# Integrated Proteome and Phosphoproteome Analyses Reveal Early and Late-stage Protein Network of Traumatic Brain Injury

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# Abstract

Traumatic brain injury (TBI) is a major public health concern all around the world. Accumulating evidence suggests that pathological processes after brain injury continuously evolve. Here, we identified the differentially expressed proteins (DEPs) and differentially expressed phosphoproteins (DEPPs) in the early and late stages of TBI in mice using TMT labeling, enrichment of Phos affinity followed, and high-resolution LC-MS/MS analysis. Subsequently, integrative analyses, including functional enrichment-based clustering analysis, motif analysis, cross-talk pathway/process enrichment analysis, and protein-protein interaction enrichment analysis were performed to further identify the different and similar pathophysiologic mechanisms in the early and late stage. Our work reveals a map of early and late-stage protein networks in TBI, which shed light on useful biomarkers and the underlying mechanisms in TBI and its sequelae.


# 1. Introduction

Traumatic brain injury (TBI) is a major public health concern all around the world. In particular, China has more patients with TBI than most other countries in the world, with a mortality of approximately 13 cases per 100,000 people[1]. TBI may lead to various clinical states in the early and late stages, such as acute seizures and epilepsy in the early stage, neuroendocrine dysfunction, depression, post-traumatic stress disorder, chronic traumatic encephalopathy, and so on in the late stage[2–5]. Accumulating evidence suggests that pathological processes after brain injury continuously evolve[5–7]. Therefore, it's necessary to further illustrate the development of TBI, to compare the different and similar pathophysiologic mechanisms in the early and late stages.

Brain damage in TBIs is caused by rotational or linear acceleration forces, or by blunt trauma with impact deceleration[8], and sometimes even by penetrating head injury[9]. No matter what kind of damage, proteins play a critical role in the development of pathophysiologic progress after TBI because proteins are the direct executors of biological functions. For example, postsynaptic scaffold protein Preso promotes signaling which goes through from NMDAR to NO, and thus facilitates excitotoxicity after neuronal injury[10]. In addition, cis phosphorylated protein tau contributes to the pathology development of TBI, and is important for the progress of sequelae after TBI[11]. The interactions among proteins or phosphoproteins link the different biological functions together, and may be helpful for illustration of the pathophysiologic mechanisms after TBI and the therapies of TBI and its sequelae.

In this study, differentially expressed proteins (DEPs) and differentially expressed phosphoproteins (DEPPs) were identified in the early and late stages of TBI in mice using TMT labeling, enrichment of Phos affinity, and high-resolution LC-MS/MS analysis. Integrative analyses, including functional enrichment-based clustering analysis, motif analysis, cross-talk pathway/process enrichment analysis, and protein-protein interaction enrichment analysis were performed to further identify the different and similar pathophysiologic mechanisms in the early and late stage, thereby contributing to a map of early

and late-stage protein network in TBI. The interacting proteins may shed light on the process of pathophysiologic development, and may be helpful for future therapies.

## 2. Materials And Methods

# 2.1. Animals and Traumatic Brain Injury Model

Eighteen C57BL/6 mice (twelve weeks, 25~30g, each group: 3, total: 18) that were provided by Fourth Military Medical University were fed in an air-conditioned house (27°C) for seven days and exposed to a 12 hours light/dark cycle. The animal study was performed under the guidance of the National Institutes of Health Guide for the Care and Use of Laboratory Animals at the Fourth Military Medical University.

The cortical controlled impact (CCI) model was selected as the TBI model *in vivo*. The mice were placed in the stereotactic frame after anesthesia (oxygen flow: 800 mL/min, isoflurane concentration: 4%). Incise the skin in the middle, expose the skull, and keep the dura intact carefully. The right cortex was hit with an actuator (3 mm in diameter) at a rate of 3 m/s and 1.5 mm which was compressed to cause damage. Subsequently, the vulnus was sutured and the wound was infiltrated with lidocaine cream. At the end of the surgery, mice were returned to their cages with free access to water and food. After 1 day and 7 days, brain samples from the injured area were extracted and used for further experiments.

# 2.2. Trypsin Digestion

After common protein extraction[23], the DTT (10mmol/L) was used to reduce the protein liquid (37°C, 1 hour). Subsequently, the IAA (20mmol/L) was used to alkylate the mixture (40 min, 25°C, keep in the dark). The TEAB (100mmol/L) was added to the protein solution to dilute the urea until 2mol/L. After this, the trypsin (trypsin: protein = 1: 50, mass ratio) was added to the protein solution for the first digestion (8 hours). The second digestion (4 hours) was used the trypsin (trypsin: protein = 1: 100, mass ratio).

# 2.3. TMT Labeling and HPLC Fractionation

The Strata X C18 SPE column was used to desalt the peptide that was digested by trypsin in the 5.2 part. After vacuum-drying, the TEAB (0.5 mol/L) was used to reconstitute the peptide. Subsequently, the 6-plex TMT kit was used to process it following the manufacturer's protocol (the quantity of the TMT reagent which was enough for labeling 100 μg of the protein was defined as one unit). The mixtures were desalted and vacuum-dried after the incubation of 2 hours (27°C). The Agilent 300Extend C18 column, whose internal diameter is $4.6\times10^{-3}$ m, length is 0.25 m, and particle size is $5\times10^{-6}$ m, was used to separate the specimens through high pH reverse-phase HPLC according to the manufacture's protocol.

# 2.4. Affinity Enrichment

For the identification of phosphoproteins only. The IMAC microspheres were added to the peptide solution following the manufacture's protocol and then the mixture was incubated vibrantly. After removing the supernatant, the precipitation was collected through centrifugation following the manufacture's protocol. Subsequently, the precipitation was washed by the ACN/TFA solution (50%: 6%, and then 30%:0.1%,

respectively), according to the manufacture's protocol. Then the 10% $NH_3$•$H_2O$ elution buffer was added to the washed precipitation and the mixture was incubated vibrantly following the manufacture's protocol. Finally, the supernatant was collected and lyophilized.

