Comparative analysis of the transcriptome and proteome during mouse placental development

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SUPPLEMENTARY INFORMATION

The following files are available free of charge.

- Supplementary Materials and Methods and Supplementary Figures: Supplementary Scheme S1 and Supplementary Figures S1 and S2 (file type: DOCX):
    - Supplementary Scheme S1. General overview of sample processing steps until LC-MS/MS analysis.
    - Supplementary Figure S1. Pairwise correlation scatterplots of placental proteome samples between E7.5 replicates (a) and E9.5 replicates (b), with Pearson correlations.
    - Supplementary Figure S2. Pairwise correlation scatterplots of placental phosphoproteome samples between E7.5 replicates (a) and E9.5 replicates (b), with Pearson correlations.
- Supplementary Tables: Supplementary Tables S1-S8 (file type: XLSX):

- Supplementary Table S1: Transfer of peptides from C18 loading (trap) column onto SCX.
- Supplementary Table S2: Elution of peptides from SCX to 2nd C18 column (nanospray tip) and reverse-phase separation into the MS.
- Supplementary Table S3: Whole proteome dataset after filtering and normalization.
- Supplementary Table S4: Placenta whole proteome studies and number of proteins quantified in each study.
- Supplementary Table S5: Differentially expressed proteins.
- Supplementary Table S6: Class I phosphosites after filtering and normalization.
- Supplementary Table S7: Differentially accumulating Class I phosphosites.
- Supplementary Table S8: Enrichment of overrepresented kinase motifs among differentially accumulating phosphosites and the putative kinases associated with them.

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SUPPLEMENTARY MATERIALS AND METHODS

Materials

Urea (Acros Organics, Cat # 140750010), tris-hydrochloric acid (HCl) pH 7.5 (Teknova, Cat # T1075), tris(2-carboxyethyl)phosphine (TCEP) (Thermo Scientific, Cat # 20490), iodoacetamide (IAA) (Sigma, Cat # I1149), ammonium bicarbonate (NH_4HCO_3) (Acros Organics, Cat # 393210010), trypsin (1 μg/μl) (Roche, Cat # 03708969001), LysC (0.1 μg/μl) (Wako, Cat # 125-05061), formic acid (Fisher Chemical, Cat # A117-50), hydroxylamine (Thermo Scientific, Cat # 90115), lactic acid (Fisher Chemical, Cat # 50-21-5), acetonitrile (Fisher Chemical, Cat # A996-4), trifluoroacetic acid (TFA) (Alfa Aesar, Cat # 44630), ammonium hydroxide (Fisher Chemicals, Cat # A669S-500), zirconium oxide beads (Next Advance, Cat # ZrOB10), Bradford assay kit (Thermo Fisher, Cat # 1863028), filter units (Millipore Sigma, Cat # MRCF0R030), Bicinchoninic Acid (BCA) assay (Thermo Scientific, Cat # 23225), Sep-Pak C18 1 cc 50 mg cartridges (Waters, Cat # WAT054960), TMTsixplex™ label reagents (ThermoFisher, Lot #SC244363), Titansphere Phos-TiO2 beads (GL Sciences, Cat # 5010–21315)

Protein extraction and digestion

Protein extraction and digestion were carried out according to previously published methods[1,2] with minor changes in buffer volumes and length/number of centrifugations. Scheme S1 gives a general overview of sample processing until liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Urea Extraction

Fresh lysis buffer (400 μl; 8 M urea, 0.1 M Tris-HCl pH 7.5, 5 mM tris(2-carboxyethyl)phosphine (TCEP)) was added to each sample tube, followed by mechanical disruption using one mm zirconium oxide beads (Next Advance) and a MiniG shaker (3 minutes, 1,500 rpm). The six tubes (three biological replicates for E7.5 and three for E9.5)

were centrifuged at 4,000 g for 5 minutes at room temperature. Bradford assay (ThermoFisher) was performed according to kit instructions to estimate protein yield (~400-500 μg/ml protein per replicate at E7.5 and ~1000-1200 μg/ml protein per replicate at E9.5). Samples were then sonicated in a water bath for 15 minutes.

