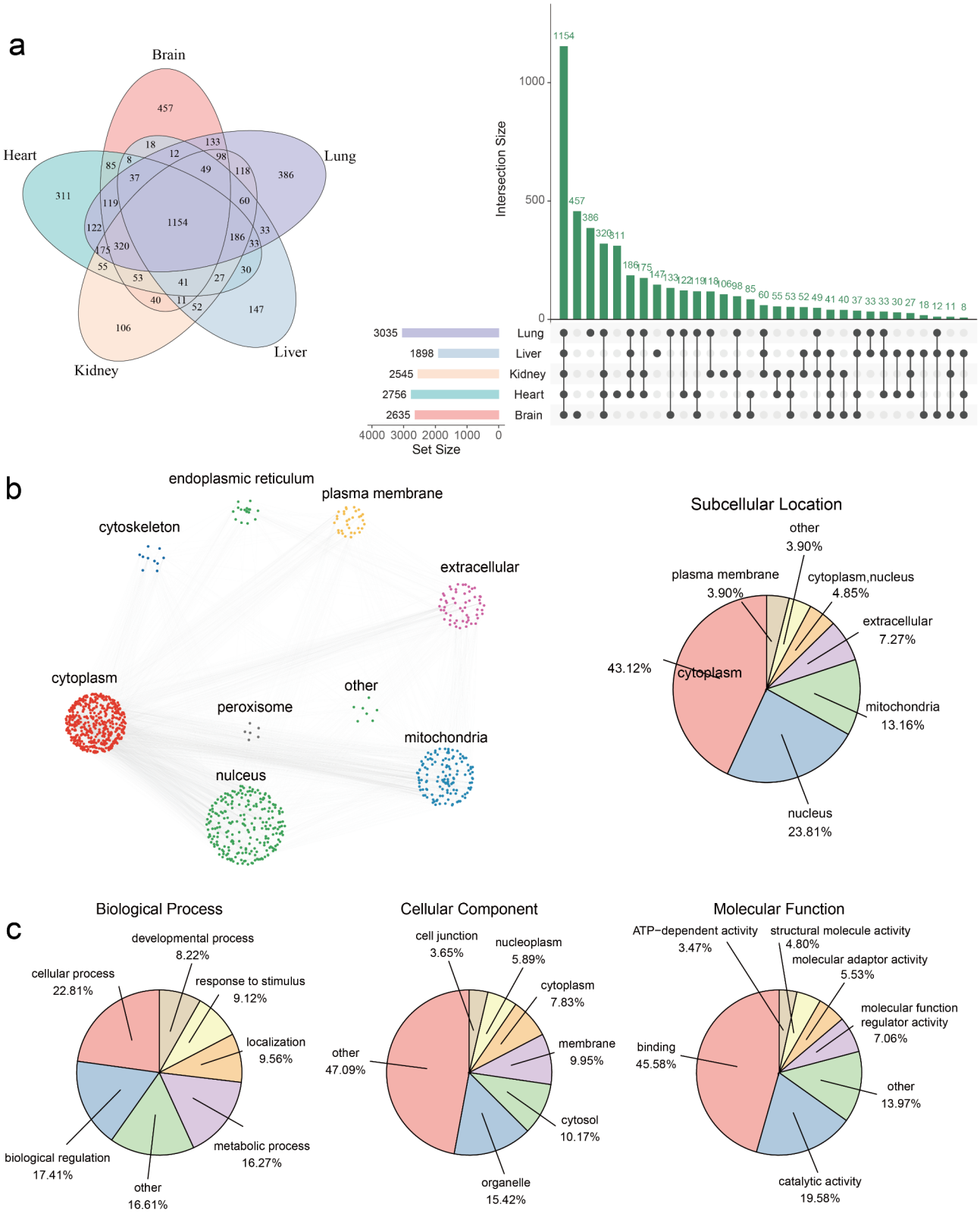


Fig. 2 Functional annotation of co-expression Kcr proteins in brain, heart, kidney, liver and lung organs. (**a**) Venn analysis screening for co-expression Kcr proteins in brain, heart, kidney, liver and lung organs. (**b**) Subcellular localization analysis of co-expression Kcr proteins. (**c**) GO classification of co-expression Kcr proteins

