Omics profiling identifies MAPK/ERK pathway as a gatekeeper of nephron progenitor metabolism

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ABSTRACT

Nephron endowment is defined by fetal kidney growth and critically dictates renal health in adults. Despite the advances in understanding the molecular regulation of nephron progenitors, the causes for low congenital nephron count and contribution of basic metabolism to nephron progenitor biology remain poorly understood. Here we characterized the metabolic consequences of MAPK/ERK-deficiency in nephron progenitors, whose maintenance and propagation in developing kidney critically depends on ERK activation. Our LC/MS-based metabolomics profiling identified 42 reduced metabolites, of which 26 were further supported by *in vivo* transcriptional characterization of MAPK/ERK-deficient nephron progenitors. This revealed a severe shortage of energy and nucleotide biosynthesis precursors, blockage in glycolysis and diminished pyruvate and proline metabolism. Utilization of *in vitro* kidney cultures demonstrated a dosage-specific function for glycolytic pyruvate as an energy source that controls the shape of the ureteric bud tip to serve as a niche for nephron progenitor regulation. Analysis of the proline biosynthesis effects in developing kidney *in vivo* revealed premature loss of nephron progenitor maintenance in the absence of *Pycr1/2* functions. Our results suggest that MAPK/ERK-dependent nephron progenitor metabolism functionally contributes to progenitor preservation by controlling pyruvate availability and proline metabolism in developing kidneys.

INTRODUCTION

The differentiation capacity of embryonic nephron progenitors (NP) is a crucial factor for renal health later in life as the extent of fetal nephrogenesis determines an individual's final nephron number, and low nephron count predicts a higher risk for renal complications (1, 2). Embryonic kidney development is orchestrated by inter- and intracellular signaling taking place between two mesodermal derivatives, the ureteric bud (UB) and metanephric mesenchyme (MM) (3, 4). The process of UB branching patterns and expands kidney in size while the MM hosts NPs, which give rise to all segments of functional nephron (5). Together, the UB and MM form the NP niche, where balanced propagation and differentiation of MM-residing progenitors are guided by niche-intrinsic and extrinsic cues (6).

The total NP lifespan is limited to the fetal period in humans and to the first postnatal days of life in mouse (7-9). The molecular regulation of NP maintenance and differentiation has been extensively studied (10, 11), but the factors contributing to the cessation of the nephrogenic program are only evolving and suggest involvement of changes in signal transduction activities (12-14). Moreover, information is only emerging about metabolic events and especially mitochondrial metabolism, underlying the cell type-specific behaviors of NPs during their propagation and differentiation switches (15, 16). A recent study demonstrated higher glycolysis dependence in younger versus older NPs and showed that glycolysis inhibition promotes differentiation being in line with the requirement for aerobic glycolysis (also known as Warburg effect) in maintenance of pluripotency (17, 18). Additionally, Von Hippel-Lindau (VHL) tumor suppressor appears important for mitochondrial respiration in NPs, which show mild differentiation deficiency in the NP-specific absence of *Vhl* (19).

Although one of the major energy precursors for mitochondria, namely pyruvate, is essential for normal early embryonic development and is suggested to contribute to the cell fate switches in embryonic stem cells (20-22), general understanding of its functions in organogenesis is missing. The last step of pyruvate production is regulated by the embryonic kidney expressed glycolytic enzyme pyruvate kinase isozyme M2 (PKM2), whose function is controlled by the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and whose reduced activity is shown to protect against kidney injury (23, 24). Similar to MAPK/ERK, PKM2 regulates cell cycle in embryonic cardiomyocytes (25-28).

We have recently demonstrated the importance of MAPK/ERK activity to the transmission of extracellular signals for NP regulation, where it on one hand maintains NP identity and on the other hand propels differentiation in nephron precursors (4, 11, 29). Interestingly, MAPK/ERK target p53 contributes to metabolic fitness in propagating NPs (30). The p53 target Rrm2b, encoding a catalytic subunit of ribonucleotide reductase (p53R2), is required for nephron function and interestingly interacts with mitochondrial proline synthesis enzymes pyrroline-5-carboxylate reductase (PYCR) 1 and 2 (31-33). Proline metabolism is heavily utilized in cancer cells and deficiencies in its metabolism are causative for many congenital disorders including different types of *cutis laxa* with rare renal manifestation (34-36). Emerging evidence also supports a regulatory role for proline in the maintenance of pluripotency at least in embryonic stem cells (37, 38). Single and double knockout studies of *Pycr1* and -2 demonstrated essential involvement of proline metabolism in neuronal differentiation (39, 40), but its other organ- and tissue-specific requirements remain elusive.