## 2.5. LC-MS/MS Analysis

The 0.1% FA was used to dissolve the collected peptides. The solution was loaded onto the reversed-phase pre-column. The reversed-phase analytical column was used to separate peptides. The concentration gradient was from a concentration of 7%~20% B solvent (98% CAN, 0.1% FA) for 25 min, to a concentration of 20%~35% for 8 min, and then up to a concentration of 80% for 3 min. Finally, the concentration was kept for 5 min. The study was performed using the EASY-nLC 1000 UPLC system, which controlled the flow rate of 300 ml/min. The Q ExactiveTM plus hybrid quadrupole-Orbitrap mass spectrometer (ThermoFisher Scientific) was used to analyze the result.

The tandem mass spectrometry (MS/MS) in Q ExactiveTM plus which was coupled online to the UPLC was used to process the peptides after the acceptance of the NSI source. The NCE was set to 30 for LC-MS/MS peptides detection, and the resolution was set to 70,000 to text the intact peptides while the resolution was set to 17,500 to detect the ion fragments in the Orbitrap. The top 20 precursor ions that exceed the threshold of $2\times10^4$ were scanned with 30 s dynamic exclusion in the MS survey system. And the procedure based on data that commutes from one MS scan to 20 LC-MS/MS scan was used to identify the top 20 precursor ions. The electrospray voltage was set to 2 kV. To keep the ion trap from overfilling, automatic gain control (AGC) was applied. The application of LC-MS/MS spectra was thresholded by the amount of $5\times10^4$ ion. The m/z scan range was set from 350 to 1800 in MS scan while the fixed first mass was set to 100m/z.

## 2.6. Database Search

The Mascot search engine (v.2.3.0) for proteome and MaxQuant with an integrated Andromeda search engine (v.1.4.1.2) for phosphoproteome were used to analyze proteome and phosphoproteome respectively. The Swissport Mouse Database was used as the reference resource. For proteome, trypsin/P, which was recognized as a special cleavage enzyme, was set up to 2 missing cleavages. For phosphoproteome, the trypsin/P was set up to two missing cleavages, five modifications per peptide, and five charges. The error was set to 10 ppm and 0.02 Da for precursor ions and fragment ions respectively. The false discovery rate (FDR) which was used for the identification of peptides, modification sites was set to 0.01. The TMT-6-plex was used for protein quantification. The peptides whose length was less than 7 amino acids were ignored.

## 2.7. Quality Control

The MS data validations for proteome (Supplementary Figure S1A&B) and phosphoproteome (Supplementary Figure S1C&D) were performed. The distribution of mass error is around zero, most of which are less than 0.02 Da (Supplementary Figure S1A&C). Furthermore, the length in proteome

distributed from 8 to 16 (Supplementary Figure S1B) while the length distributed from 8 to 20 (Supplementary Figure S1D). The results revealed that the samples meet the criteria.

## 2.8. Functional Enrichment-based Clustering Analysis

The functional enrichment analysis was based on the KEGG Pathway database, GO Biological Process database, and InterProscan database. The p-value which less than 0.05 was deemed as significant (two-tailed Fisher's exact test). The functional enrichment categories whose p-value was less than 0.01 were further transformed through the function

$$x = -\log_{10}\left(p_{value}\right)$$

And then the results were further transformed for standard Z-Distribution. Finally, the Euclidean distance and average linkage clustering were used to re-cluster the transformed z-scores through one-way hierarchical clustering. The results were depicted using the 'heatmap.2' function in the 'gplots' package in R software.

## 2.9. Motif Analysis

Soft motif-x was applied for motif analysis. The sequence sites around the modify-21-mers, which come from the -10 position to the +10 position, were identified. All parameters were set as default.

## 2.10. Cross-talk Pathway and Process Enrichment Analysis

Cross-talk pathway and process enrichment analysis were performed based on the KEGG Pathway database, GO Biological Processes database, Reactome Gene Sets database, CORUM database, TRRUST database, and PaGenBase database. P-value was set to 0.01, the minimum count was set as 5, and the enrichment factor was set to 1.5. The results were grouped and categorized into different clusters according to the similarities which were set to 0.3 and calculated using Kappa scores. The clusters that share a similarity were connected by edges. The map is depicted using Metascape[24] and Cytoscape[25].

## 2.11. Protein-protein Interaction Enrichment Analysis

The protein-protein interaction enrichment analysis was performed using the BioGrid database[26]. The Molecular Complex Detection (MCODE) algorithm[27] was used to construct and refine the protein-protein interaction network. The protein-protein interactions are depicted using Metascape[24] and Cytoscape[25].

# 3. Results

*3.1. Identification of DEPs and DEPPs in Early and Late stages of TBI*

In proteome profiling, 4586 protein groups were identified. The cutoff of fold-change was set as more than 1.2 or less than 0.83. Totally 3423 proteins were quantified, where 123 proteins are high-expressed

and 49 proteins are low-expressed in group 1d/C, and 109 proteins are high-expressed and 9 proteins are low-expressed in group 7d/C, and 129 proteins are high-expressed and 88 proteins are low-expressed in group 7d/1d when compared to the control sample (Figure 1A).

The TMT labeling, enrichment of Phos affinity, and high-resolution LC-MS/MS analysis were applied to quantitative phosphoproteomics analysis. Among the 2229 protein groups, 5961 phosphorylation sites were recognized, where 4095 sites in 1656 proteins were quantified. The cutoff of fold-change was set as more than 1.2 or less than 0.83. Among the quantified proteins, 315 proteins are high-expressed and 453 proteins are low-expressed in group 7d/C, 360 proteins are high-expressed and 738 proteins are low-expressed in group 1d/C, and 682 proteins are high-expressed and 508 proteins are low-expressed in group 7d/1d when compared to the control sample (Figure 1B).

*3.2. Functional Enrichment-based Clustering Analyses of DEPs and DEPPs at Early and Late stage in TBI*

The functional enrichment analyses based on DEPs (Figure 1C) or DEPPs (Figure 1D), including biological process, molecular function, cellular component, and KEGG pathway analyses, were clustered to compare the difference in group 1d/C, 7d/1d, and 7d/C.