Filter Aided Sample Preparation (FASP)-based on filter digestion

FASP was used to purify the proteins with minimal loss. Since the protein concentration exceeded filter (Micocon-30kDA Centrifugal Filter Unit with Ultracel-30 membrane, Cat # MRCF0R030) capacity, we divided each replicate between three filters and then made up the volume in each to 230 μl. Filters were centrifuged at 12,000 g for 10 minutes. Then, 200 μl UA buffer (8 M urea, 0.1 M Tris-HCl pH 7.5) was added and the samples were centrifuged again. Iodoacetamide (IAA) was diluted in UA buffer to 0.05 M. 200 μl of 0.05 M IAA was added to each tube and tubes were incubated in the dark for 1 minute while shaking at 600 rpm, then incubated for another 20 minutes in the dark without shaking.

Samples were centrifuged for 10–20 minutes at 12,000 g, then 100 μl of UA buffer was added and tubes were centrifuged again. Samples were washed with 0.05 M ammonium bicarbonate (NH_4HCO_3) and then centrifuged at 12,000 g for 10–15 minutes. This step was repeated twice, once with 200 μl and once with 100 μl.

Filter units were transferred to new 1.5 ml collection tubes. One μg/µl trypsin was diluted 1:50 in 0.05 M NH_4HCO_3 and added to tubes, then incubated overnight at 37ºC. Afterwards, an aliquot of the trypsin-digested samples was used in Bradford assay to estimate the extent of digestion, as the Bradford assay only estimates proteins, not peptides. Based on the reading, trypsin (1 µg/µl) was mixed with LysC (0.1 µg/µl) in 2:1 ratio of trypsin:LysC. Next, 50 μl was added to each filter, and then samples were incubated at 37ºC for an additional 4 hours.

Samples were centrifuged at 12,000 g for 5 minutes, and 50 μl of 0.05 M NH_4HCO_3 was added, mixed, and then samples were centrifuged again. Then, 100 μl of 0.05 M NH_4HCO_3 was

added, mixed, and samples were centrifuged again for 10 minutes. Flow throughs for each replicate tube were combined to obtain six tubes (three biological replicates for E7.5 and three biological replicates for E9.5). The Bicinchoninic Acid (BCA) assay (Pierce) was performed to determine peptide concentration (~100 μg total peptides from each E7.5 replicate and ~200 μg total peptides from each E9.5 replicate). Samples were stored at -80ºC until C18 desalting. Sep-Pak C18 1 cc 50 mg cartridges (Waters) were used for desalting. Eluted peptides were concentrated via SpeedVac and resuspended in 0.1% formic acid (FA). Peptide concentration was determined using BCA assay.

Tandem Mass Tag (TMT) Labeling

TMTsixplex™ label reagents (ThermoFisher, Lot #SC244363) were used to label the samples according to manufacturer's recommended peptide to TMT reagent ratio. 59 μg of vacuum dried peptides from each sample were resuspended with 100 μl 50 mM TEAB buffer and vortexed for 10 minutes at room temperature. 41 μl acetonitrile was added to each tube of the TMT labels (0.8 mg), then vortexed and incubated at room temperature for 5 minutes to resuspend the labels. The peptide solutions were added to each tube of the TMT labels, pipetted up and down several times and vortexed to mix them well. After 2 hours incubation at room temperature, 8 μl of 5% hydroxylamine were added to each tube, vortexed and incubated at room temperature for 15 minutes to quench the labeling reaction. Next, the six samples were mixed together and stored at -80ºC.

Phosphopeptide enrichment

The TMT-labeled phosphopeptides were enriched using Titansphere Phos-TiO_2 beads (GL Sciences 5010–21315), based on previously published methods[3,4]. The beads were prepared by resuspending in 1.5 ml wash and binding buffer (2 M lactic acid in 50% acetonitrile), vortexing, and then centrifuging at 3,000 g for 1 minute; this was repeated a total of three times. At the last step of washing, 1 mg and 1.5 mg TiO_2 beads were aliquoted to new tubes before