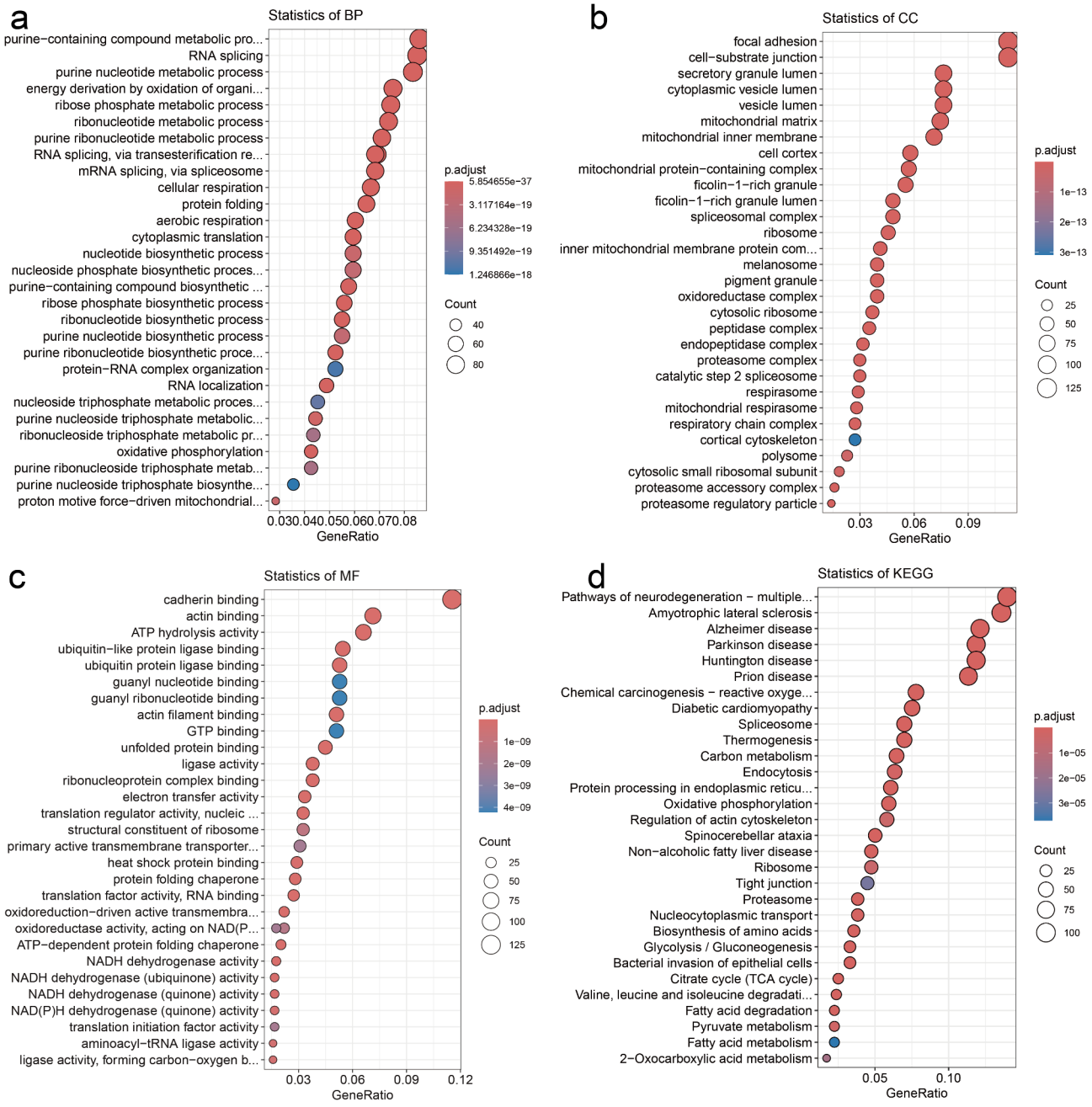


Fig. 3 Enrichment analysis of co-expression Kcr proteins. GO enrichment analysis of co-expression Kcr proteins, including biological processes (**a**), cellular components (**b**) and molecular functions (**c**). (**d**) KEGG enrichment analysis of co-expression Kcr proteins

amino acids upstream and downstream of the identified modification sites (Fig. 5b-c). Lysine (K), alanine (A), and glutamic acid (E) residues all occur with high frequency in the top 10 positions upstream and downstream of the modification site. In contrast, cysteine (C) and serine (W) residues occur less frequently.

Functional enrichment analysis of specifically expressed proteins

To investigate the heterogeneity of the brain, heart, kidney, liver, and lung during development, we used heat map analysis of specific modification sites and functional enrichment analysis of specific expressed proteins from the five organs (Fig. 6). The figure depicts 982 specific Kcr modification sites on brain-specific expression proteins, 1,570 specific Kcr modification sites on heart-specific expression proteins, 158 specific Kcr modification sites