Based on DEPs, the different biological processes in group 1d/C are multi-organism process, regulation of hydrolase activity, and acute-phase response, etc, while the different biological processes in group 7d/C are neuron projection regeneration, regulation of protein modification, etc, and the different biological processes in 7d/1d are regulation of cell growth and cholesterol transport, etc, suggesting a process from acute-phase response to neuron repairing after brain trauma. The different molecular functions in group 1d/C are serine-type endopeptidase inhibitor activity, phospholipase inhibitor activity, etc, while the different molecular functions in group 7d/C are phosphatidylcholine-sterol O-acyltransferase activator activity, etc, suggesting a modification of phosphorylation after brain trauma. The different cellular components in group 1d/C are extracellular vesicles and exosomes, etc, while the different cellular components in group 7d/C are fibrinogen and chylomicron, etc, suggesting a transformation from early cell-cell communications to late coagulation.

Based on DEPPs, the different biological processes in group 1d/C are the establishment of protein localization to the plasma membrane, cell migration, actin filament bundle assembly, etc, while the different biological processes in group 7d/C are neuron projection, signaling, etc, and the different biological processes in group 7d/1d are negative regulation of MAPK activity, etc, suggesting an early cell-cell communication that mediates signalings to result in neuron repairment. The different cellular components in group 1d/C are leading edge membrane, etc, while the different cellular components in group 7d/C are adhering junction, synapse, etc. The different KEGG pathways in group 1d/C are VEGF signaling, oxytocin signaling, Rap1 signaling, etc, while the different KEGG pathways in group 7d/C are calcium signaling, NOD-like receptor signaling, retrograde endocannabinoid signaling, etc, and the different KEGG pathway in group 7d/1d is insulin resistance.

*3.3. Motif Analysis of The Phosphosites*

We identified 34 conserved motifs based on serine (S) phosphosites, 8 conserved motifs based on threonine (T) phosphosites, and 3 conserved motifs based on tyrosine (Y) phosphosites (Figure 2A). In particular, motif [xxxxxxxxxx_pS_PExxxxxxxx], [xxxxxxxRRx_pS_xxxxxxxxxx], [xxxxxxxxxx_pS_DxExxxxxxx], [xxxxxxxxxx_pS_EEExxxxxxx], [xxxxxxxxxx_pS_xDExxxxxxx], [xxxxxxxRxx_pS_Lxxxxxxxxx], and [xxxxxxxxxx_pT_SPxxxxxxxx] were strikingly conserved with motif score > 30. Among these motifs, [xxxxxxxxxx_pS_EEExxxxxxx] and [xxxxxxxxxx_pT_SPxxxxxxxx] are most significant with fold change > 10. The motif [xxxxxxxxxx_pS_EEExxxxxxx] was identified as the Casein Kinase II substrate motif[12], while the motif [xxxxxxxxxx_pT_SPxxxxxxxx] was a novel motif.

Further, the heatmaps were depicted to illustrate the enrichment or depletion of specific amino acids around the phosphosites of the serine (S) (Figure 2B), threonine (T) (Figure 2C), and tyrosine (Y) (Figure 2D). The amino acids asparticacid (D), glutamicacid (E), lysine (K), and arginine (R) tended to be present in the proximity of serine phosphosites. The amino acids asparticacid (D), glutamicacid (E), proline (P), serine (S), and arginine (R) tended to be present in the proximity of threonine phosphosites. The amino acids asparticacid (D), glutamicacid (E), lysine (K), valine (V), and arginine (R) tended to be present in the proximity of tyrosine phosphosites. Besides, Arginine (R) was increasingly presented at the sites around the serine phosphosites and threonine phosphosites but was strikingly depleted at +1 and +2 positions (Figure 2B&C). Interestingly, Arginine (R) was inclined to be presented at the sites surrounding the tyrosine phosphosites while being depleted at -1 and +1 positions (Figure 2D). Besides, lysine (K) was greatly presented at the sites surrounding the tyrosine phosphosites but was strikingly depleted at +1 and +3 positions (Figure 2D). Considering that we identified the different molecular functions in group 1d/C are serine-type endopeptidase inhibitor activity, phospholipase inhibitor activity, while the different molecular functions in group 7d/C are phosphatidylcholine-sterol O-acyltransferase activator activity, these special amino acids near the phosphosites may reflect the preferable enzymes that catalyze phosphorylation after the brain trauma.

### *3.4. Common Cross-talk Pathway/Process and Protein-protein Interacting Analyses of DEPs and DEPPs at Early and Late stage in TBI*

The common cross-talk pathway/process enrichment analysis of DEPPs (Figure 3A) and DEPs (Figure 3B), and protein-protein interaction enrichment analysis of DEPPs (Figure 3C) and DEPs (Figure 3D) were performed to further identify the connections and similarities among different functional clusters.

For cross-talk pathway and process enrichment analysis of DEPPs (Figure 3A), the identified pathways and processes were clustered according to their similarity functions. The top 20 clusters further clustered into four separate super-clusters in group 1d/C and five separate super-clusters in group 7d/C. For cross-talk pathway and process enrichment analysis of DEPs (Figure 3B), the top 20 clusters further clustered into a super-cluster in group 1d/C and three separate super-clusters in group 7d/C.

For protein-protein interaction enrichment analysis of DEPPs (Figure 3C), the interactive proteins clustered into three main clusters in group 1d/C, where PCE/CE pathway, WNT signaling, chemical synapse

transmission, small GTPase mediated signaling, and RAS signaling are important. In group 7d/C, the main cluster is the synapse and postsynapse-related proteins, suggesting the importance of chemical synapse transmission. In addition, in group 7d/C, the cluster regulation of NMDA receptors and neuron projection is also important. For protein-protein interaction enrichment analysis of DEPs (Figure 3D), in group 1d/C, the main clusters are post-translation protein phosphorylation, platelet degranulation, acute response to elevated platelet cytosolic Ca2+, and respiratory complex biogenesis, suggesting early protein phosphorylation and acute-phase response. In group 7d/C, the main clusters are post-translational protein phosphorylation, platelet degranulation, regulation of insulin-like growth factor (IGF) transport and uptake.