We have utilized a systems biology approach to study the cellular functions that MAPK/ERK activity effectuates in NPs. Our combined RNA sequencing and metabolomics characterization of control and MAPK/ERK-deficient NPs revealed cellular metabolism as one of the most important contributing factors in their regulation. These results establish that the MAPK/ERK

pathway guides nephrogenesis in developing kidney through regulating energy metabolism in NPs.

RESULTS

Transcriptional profiling identifies mitochondrial metabolism as the most MAPK/ERK-dependent cellular process in nephron progenitors

Recently, our group utilized a genetic approach to reveal critical roles for NP-specific MAPK/ERK signaling in progenitor maintenance and propagation (20). NP-specific loss of MAPK/ERK activation due to conditional inactivation of both *Mek* genes (*Six2-TGC^{tg/+};Mek1^{fl/fl};Mek2^{-/-}*) results in rapid NP deprivation due to cell-intrinsic proliferation defects and molecular changes in the niche composition. Transcriptional profiling of MAPK/ERK-deficient NPs isolated from E13.5 kidneys (before the onset of significant NP loss) identified over 5,000 differentially expressed genes (DEGs), which represent transcripts with significant effects on mitochondrial energy metabolism (Kurtzeborn et al, manuscript in revision). To further investigate the mitochondrial metabolism in NP populations, we now performed the biological interpretation analysis of the NP RNA-Seq data using the Cytoscape plug-in ClueGO (41, 42).

An overview of the gene ontology (GO) analysis of DEGs ($\text{Padj} < 0.05$ and $|\log_2\text{fold change}| \geq 2$) identified in MAPK/ERK-deficient NPs is shown in Figure 1A. This shows that oxidative phosphorylation (18.87%) and mitochondrion (11.32%), which represent mitochondrial metabolism, account for a total of 30.19% of cellular functions affected by MAPK/ERK-deficiency and demonstrates that mitochondrial metabolism represents the largest portion (nearly one-third) of cellular pathways that depend on MAPK/ERK. Further characterization of down-regulated DEG results by integrating “cellular component” and Kyoto Encyclopedia of Genes and Genomes (KEGG) database pathway (43, 44) demonstrated that mitochondrial inner membrane (25.0%) and mitochondrion (15.0%) account 40.0% of the

down-regulated transcripts (Figure 1B) supporting diminished mitochondrial metabolism in the absence of MAPK/ERK activation. The up-regulated genes were so scattered and few that their pathway analysis failed to provide a meaningful outcome (Table S1).

Metabolomics analysis of MAPK/ERK-deficiency

We next applied the LC/MS-based metabolomics approach to assess MAPK/ERK-affected metabolism in more detail. An untargeted metabolomics approach was applied to the embryonic kidney mesenchyme cell line (mK4) treated with the MEK inhibitor U0126 to mimic genetic MAPK/ERK-deficiency *in vitro*. The LC/MS data of the non-treated cells, DMSO-treated cells, and the MEK-inhibited cells were analyzed by the partial least square's discriminant analysis (PLS-DA), a multivariate statistical analysis technique. As a result, each group showed clear discrimination in both positive and negative detection mode, while clustering well within their treatment group (Figure 2). Comparison of the untargeted MS2 spectrum patterns with an online MS database revealed a total of 38 significantly different metabolites, summarized in Table 1. Of these, the majority of metabolites were down-regulated and only four (acetylasparagine, methylglutamic acid, xanthine, and oxidized glutathione) were up-regulated.

We next performed targeted metabolomics analysis aiming at identification of metabolic intermediates from glycolysis and mitochondrial metabolism, and quantification of principally altered metabolites. This revealed eight statistically different intermediate metabolites upon MAPK/ERK-deficiency: ATP, ADP, phosphoenolpyruvate, ribose-5-Phosphate, 3-phosphoglyceric acid, citrate, glucose, and glucose-6-phosphate, which all are down-regulated (Table 1). This quantitative intermediate metabolite result is well in line with our RNA-Seq results (Kurtzeborn et al, manuscript in revision) further analyzed here (Fig. 1), which indicated down-regulation of mitochondrial functions.

Metabolic set enrichment analysis (MSEA) of all identified metabolites by MetaboAnalyst revealed pentose phosphate pathway, Warburg effect, Glycine/Serine metabolism, and Purine metabolism as the most altered metabolic pathways in the absence of MAPK/ERK activation (Figure 3A). Visualization of the actual metabolite level changes by heatmaps shows that most of the metabolic pathways, but particularly energy-expensive metabolisms including nucleotide synthesis, aerobic glycolysis, and Glycine/Serine metabolism, were down-regulated upon MAPK/ERK-deficiency (Figure 3B). Table 2 summarizes MSEA analysis results and includes a list of the contributing metabolites.