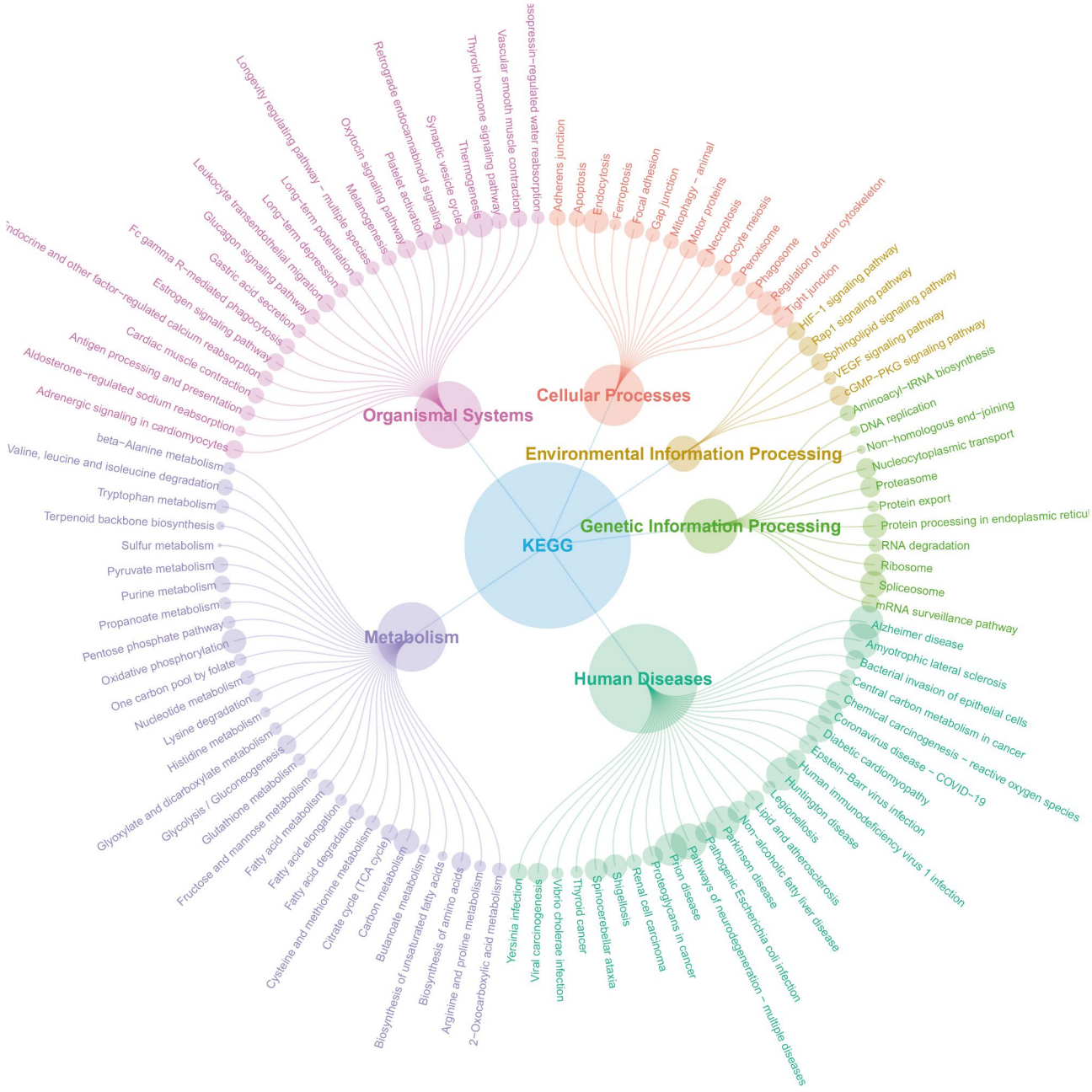


Fig. 4 Based on the results of KEGG enrichment analysis, a hierarchical network diagram depicts the six biological categories of co-expression Kcr proteins and their interrelationships in the brain, heart, kidney, liver, and lung organs

on kidney-specific expression proteins, 300 specific Kcr modification sites on liver-specific expression proteins, and 501 specific Kcr modification sites on liver-specific expressed proteins.

GO-BP analysis revealed that brain-specific expressed proteins were substantially linked with synapse organization axonogenesis, regulation of neuron projection development、modulation of chemical synaptic transmission, and regulation of transsynaptic signaling. Myofibril assembly mitochondrial gene expression, striated muscle cell development, cellular component assembly involved in morphogenesis, and mitochondrial translation biological processes functioned with significant correlation in the heart. Kidney-specific expression of proteins was significantly associated with the establishment or maintenance of epithelial cell apical/basal polarity, establishment or maintenance of apical/basal cell polarity, establishment or maintenance of bipolar cell polarity, positive regulation of T cell receptor signaling pathway, and positive regulation of antigen