### *3.5. Special Cross-talk Pathway/Process and Protein-protein Interacting Analyses of DEPs and DEPPs at Early and Late stage in TBI*

The special cross-talk pathway/process and protein-protein interacting analyses of DEPPs (Figure 4A) and DEPs (Figure 4B) were performed to further identify the in-depth connections behind the cross-talk pathway and process.

For DEPPs, the special cross-talk pathway/processes that are only presented in group 1d/C (involving 468 proteins) are cell part morphogenesis, membrane trafficking, synapse organization, etc, where the main protein-protein interactions are involved in UCH proteinases, mitotic prometaphase, vesicle docking, etc. The special cross-talk pathway/processes that are only presented in group 7d/C (involving 191 proteins) are the regulation of cell morphogenesis, actin filament-based process, small GTPase mediated signal transduction, etc. The special cross-talk pathway/processes that are both presented in group 1d/C and 7d/C (involving 505 proteins) are neuron projection morphogenesis, modulation of chemical synaptic transmission, etc, where the main protein-protein interactions are involved in localization with/to the membrane, modulation of chemical synaptic transduction, signaling by RhoGTPase, cellular responses to stress, etc.

For DEPs, the special cross-talk pathway/processes that are only presented in group 1d/C (involving 125 proteins) are regulation of the microtubule-based process, supramolecular fiber organization, neutrophil degranulation, etc, where the main protein-protein interaction is involved in respiratory chain complex biogenesis. The special cross-talk pathway/processes that are only presented in group 7d/C (involving 71 proteins) are cell junction organization, negative regulation of cellular component organization, vacuole organization, etc. The special cross-talk pathway/processes that are both presented in group 1d/C and 7d/C (involving 47 proteins) are platelet degranulation, complement and coagulation cascades, negative regulation of hydrolase activity, etc, where the main protein-protein interactions are involved in post-translational protein phosphorylation, platelet degranulation, regulation of insulin-like growth factor (IGF) transport and uptake.

## 4. Discussion

# 4.1. Neuron Projection and Recovery

The cluster neuron projection are represented both in 1d/C and 7d/C groups of DEPPs (Figure 3A), suggesting that it's important through the early and late stage after brain trauma, which was consistent with that neuronal swelling may exert protective effects against damaging excitability in the aftermath of TBI[13]. Small GTPase mediated signal transduction is isolatedly represented in group 7d/C while absent in group 1d/C of DEPPs (Figure 3A), suggesting that small GTPase mediated signaling may play a more separate function in the late stage after brain trauma[14]. A recent study also shows that knockdown of small GTPase RAC1 could strikingly facilitate the recovery of functions in neurons [15]. The cluster gliogenesis is special in group 7d/C of DEPs (Supplementary Figure S3A), suggesting a neuron repairment at the late stage after brain trauma. In fact, a recent study also reveals that IGF-1 promotes gliogenesis and improves cognitive function[16]. It's necessary to research whether the early-stage protein phosphorylation leads to late-stage gliogenesis in the future. For protein-protein interaction enrichment analysis of DEPPs (Figure 3B), in group 7d/C, the cluster regulation of NMDA receptors and neuron projection is also important, which is consistent with our former work[10]. More work and studies are needed to illustrate whether the early-stage PCE/CE pathway, WNT signaling, chemical synapse transmission, small GTPase mediated signaling, and RAS signaling lead to the late-stage neuron projection. Regulation of IGF transport and uptake-related proteins were the most important interacting protein classes that were presented both in group 1d/C and 7d/C of DEPs (Supplementary Figure S4), suggesting it may serve as a therapeutic target for early injury and late restoration. Recent research shows that growth hormone (GH) and IGF deficiency after TBI may inhibit the recovery of the axon and neurons, and thus GH/IGF-I system would be benefit for the therapy of TBI[17–19].

# 4.2. Neurodegeneration Diseases

The cluster chemical synaptic transmission are represented both in 1d/C and 7d/C groups of DEPPs (Figure 3A), suggesting that it may play a critical role through the early and late stage after brain trauma, which was consistent with that synaptic dysfunction plays a critical role in neurodegeneration diseases after TBI[20]. Modulation of chemical synaptic transmission-related proteins were identified as one of the most important interacting protein classes that were presented both in group 1d/C and 7d/C of DEPPs (Figure 4), suggesting signaling mediated by chemical synapse was important through early and late stage of TBI. Recent studies have also shown that TBI plays a critical role in the regulation of synapse function in the early and late stages, resulting in multiple secondary injury processes, such as excitotoxicity, inflammation, oxidative stress, and thus dementia later in life[21].

# 4.3. Others

UCH proteinases were identified as one of the most important interacting proteins which were only presented in group 1d/C of DEPPs (Figure 4), suggesting they are important in the early stage of TBI and have an early diagnosis potential. A recent study reveals that serum GFAP and UCH-L1 are sensitive for the prediction of intracranial injuries[22]. Cluster platelet degranulation represents both in group 1d/C and

7d/C of DEPs (Supplementary Figure S3A), suggesting a continuous hemorrhage. The cluster post-translational protein phosphorylation is special in group 1d/C of DEPs (Supplementary Figure S3A), suggesting protein phosphorylation at the early stage after brain trauma. Respiratory chain compex□biogenesis-related proteins (NDUFV3, NDUFS6, NDUFB7) were the most important proteins that were only represented in group 1d/C of DEPs (Supplementary Figure S4), suggesting that energy supply is critical in the early stage in TBI. However, there's little research that focuses on these proteins in TBI. Further studies are needed to research the functions of these proteins in TBI.