Integration of metabolomics and RNA-Seq results to the systems biology approach

To conduct a more comprehensive analysis at the level of whole systems biology, we next attempted to integrate the information from our metabolomics results and recent RNA-Seq data. The basic idea of metabolic pathways is a kind of catabolism regulated by enzymes, where these metabolic pathways are composed of regulating enzymes and the metabolites as end products. Here, we have obtained quantitative metabolite level changes in MAPK/ERK-deficient cells through a metabolomics approach and gene expression level changes in MAPK/ERK-deficient nephron progenitors by RNA-Seq. The mutually meaningful relationship between metabolite and gene expression levels was investigated by integrating the two analysis results, and the combined results are summarized in Figure 4.

The integrated systems biology approach identified several metabolic pathways affected by MAPK/ERK-deficiency. Among the 46 originally identified metabolites, 18 metabolites were correlated to 25 differentially expressed genes (Figure 4, Table 3) in our RNA-Seq data (Kurtzeborn et al, manuscript in revision). The most significant findings underline and confirm the severe shortage of energy, which is detected as diminished levels of ATP, ADP, Phosphoenolpyruvate, Ribose-5-phosphate, and 3-Phosphoglycerate and transcripts encoding their metabolic enzymes (Table 3). Interestingly, many of the diminished metabolic pathways

were also related to amino acid metabolism. A few of these are tryptophan, shown to regulate pluripotent stem cell proliferation and expression of pluripotency regulator *Oct4*; glutamine, which regulates reversibility of hair follicle stem cells; and glycine, which is the major regulatory metabolite with functions in antioxidant response and neuronal specification (45-48). One additionally interesting metabolite identified here is proline, which not only regulates stem cell pluripotency but also contributes to the control of neuronal differentiation (38-40, 49-51). Proline metabolism is down-regulated in MAPK/ERK-deficient cells, and its direct regulators *Pycr2* (mitochondrial) and *Pycr1* (cytosolic) are also down-regulated in MAPK/ERK-deficient NPs. The correlations of metabolite-to-gene expression changes identified here are visualized in Figure 5.

Pyruvate dose-dependently affects nephron progenitor niche morphology

To investigate the physiological consequences of identified metabolic changes, we first assayed the effect of pyruvate on early kidney development. Embryonic day 11.5 (E11.5) kidneys were cultured for 72h *in vitro* as described earlier (52) under normal (1mM) and different pyruvate concentration conditions. Cultured kidneys were immunostained with calbindin to visualize ureteric bud tips, which form an extrinsic niche for NPs (10), and branching morphogenesis (Figure 6A). Without pyruvate, kidney growth through branching morphogenesis was severely compromised (50-80% less tips), the ratio of ureteric bud tips-to-trunks was obscured, and the tips were enlarged, especially with 0.5mM pyruvate concentrations. Pyruvate supplementation with low doses, concentrations less than 1.0 mM, improved the branching rate and pattern but branching was not completely rescued to the level typical for control kidneys (1.0 mM pyruvate). Interestingly, pyruvate concentration higher than 1mM resulted in diminished branching and tip amounts but with a different pattern than in low concentrations (0-0.5mM) and without a dramatic effect on tip morphology.

Proline metabolism is required for nephron progenitor maintenance

Our integrated metabolomics and transcriptomics data revealed that the majority of pathways involved in energy metabolism are down-regulated upon MAPK/ERK-deficiency. Down-regulation of proline metabolism is of particular interest as in addition to reduction of the metabolite itself, the expression of its regulatory genes *Pycr2* and *Pycr1* was significantly diminished (Figure 4).

To assess the physiological *in vivo* requirement of proline metabolism in kidney development, we utilized conventional inactivation of *Pycr1* and -2 genes in mouse. We found that inactivation of *Pycr* genes resulted in Mendelian ratios of homozygous *Pycr1*^{-/-}, *Pycr2*^{-/-} and double knockout *Pycr1*^{-/-};*Pycr2*^{-/-} pups with grossly normal kidney morphology and histology (Supplementary Figure 1). Further characterization of *Pycr1*^{-/-};*Pycr2*^{-/-} mouse kidneys at postnatal day 1 (P1) revealed defects in NP population as detected by a reduced number of PAX2-positive cells per ureteric bud and by premature differentiation of postnatal SIX2-positive NPs (Figure 6B). These results demonstrate that proline metabolism, which we identified here to depend on MAPK/ERK activation, is essential for the maintenance of NP population in the developing kidney

DISCUSSION

Metabolomics in combination with systems biology is powerful approach to study functional linkages of metabolic and transcriptional changes (53, 54). Metabolomics has the potential to link genotype to phenotype (55), and it has been broadly and successfully used in systems biology studies (56) to gain a better understanding of complex biological systems, as exemplified by metabolomics studies of mitochondrial dysfunction in kidney diseases (57-61). We have previously demonstrated the importance of MAPK/ERK pathway in NP maintenance and differentiation during embryonic kidney development (29). Here we combined metabolomics and transcriptomics characterization of MAPK/ERK-deficient NP cells to advance the understanding of cellular defects contributing to congenital kidney anomalies.