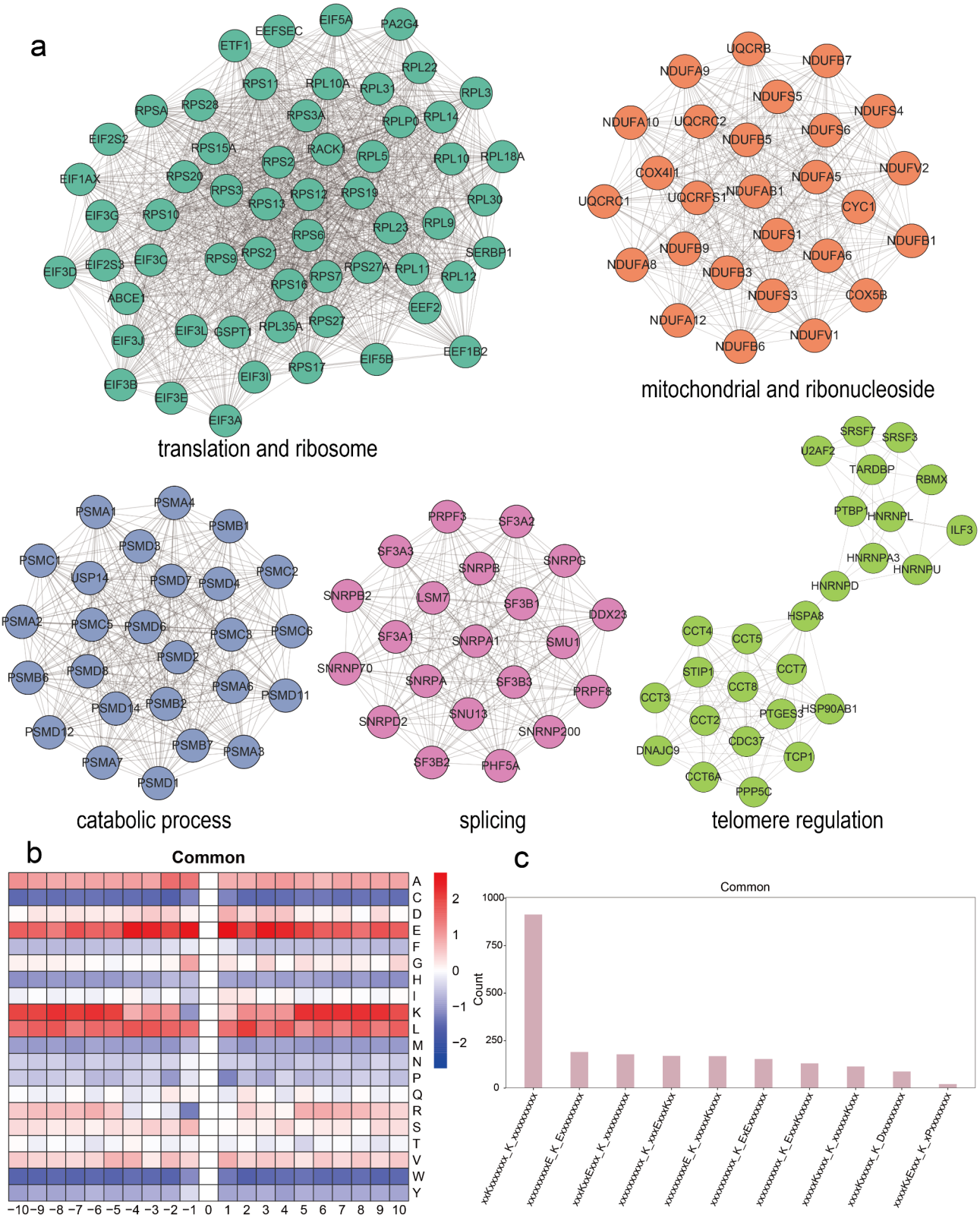


Fig. 5 PPI network analysis (**a**) and site motif analysis (**b**) of co-expression Kcr proteins. (**c**) Number of the Kcr peptides in each motif