# 5. Conclusions

We identified the DEPs, DEPPs, cross-talk pathways/processes, and interacting proteins in the early and late stages of TBI. PCE/CE pathway and β-catenin independent Wnt signaling (PSMA, SYN, etc), chemical synapse signaling (DLG, LQSEC, etc), and small GTPase mediated signaling transmission pathway (GRIA, etc), UCH proteinase signaling (PSMA, PSMB, etc)-related proteins are the most important interacting proteins in the early stage of TBI while the NMDA signaling-related proteins (DNM, etc) are the most important interacting proteins in the late stage TBI. The DEPs or DEPPs in the early stage may contribute to the acute phase response of TBI and the DEPs or DEPPs in the late stage may play a critical role in TBI sequelae.

# 6. Abbreviation

Traumatic brain injury (TBI), differentially expressed proteins (DEPs), differentially expressed phosphoproteins (DEPPs), serine (S), threonine (T), tyrosine (Y), asparticacid (D), glutamicacid (E), lysine (K), arginine (R), proline (P), valine (V), insulin-like growth factor (IGF), growth hormone (GH), cortical controlled impact (CCI).

# 7. Declarations

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**Conflicts of Interest:**

The authors declare no conflict of interest.

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Not applicable.

**Code availability:**

Not applicable.

**Ethics approval:**

The animal study was performed under the guidance of the National Institutes of Health Guide for the Care and Use of Laboratory Animals at the Fourth Military Medical University.

**Consent to participate:**

Not applicable.

**Consent for publication:**

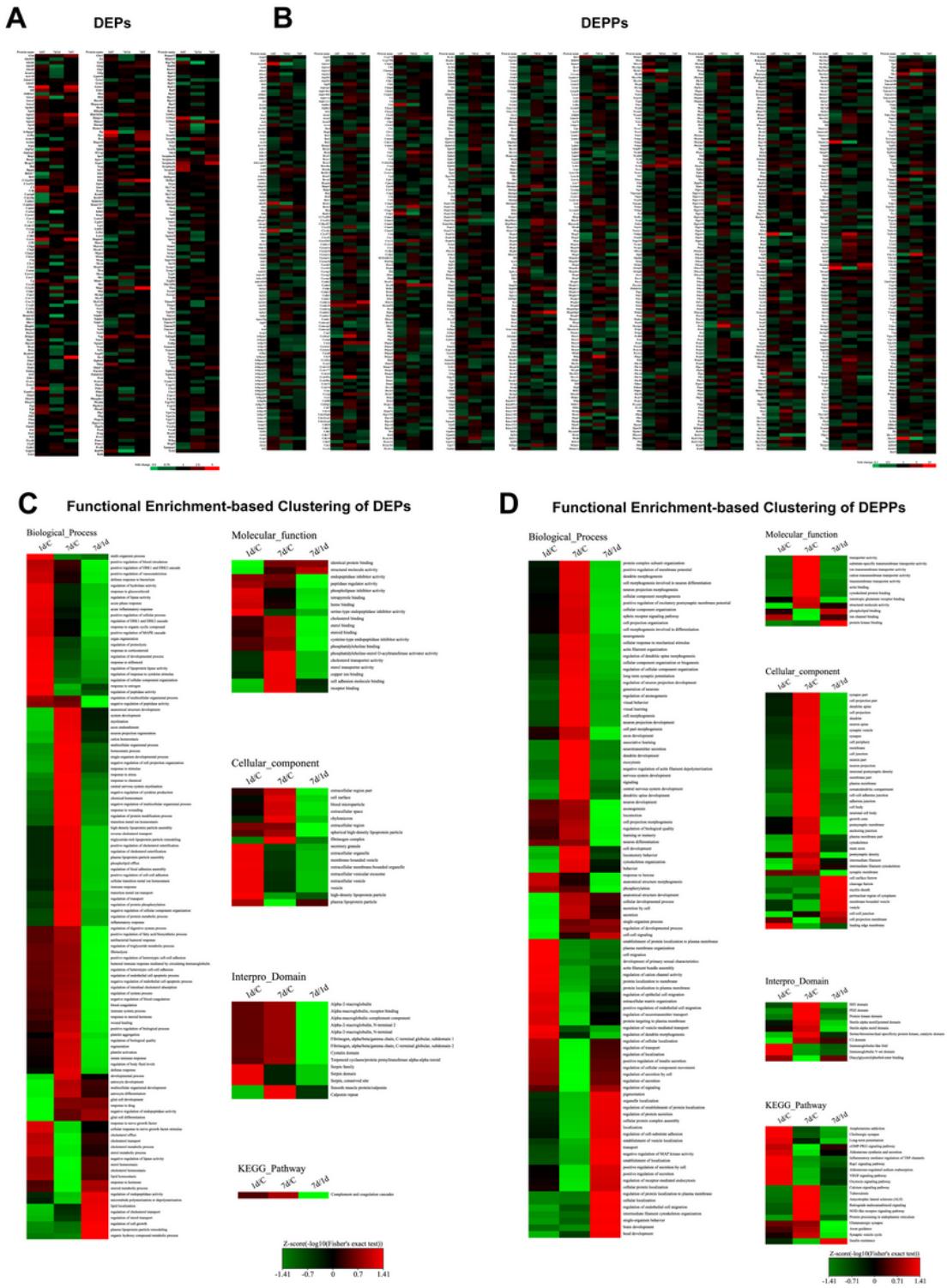
Not applicable.