centrifugation. After centrifugation, the wash and binding buffer were removed and the TiO_2 beads were saved for the phosphopeptide enrichment. 300 μg TMT6-labeled and vacuum dried peptides were resuspended with 300 μl wash and binding buffer and then added to 1.5 mg TiO_2 beads, rotated at room temperature for 1 hour, and then centrifuged at 3,000 g for 1 minute. The supernatant was processed with a second round of enrichment using 1 mg of TiO_2 beads. 1.8 ml wash and binding buffers were added to each tube of the two enrichment steps, vortexed and centrifuged at 3,000 g for 1 minute. This wash was repeated once. Next, the TiO_2 beads were washed twice with 1.8 ml of 50% acetonitrile in 0.1% trifluoroacetic acid (TFA). After the wash steps, 500 μl of 3% ammonium hydroxide was added to each tube of the two enrichment steps, vortexed and centrifuged at 3,000 g for 1 minute. The eluted supernatants were combined. One more elution step was performed with 5% ammonium hydroxide. All the supernatants from the two elution steps were combined and SpeedVac'ed to dry, and then the phosphopeptides were resuspended in 0.1% FA, and stored at -80ºC until the LC/MS-MS run.

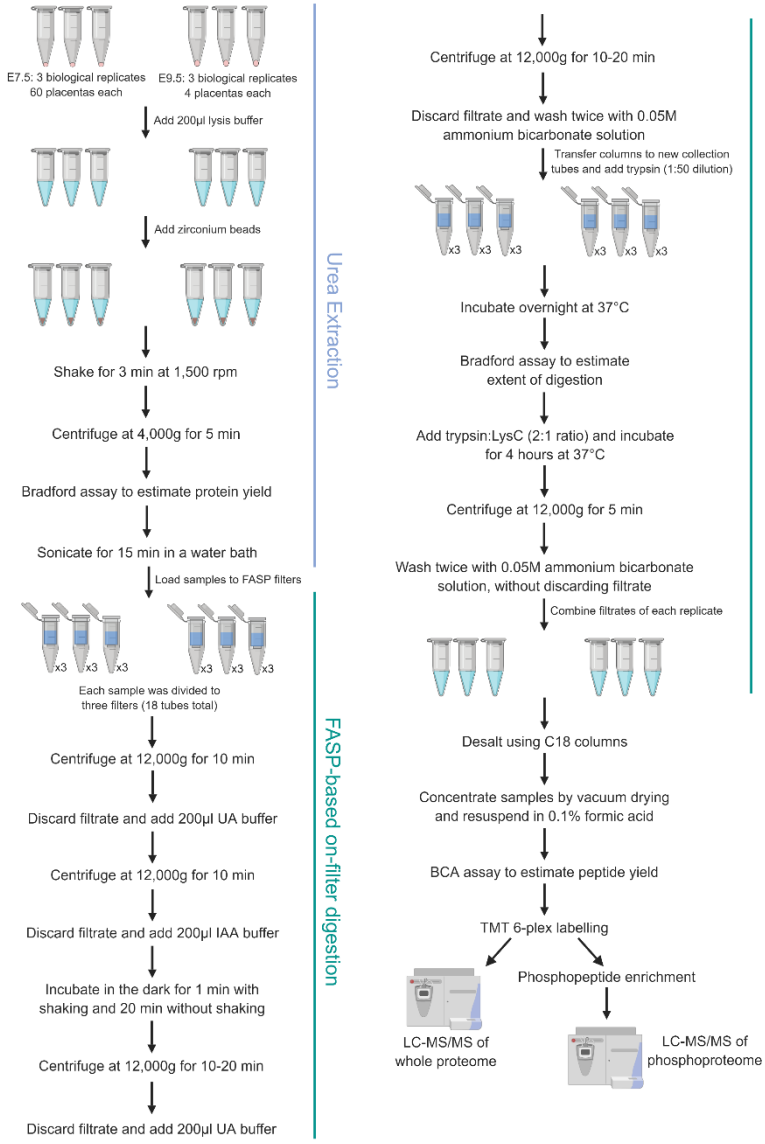
LC/MS-MS

An Agilent 1260 quaternary HPLC was used to deliver a flow rate of ~600 nL min-1 via a splitter. All columns were packed in house using a Next Advance pressure cell, and the nanospray tips were fabricated using a fused silica capillary that was pulled to a sharp tip using a laser puller (Sutter P-2000). 