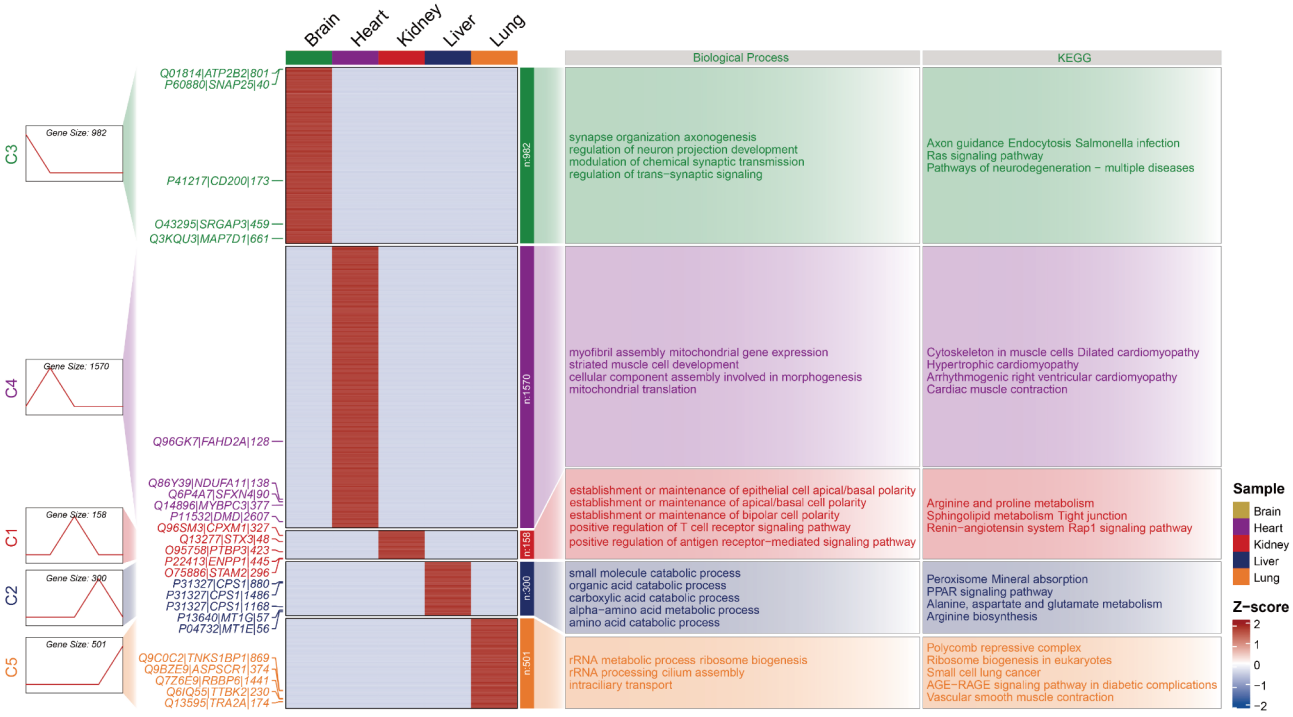


Fig. 6 Biological process analysis and KEGG analysis were used to examine the functional enrichment of Kcr proteins expressed in various organs

receptor-mediated signaling pathway. Liver-specific expressed proteins were significantly associated with small molecule catabolic process, organic acid catabolic process、carboxylic acid catabolic process, alpha-amino acid metabolic process, and amino acid catabolic process.

In addition, Axon guidance Endocytosis Salmonella infection, Ras signaling pathway, and Pathways of neurodegeneration-multiple diseases were specifically enriched in the brain according to KEGG enrichment analysis. Cytoskeleton in muscle cells Dilated cardiomyopathy, Hypertrophic cardiomyopathy, Arrhythmogenic right ventricular cardiomyopathy, and Cardiac muscle contraction were specifically enriched in the heart. Specifically expressed proteins in the kidney were significantly enriched in Arginine and proline metabolism, Sphingolipid metabolism Tight junction, and Renin-angiotensin system Rap1 signaling pathway. specifically expressed proteins in the liver were significantly enriched in Peroxisome Mineral absorption, PPAR signaling pathway, Alanine, aspartate and glutamate metabolism, and Arginine biosynthesis. Polycomb repressive complex, Ribosome biogenesis in eukaryotes, small cell lung cancer, AGE-RAGE signaling pathway in diabetic complications, and Vascular smooth muscle contraction were specifically enriched in lung.

Motif analysis of specifically expressed proteins

Motif analysis revealed that, overall, Kcr modification sites had comparable characteristics in all five organs, with considerable enrichment in glutamate (E), lysine (K), and valine (L) residues (Fig. 7). However, there were variations in the frequency of site enrichment. In brain organs, E residues were more common at modification sites 1, -1, and − 2, K residues at modification sites 8, -7, and − 9, and L residues at modification sites − 3 and − 4. In cardiac organs, E residues appeared in higher frequency at modification sites 1, -1, -2 and K residues appeared in higher frequency at modification sites 6, 7, -6, -9. In heart organs, E residues appeared in higher frequency at position − 1 of the modification site, K residues appeared in higher frequency at position 6 of the modification site, and L residues appeared in higher frequency at positions 1, 5, -4, -6, and − 7 of the modification site. In the liver organ, E residues appeared in higher frequency at modification sites 1 and − 1, K residues appeared in higher frequency at modification site 5, and L residues appeared in higher frequency at modification site 2. In lung organs, E residues appeared at higher frequency at modification site 3, K residues at modification site − 5, L residues at modification sites 4, -2, -3, -4, -7, and R residues at modification sites 5 and − 9.

Screening and motif analysis of highly modified sites for Kcr

Venn analysis results revealed a total of 5 significantly changed proteins in 5 organs: SPTBN1, HSP90AA1, MYH9, MYH10, and SPTAN1 (Fig. 8a). In addition, 12 highly modified proteins were specifically expressed in