# 8. References


1. Jiang JY, Gao GY, Feng JF *et al*: **Traumatic brain injury in China**. *Lancet Neurol,* 2019, **18**(3):286-295.
2. Morganti-Kossmann MC, Semple BD, Hellewell SC *et al*: **The complexity of neuroinflammation consequent to traumatic brain injury: from research evidence to potential treatments**. *Acta Neuropathol,* 2019, **137**(5):731-755.
3. Graham NSN, Jolly A, Zimmerman K *et al*: **Diffuse axonal injury predicts neurodegeneration after moderate-severe traumatic brain injury**. *Brain,* 2020, **143**(12):3685-3698.
4. Stein MB, Jain S, Giacino JT *et al*: **Risk of Posttraumatic Stress Disorder and Major Depression in Civilian Patients After Mild Traumatic Brain Injury: A TRACK-TBI Study**. *JAMA Psychiatry,* 2019, **76**(3):249-258.
5. Wilson L, Stewart W, Dams-O'Connor K *et al*: **The chronic and evolving neurological consequences of traumatic brain injury**. *Lancet Neurol,* 2017, **16**(10):813-825.
6. Green RE: **Editorial: Brain Injury as a Neurodegenerative Disorder**. *Front Hum Neurosci,* 2015, **9**:615.
7. Palacios EM, Owen JP, Yuh EL *et al*: **The evolution of white matter microstructural changes after mild traumatic brain injury: A longitudinal DTI and NODDI study**. *Sci Adv,* 2020, **6**(32):eaaz6892.
8. Blennow K, Brody DL, Kochanek PM *et al*: **Traumatic brain injuries**. *Nat Rev Dis Primers,* 2016, **2**:16084.
9. Luo P, Fei Z: **Twisted steel-induced penetrating head injury**. *Neurology,* 2015, **84**(18):1909.
10. Luo P, Li X, Wu X *et al*: **Preso regulates NMDA receptor-mediated excitotoxicity via modulating nitric oxide and calcium responses after traumatic brain injury**. *Cell Death Dis,* 2019, **10**(7):496.

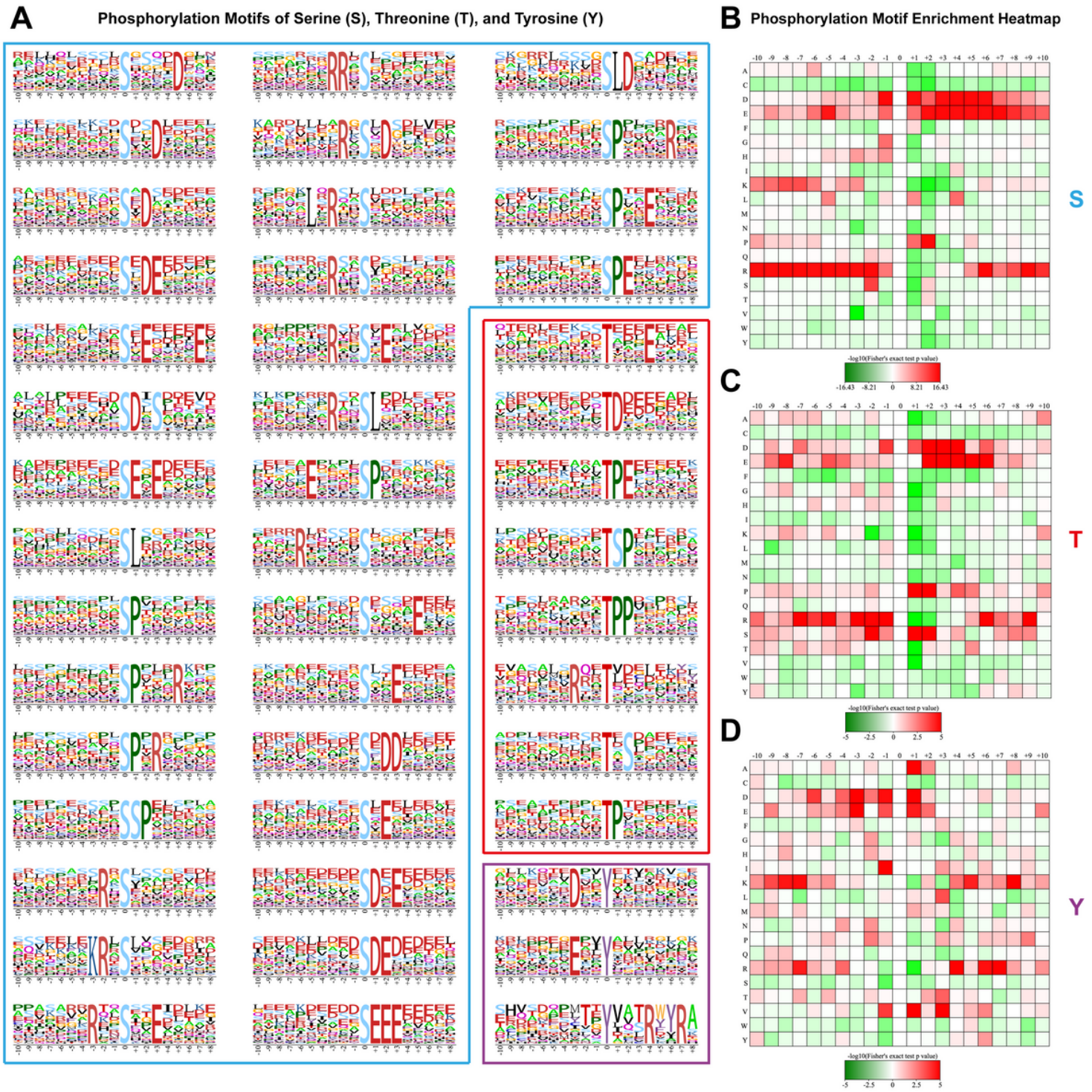
11. Vadhan JD, Speth RC: **The role of the brain renin-angiotensin system (RAS) in mild traumatic brain injury (TBI)**. *Pharmacol Ther,* 2021, **218**:107684.
12. Schwartz D, Gygi SP: **An iterative statistical approach to the identification of protein phosphorylation motifs from large-scale data sets**. *Nat Biotechnol,* 2005, **23**(11):1391-1398.
13. Sawant-Pokam PA, Vail TJ, Metcalf CS *et al*: **Preventing neuronal edema increases network excitability after traumatic brain injury**. *J Clin Invest,* 2020, **130**(11):6005-6020.
14. Mulherkar S, Tolias KF: **RhoA-ROCK Signaling as a Therapeutic Target in Traumatic Brain Injury**. *Cells,* 2020, **9**(1).
15. Huang D, Siaw-Debrah F, Wang H *et al*: **Transplanting Rac1-silenced bone marrow mesenchymal stem cells promote neurological function recovery in TBI mice**. *Aging (Albany NY),* 2020, **12**.
16. Littlejohn EL, Scott D, Saatman KE: **Insulin-like growth factor-1 overexpression increases long-term survival of posttrauma-born hippocampal neurons while inhibiting ectopic migration following traumatic brain injury**. *Acta Neuropathol Commun,* 2020, **8**(1):46.
17. Feeney C, Sharp DJ, Hellyer PJ *et al*: **Serum insulin-like growth factor-I levels are associated with improved white matter recovery after traumatic brain injury**. *Ann Neurol,* 2017, **82**(1):30-43.
18. Tanriverdi F, Schneider HJ, Aimaretti G *et al*: **Pituitary dysfunction after traumatic brain injury: a clinical and pathophysiological approach**. *Endocr Rev,* 2015, **36**(3):305-342.
19. Yuen KCJ, Masel BE, Reifschneider KL *et al*: **Alterations of the GH/IGF-I Axis and Gut Microbiome after Traumatic Brain Injury: A New Clinical Syndrome?** *J Clin Endocrinol Metab,* 2020, **105**(9).
20. Li L, Liang J, Fu H: **An update on the association between traumatic brain injury and Alzheimer's disease: Focus on Tau pathology and synaptic dysfunction**. *Neurosci Biobehav Rev,* 2020.
21. Jamjoom AAB, Rhodes J, Andrews PJD *et al*: **The synapse in traumatic brain injury**. *Brain,* 2020.
22. Bazarian JJ, Biberthaler P, Welch RD *et al*: **Serum GFAP and UCH-L1 for prediction of absence of intracranial injuries on head CT (ALERT-TBI): a multicentre observational study**. *Lancet Neurol,* 2018, **17**(9):782-789.
23. Xu H, Li X, Wu X *et al*: **Iduna protects HT22 cells by inhibiting parthanatos: The role of the p53-MDM2 pathway**. *Exp Cell Res,* 2019, **384**(1):111547.
24. Zhou Y, Zhou B, Pache L *et al*: **Metascape provides a biologist-oriented resource for the analysis of systems-level datasets**. *Nat Commun,* 2019, **10**(1):1523.
25. Shannon P, Markiel A, Ozier O *et al*: **Cytoscape: a software environment for integrated models of biomolecular interaction networks**. *Genome Res,* 2003, **13**(11):2498-2504.
26. Stark C, Breitkreutz BJ, Reguly T *et al*: **BioGRID: a general repository for interaction datasets**. *Nucleic Acids Res,* 2006, **34**(Database issue):D535-539.
27. Bader GD, Hogue CW: **An automated method for finding molecular complexes in large protein interaction networks**. *BMC Bioinformatics,* 2003, **4**:2.