45 µg of TMT-labeled peptides (non-modified proteome), or all TiO_2 enriched peptides (phosphoproteome), were loaded onto 20 cm capillary columns packed with 5 µM Zorbax SB-C18 (Agilent), which was connected using a zero dead volume 1 µm filter (Upchurch, M548) to a 5 cm long strong cation exchange (SCX) column packed with 5 µm PolySulfoethyl (PolyLC). The SCX column was then connected to a 20 cm nanospray tip packed with 2.5 µM C18 (Waters). The 3 sections were joined and mounted on a custom electrospray source for on-line nested peptide elution. A new set of columns was used for every sample. Peptides were eluted from the loading column onto the SCX column using a 0 to 80%

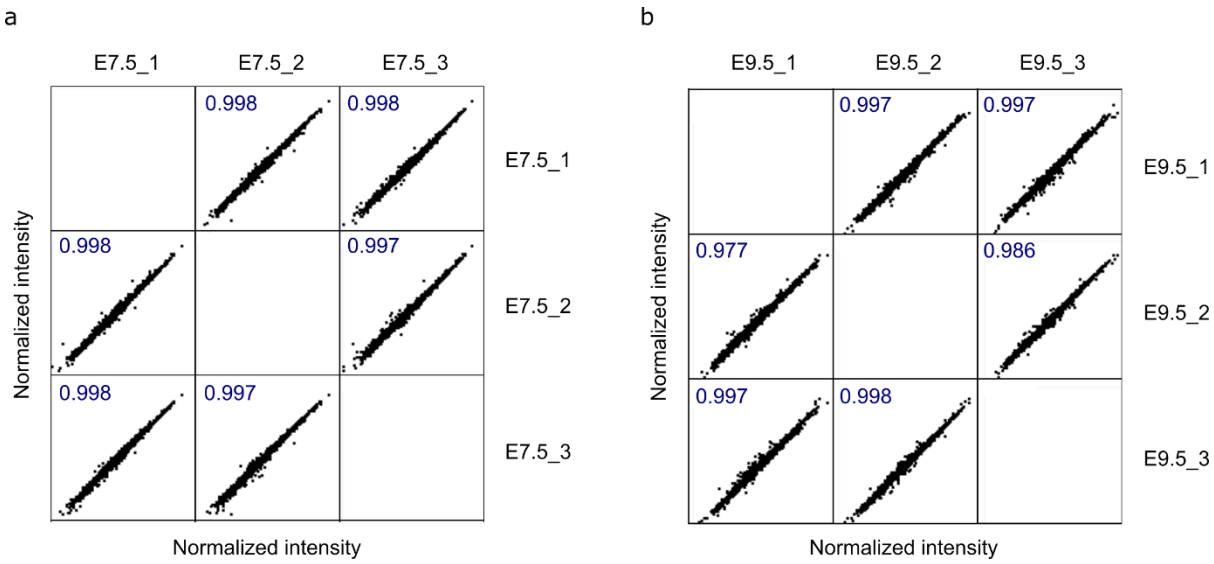
acetonitrile gradient over 60 minutes. Peptides were then fractionated from the SCX column using a series of salt steps. For the non-modified proteome, the following ammonium acetate salt steps were used: 10, 25, 30, 32.5, 34, 36, 38, 42, 44, 46, 48, 50, 52, 55, 57, 60, 65, 70, 75, 80, 90, 98, 100, 110, 130, 150, and 1000 mM. For the phosphoproteome analysis, ammonium acetate steps of 30, 80, and 100 mM were used. For these analyses, buffers A (99.9% H_2O, 0.1% formic acid), B (99.9% ACN, 0.1% formic acid), C (100 mM ammonium acetate, 2% formic acid), and D (2 M ammonium acetate, 2% formic acid) were utilized. For each salt step, a 150-minute gradient program comprised of a 0–5 minute increase to the specified ammonium acetate concentration, 5–10 minutes hold, 10–14 minutes at 100% buffer A, 15–120 minutes 5–35% buffer B, 120–140 minutes 35–80% buffer B, 140–145 minutes 80% buffer B, and 145–150 minutes buffer A was employed.