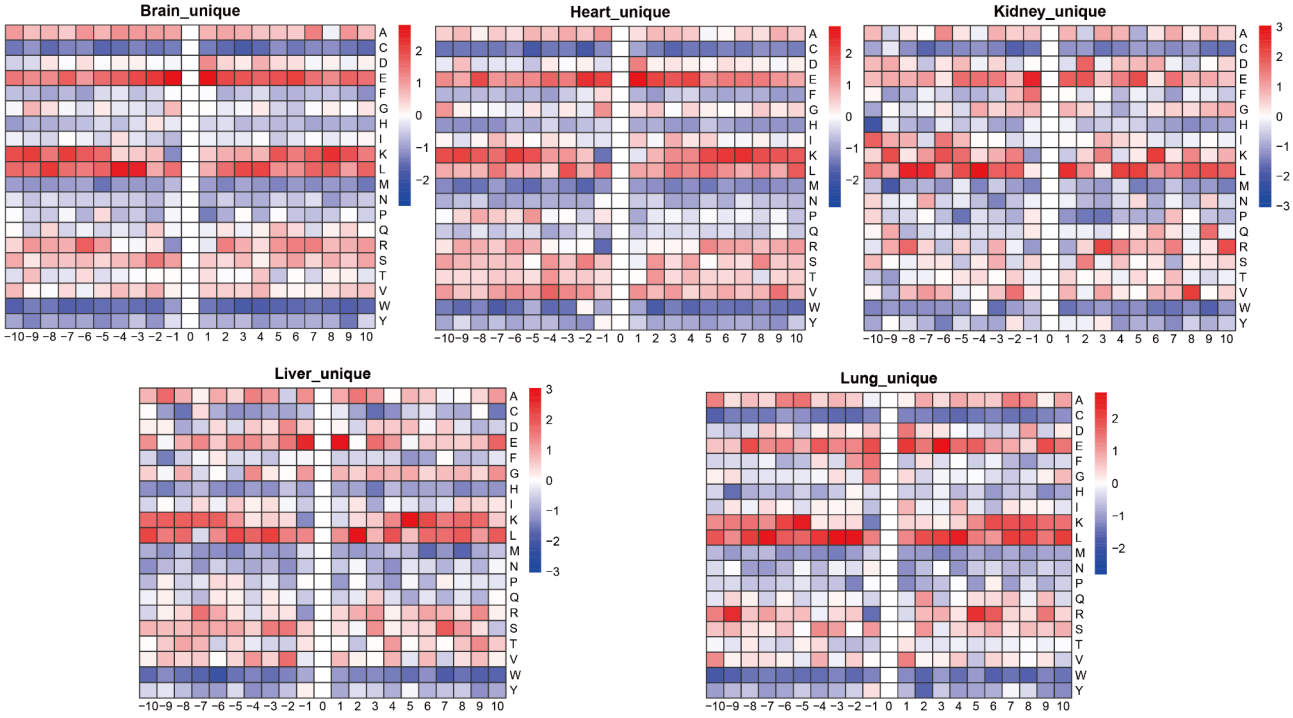


Fig. 7 Heatmap illustrating the amino acid composition around the Kcr modification site, as expressed in various organs, showing the frequency of the first ten different types of amino acids upstream and downstream of the residue. Red represents enrichment, whereas blue signifies depletion

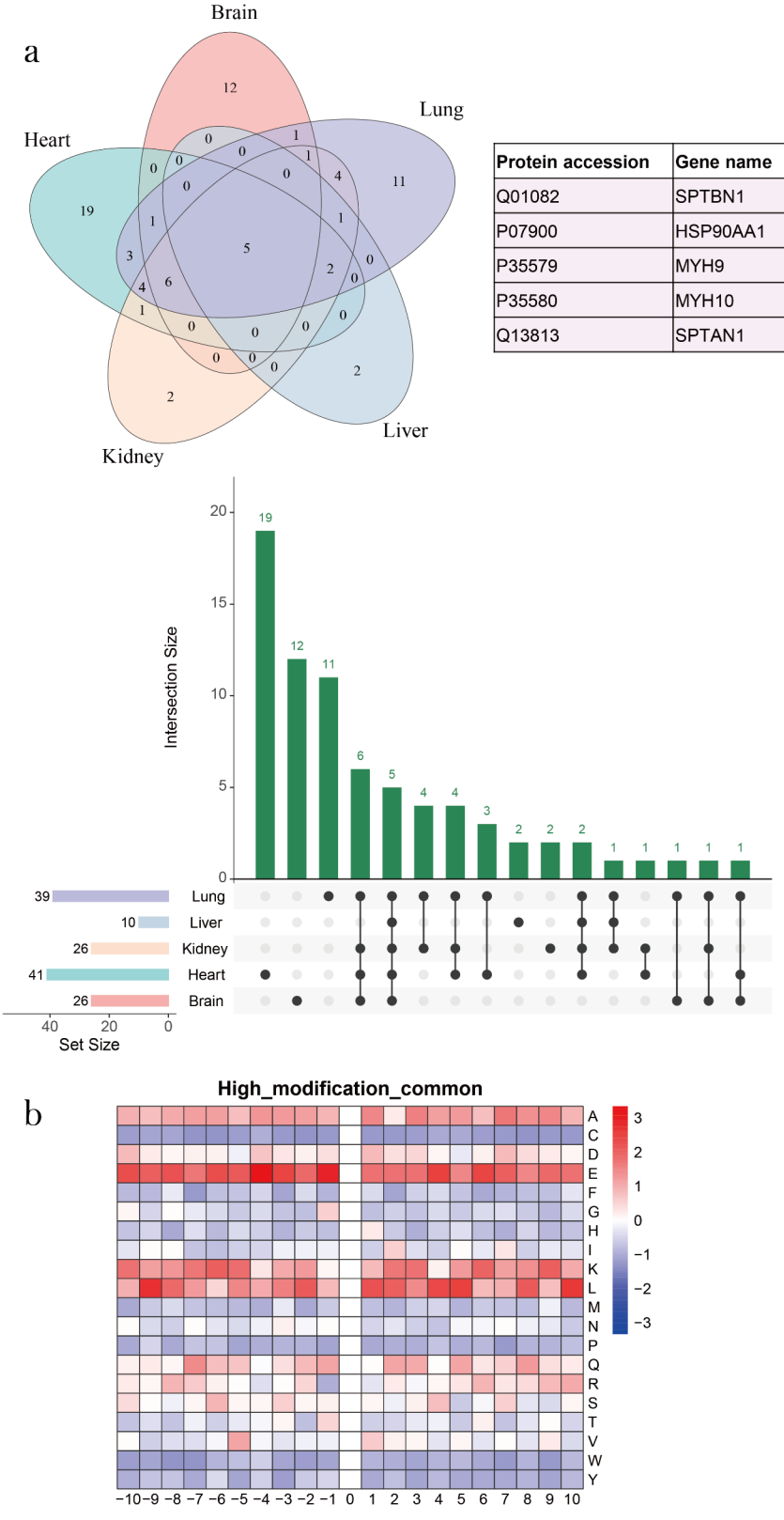
the brain, 19 in the heart, 2 in the kidney, 2 in the liver, and 11 in the lung. Meanwhile, alanine (A), glutamic acid (E), lysine (K), and valine (L) residues appeared at higher frequencies in the top 10 positions upstream and downstream of the highly modified sites, in contrast to cysteine (C) residues, which appeared at lower frequencies (Fig. 8b).