# Figures



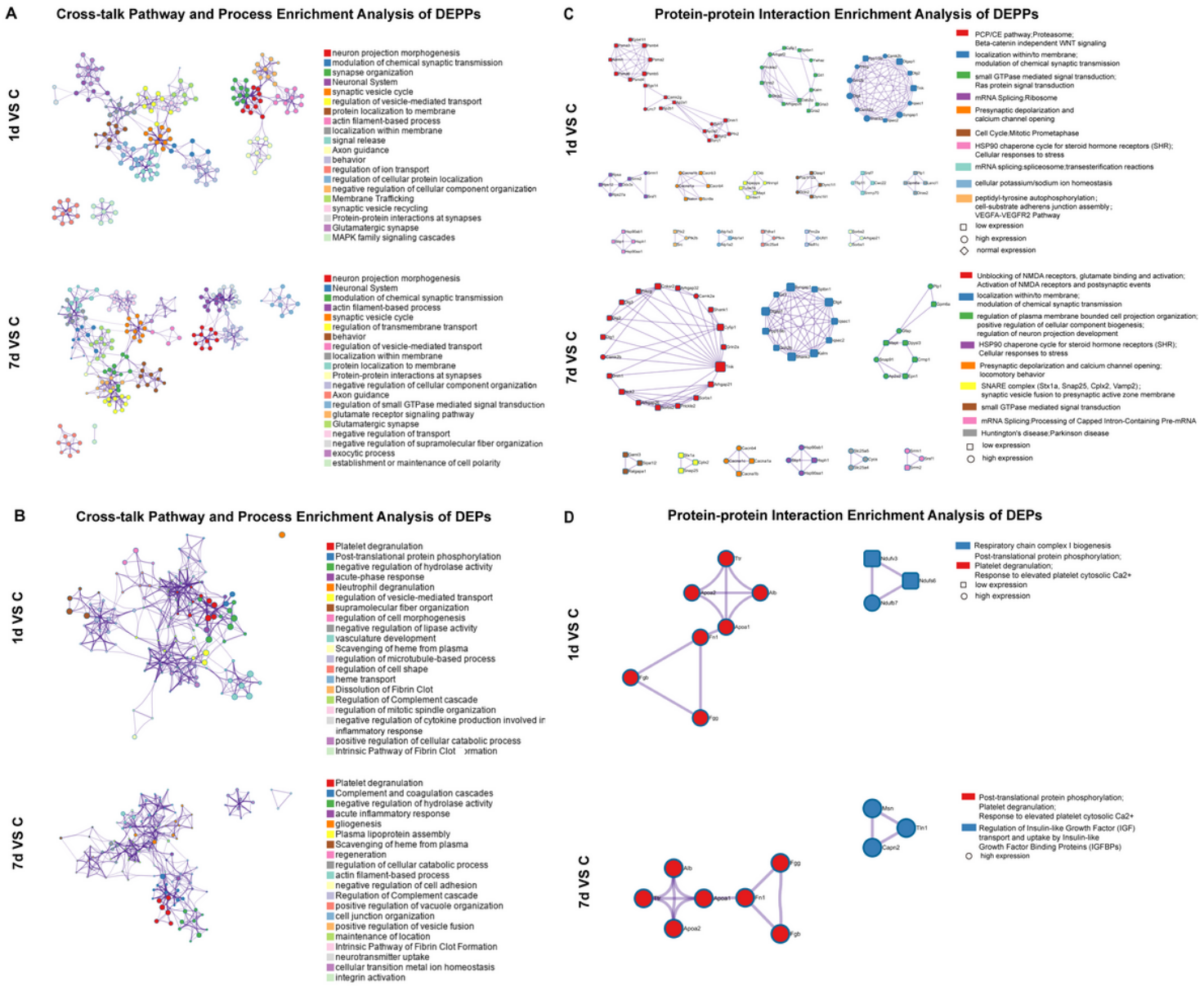

## Figure 1

Identification and functional enrichment-based clustering analyses of DEPs and DEPPs. (A), identification of DEPs. (B), identification of differentially expressed phosphoproteins (DEPPs). (C), functional enrichment-based clustering analyses of DEPs. (D), functional enrichment-based clustering of DEPPs.