Eluted peptides were analyzed using a Thermo Scientific Q-Exactive Plus high-resolution quadrupole Orbitrap mass spectrometer, which was directly coupled to the HPLC. Data dependent acquisition was obtained using Xcalibur 4.0 software in positive ion mode with a spray voltage of 2.00 kV and a capillary temperature of 275 °C and an RF of 60. MS1 spectra were measured at a resolution of 70,000, an automatic gain control (AGC) of 3e6 with a maximum ion time of 100 ms and a mass range of 400-2000 m/z. Up to 15 MS2 were triggered at a resolution of 17,500 with a fixed first mass of 120 m/z. An AGC of 1e5 with a maximum ion time of 50 ms, an isolation window of 1.3 m/z, and a normalized collision energy of 31 and 32 were used for non-modified and phospho- proteomes, respectively. Charge exclusion was set to unassigned, 1, 5–8, and >8. MS1 that triggered MS2 scans were dynamically excluded for 25 or 30 s for non-modified and phospho proteomes, respectively.

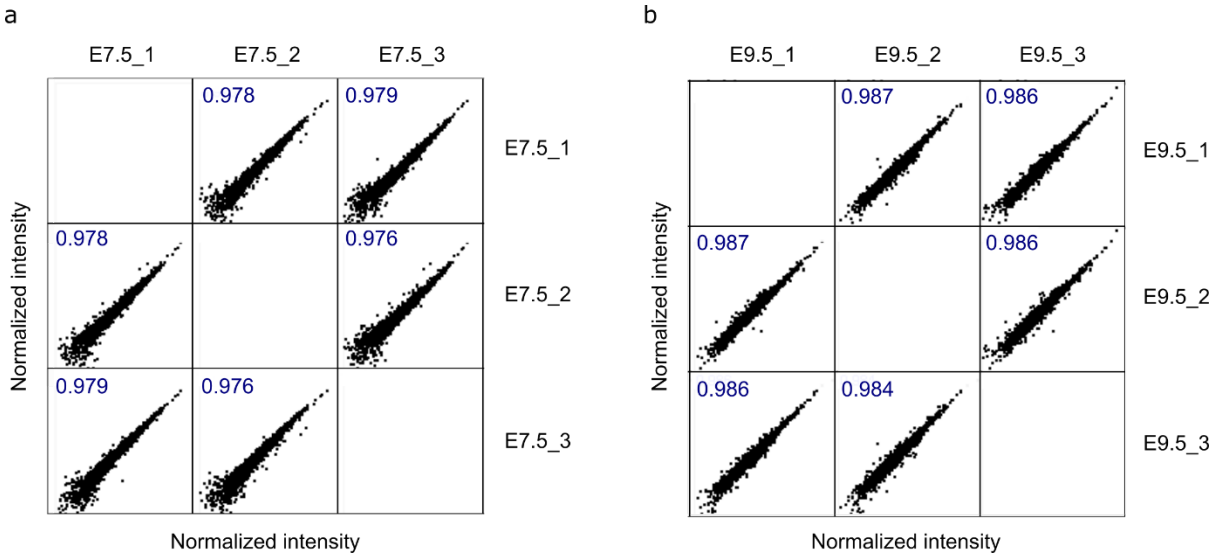
SUPPLEMENTARY FIGURES



Supplementary Scheme S1. General overview of sample processing steps until LC-MS/MS analysis. E, embryonic day; FASP, Filter-Aided Sample Preparation; UA buffer (8 M urea, 0.1 M Tris-HCl pH 7.5); IAA buffer, iodoacetamide buffer (0.05 M IAA in UA buffer); BCA, Bicinchoninic Acid; TMT, Tandem Mass Tag; LC-MS/MS, Liquid Chromatography coupled to tandem Mass Spectrometry



Supplementary Figure S1. Pairwise correlation scatterplots of placental proteome samples between E7.5 replicates (a) and E9.5 replicates (b), with Pearson correlations.



Supplementary Figure S2. Pairwise correlation scatterplots of placental phosphoproteome samples between E7.5 replicates (a) and E9.5 replicates (b), with Pearson correlations.

SUPPLEMENTARY REFERENCES

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