Discussion

Kcr is a novel histone PTM, or protein modification modality, that is primarily engaged in cellular metabolism, cell cycle, cell signaling, and cellular organization activities [7, 18, 19]. It has been proposed that methylation, acetylation, and ubiquitination in human preimplantation embryos perform complementary and fine-tuned regulatory roles in embryonic development [20]. However, no investigations of Kcr in the developing brain, heart, kidney, liver, and lung of 17-week fetus have been described. To better understand the Kcr modification of various organs in 17-week fetal development and its potential function during development, we used high-resolution LC-MS/MS and highly sensitive immunoaffinity purification. A total of 24,947 Kcr modification sites in 5,102 proteins distributed in the brain, heart, kidney, liver, and lung organs were identified (Fig. 1d), with 1,154 proteins co-expressed in five fetus organs (Fig. 2a). This is a report on lysine crotonylation protein map during 17-week fetal development in humans.

Resolving the geographic distribution of the fetus Kcr proteome at the subcellular level can help us better comprehend fetus development and illness. We mapped the in situ localization of 1,154 Kcr proteins co-expressed in brain, heart, kidney, liver, and lung organs to 30 subcellular structures at the single-cell level, revealing the Kcr proteome of nine major organelles, including the nucleus, cytoplasm, and mitochondria (Fig. 2b). Furthermore, these co-expressed Kcr proteins are involved in a wide range of biological processes, such as multiple cytoplasmic translation, protein folding, aerobic respiration, genetic material creation, and metabolic processes (Fig. 3). Kcr modifications have been shown to impact chromatin structure, transcription, translation, and nucleosome assembly, all of which impact gene expression and control the development of organs [21]. Protein interaction network study show that several interactions in these biological processes may be linked to Kcr modification. As a result, Our results show that Kcr modification in 17-week-old fetuses' brain, heart, kidney, liver, and lung may influence genetic material transcription and translation, energy production, and metabolic activities.

It's interesting that our research revealed that the Kcr proteins that are expressed in the brain, heart, kidney, liver, and lung all perform distinct tasks. Proteins that were specifically expressed in brain tissue were closely linked to developmental processes and the central nervous system; in heart tissue, they were linked to the motility and development of cardiomyocytes; in kidney



Protein accession	Gene name
Q01082	SPTBN1
P07900	HSP90AA1
P35579	MYH9
P35580	MYH10
Q13813	SPTAN1

Organ	Protein accession	Gene name
Lung	Q05682	CALD1
Lung	P12111	COL6A3
Lung	Q14247	CTTN
Lung	O43491	EPB41L2
Lung	P46940	IQGAP1
Lung	P35749	MYH11
Lung	Q7Z406	MYH14
Lung	P13667	PDIA4
Lung	Q92878	RAD50
Lung	Q14683	SMC1A
Lung	P12956	XRCC6
Liver	P31327	CPS1
Liver	P02549	SPTA1
Brain	Q01484	ANK2
Brain	Q3V6T2	CCDC88A
Brain	Q5SW79	CEP170
Brain	Q9UDT6	CLIP2
Brain	Q14203	DCTN1
Brain	P78559	MAP1A
Brain	P46821	MAP1B
Brain	P11137	MAP2
Brain	Q9Y4I1	MYO5A
Brain	P07197	NEFM
Brain	Q9NQC3	RTN4
Brain	Q9UQE7	SMC3
Kidney	Q99715	COL12A1
Kidney	P30101	PDIA3
Heart	P35609	ACTN2
Heart	P08133	ANXA6
Heart	Q6ZP82	CCDC141
Heart	P11532	DMD
Heart	Q14315	FLNC
Heart	P24043	LAMA2
Heart	P07942	LAMB1
Heart	P11047	LAMC1
Heart	Q14896	MYBPC3
Heart	P13533	MYH6
Heart	P12883	MYH7
Heart	P52179	MYOM1
Heart	O76041	NEBL
Heart	Q86VF7	NRAP
Heart	Q5VST9	OBSCN
Heart	Q92736	RYR2
Heart	P11277	SPTB
Heart	Q9Y4G6	TLN2
Heart	Q8WZ42	TTN