## Figure 2

Motif analysis of the phosphosites. (A), phosphorylation motifs of Serine, Threonine, Tyrosine. The blue, red, and purple boxes show the Serine, Threonine, and Tyrosine phosphosites respectively. (B), phosphorylation motif enrichment heatmap of Serine. (C), phosphorylation motif enrichment heatmap of Threonine. (D), phosphorylation motif enrichment heatmap of Tyrosine.




## Figure 3

Common cross-talk pathway/process and protein-protein interaction enrichment analysis. Cross-talk pathway/process enrichment analysis of DEPPs (A) and DEPs (B); different colors represent different pathways and processes; each circle which contains at least five proteins represents a subclass of the pathway and process; cross-talk functions are connected by edges. Protein-protein interaction enrichment analysis of DEPPs (C) and DEPs (D); different colors represent different functions which contain at least three proteins; interacting proteins are connected by edges.

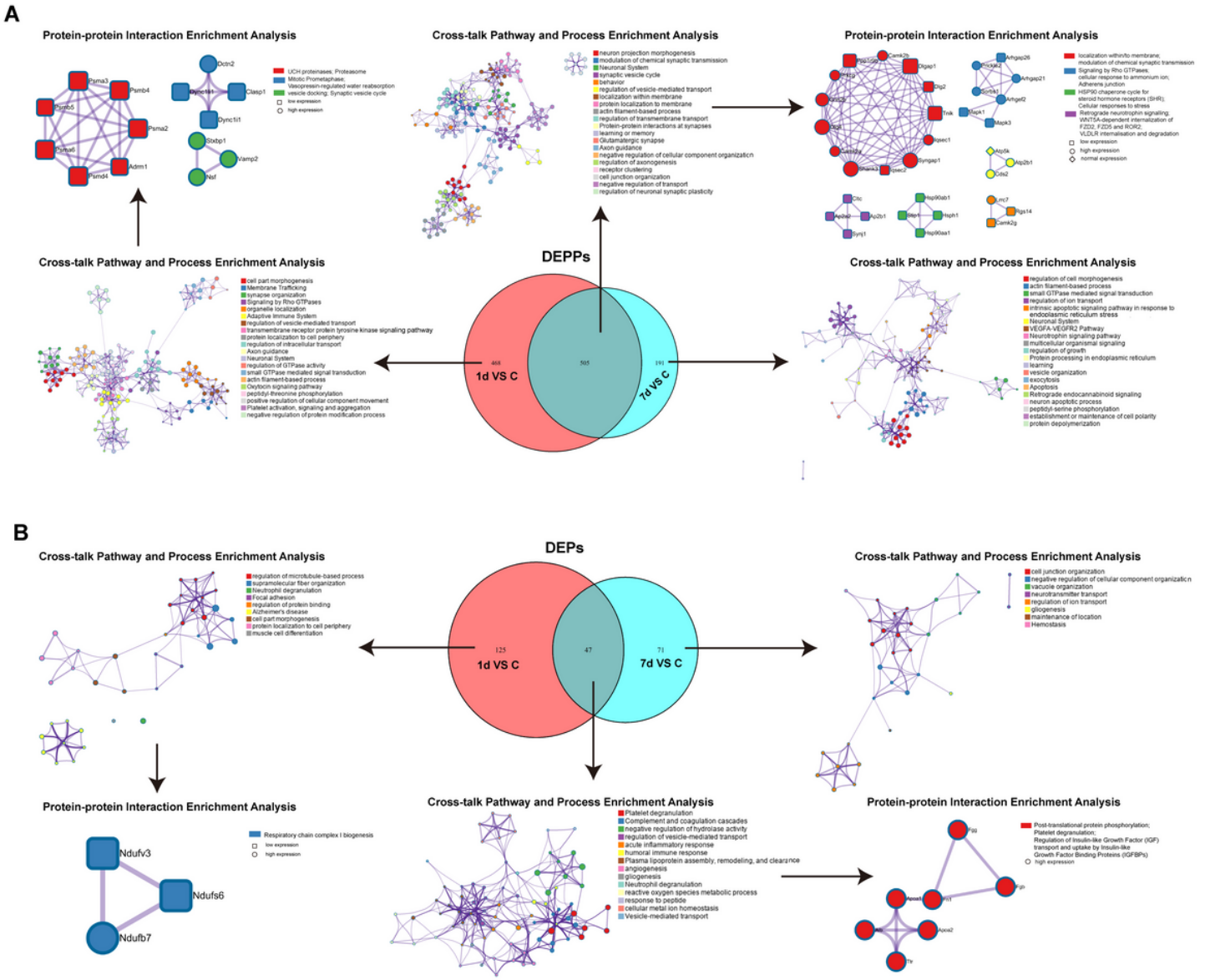



**Figure 4**

Special cross-talk pathway/process and protein-protein interaction enrichment analysis. Special cross-talk pathway/process and protein-protein interaction enrichment analysis of DEPPs (A) and DEPs (B). In cross-talk and process enrichment analysis, different colors represent different pathways and processes; each circle that contains at least proteins represents a subclass of the pathway and process; cross-talk functions are connected by edges. In protein-protein interaction enrichment analysis, different colors represent different functions that contain at least three proteins; interacting proteins are connected by edges.

# Supplementary Files

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