Fig. 8 Motif analysis of crotonylation high modification sites, with more than 20 modification sites in each organ. (**a**) Venn diagram showing the high modification sites of each organ. (**b**) A heatmap of high modification1 sites that are co-expressed in the brain, heart, kidney, liver, and lung

and liver tissues, they were primarily linked to material catabolism, absorption, and metabolic pathways; and in lung tissues, they were linked to cilia assembly, transport, and rRNA metabolic processes (Fig. 6). According to reports, Kcr are crucial for neurogenesis and synaptogenesis in the mature brain [22]. Our findings shows that Kcr during the 17-week fetal period are associated with neurogenesis and synaptogenesis in the brain. These findings indicate that Kcr plays a crucial role in fetal life-sustaining activities and development, however the precise mechanism of action requires additional investigation. Motif analysis revealed the conserved and particular nature of these proteins Kcr sites, which could be important for understanding their biological roles. In our study, we analyzed the amino acid sequence features around Kcr in various organs such as the brain, heart, kidney, liver, and lung to explore the pattern of Kcr during fetus development, and we discovered that the amino acids around Kcr had similar features in the brain, heart, kidney, liver, and lung (Fig. 7).

In our study, SPTBN1, HSP90AA1, MYH9, MYH10, and SPTAN1 highly modified Kcr proteins were discovered to be co-expressed in fetal brain, heart, kidney, liver, and lung (Fig. 8a). It has been established that maternal smoking influences the level of HSP90AA1 protein expression in fetal liver [23]. MYH9 is expressed in both the fetal and mature kidneys [24]. Abnormal MYH9 expression may cause fetal lung hypoplasia, culminating in congenital diaphragmatic hernia [25]. However, no studies on the expression levels of SPTBN1, HSP90AA1, MYH9, MYH10, or SPTAN1 crotonylation modifications have been published. In addition, a number of highly modified proteins are expressed in particular organs: 12 in the brain, 19 in the heart, 2 in the kidney, 2 in the liver, and 11 in the lung (Fig. 8a). Several investigations have found that these particular proteins perform critical functions in organ development. For example, CALD1 is associated with idiopathic pulmonary fibrosis when highly expressed in fibroblasts [26]. CTTN is a significant PTM substrate that contributes to the development of a variety of lung disorders [27]. Specific significantly changed proteins in the fetal lung are intimately linked to pulmonary fibrosis [28–30]. CPS1 is specifically modified in the liver, and its protein expression level decreases after autophagy abnormalities [31]. The expression of SPTA1 in the liver influences glucose metabolism in pregnant mice [32]. Abnormal expression of ANK2 is linked to epilepsy, and deletion of ANK2 in the mouse fetal alters the synaptic proteome, increases neuronal activity and synchronization, and causes seizures or death [33]. MAP1A and MAP1B are brain developmental regulator proteins that are primarily involved in axon production [34]. NEFM expression influences the proliferation and survival of embryonic sympathetic neuroblasts [35]. ANXA6 can restore muscular membranes in the heart [36]. It is clear that these specially expressed proteins serve critical roles during development. This highly modified Kcr protein could be useful in future fetal development research.

We used high-resolution LC-MS/MS and highly specific immunoaffinity purification techniques to determine crotonylation profiles in 17-week fetal brain, heart, kidney, liver, and lung tissues. This study provides useful information on Kcr levels in various organs of the 17-week fetus. In order to better understand the crotonylation modifications in the development of various organs in the fetus, we will further explore the dynamic changes of crotonylation in organ development at different developmental periods of the fetus and how these changes affect organ function and disease occurrence.

Conclusion

This study provides commonality and specificity of lysine crotonylation modifications in the development of brain, heart, kidney, liver, and lung organs during the 17-week fetal period, and reveals that the synthesis, transcription and translation, energy production, and metabolism processes of genetic material during the 17-week fetal period are closely related to crotonylation modifications, which will help to promote the future studies on the mechanisms of fetal development.

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Author contributions

LH wrote the manuscript. DT, SX and YD designed the experiments and supervised the study. HC and QY performed the experiments. ZZ and YW collected biological samples from the subjects. HG and WS provided clinical information. JG and JM analyzed the sequencing results. LL supervised the experiment. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shenzhen People's Hospital (LL-KY-2023080-01). Additionally, an informed consent document was signed by the patients.

Competing interests

The authors declare no competing interests.

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