Congenital anomalies of the kidney and urinary tract (CAKUT) are common birth defects caused by abnormalities in kidney morphogenesis, which contribute to the growing worldwide chronic kidney disease burden (62-64). The spectrum of CAKUT phenotypes varies from mild renal hypoplasia with reduced nephron count, to hypodysplasia and a complete lack of kidneys, a condition known as agenesis or aplasia (65). The milder CAKUT forms such as reduced nephron mass due to impaired NP maintenance or differentiation are not life-threatening at early ages but do possess increased risk of chronic kidney disease with cardiovascular co-morbidities later in life (1, 2). As the final nephron count in each individual is determined by renal morphogenesis during fetal development, any factor possibly affecting the rate and extent of nephrogenesis is of high interest.

There is increasing awareness of how energy metabolism and especially mitochondrial dysfunction contributes to various congenital, acute, and chronic pathologies. Cancer cells are known for their aerobic glycolysis, a.k.a Warburg effect, during which glucose uptake and its conversion to lactate are high and a similar metabolic profile is recognized in actively proliferating cells including stem cells and fetal neuronal tissues (66, 67). MAPK/ERK pathway regulates ATP levels and energy availability through the control of α -enolase, which is a glycolytic pyruvate producing enzyme (68). Adult kidney is a highly energetic organ that contains abundant mitochondria (69) and where malfunctioning mitochondrial energy metabolism has significant effects on the pathogenesis of adult-onset kidney diseases (70). Therefore, mitochondria have emerged as promising therapeutic options for renal diseases (71, 72).

Most metabolite-gene interactions observed here showed the same change patterns presenting decreased measures for both. For example, we identified that the proline production-related enzymes PYCRL and PYCR2 were down-regulated, and their metabolic product, L-proline, was also diminished. As another example, we identified a significant decrease in both acid

synthase (FASN) which is required to produce its end-metabolite product palmitic acid. Conversely, in a few cases, the correlation between the metabolite level and the gene expression was opposite to each other. For instance, subtypes of glutathione peroxidase family were down-regulated (GPX1, GPX3, GPX4, and GPX7), but the oxidized glutathione produced by these genes was increased demonstrating that transcriptional changes do not always translate into protein level differences. All the possible correlations between identified metabolites and DEGs are summarized in Table 3.

Our integrated metabolomics and RNA-Seq characterization of MAPK/ERK-deficient NPs revealed significant gene expression changes in mitochondrial energy metabolism (30%) including oxidative phosphorylation (19%) and mitochondrion (11%). The majority of these transcripts and metabolites were downregulated by MAPK/ERK deficiency. Our experiments revealed diminished mitochondrial counts, reduced expression of α -enolase and decreased ATP levels in NPs with MAPK/ERK-deficiency. These likely stem from the block in glycolysis, as seen by diminished phosphoenolpyruvate (PEP) levels in the absence of MAPK/ERK activation. Under normal conditions, mitochondrial oxaloacetate can be metabolized to PEP by phosphoenolpyruvate carboxykinase 2 (PCK2), but as we also observed decreased expression of *Pck2* upon MEPK/ERK-deficiency this supplementation pathway likely is unable to rescue reduced PEP levels. These results collectively demonstrated that the MAPK/ERK activity in NPs significantly contributes to cellular metabolism, particularly to mitochondrial energy metabolism.

In addition to energy metabolism, we identified a shortage of precursors for nucleotide and protein biosynthesis. Maintenance of the NP population throughout embryonic and early postnatal periods depends on their persistent proliferation that replenishes induced NPs destined for differentiation (10). We have previously reported that MAPK/ERK deficiency causes NP proliferation decline due to diminished DNA replication, which contributes to the

failure in their maintenance (29). Glucose metabolism by glycolysis and mitochondrial tricarboxylic acid (TCA) cycle are recognized as fundamental bases for DNA synthesis and post-translational modifications of proteins and nucleotides (16, 73). Their disturbance in the absence of MAPK/ERK activation contributes to the failure to maintain NP population *in vivo*. Production of pyruvate, one of the major metabolite precursors playing essential functions in mitochondrial metabolism, requires function of the MAPK/ERK-regulated glycolytic enzyme pyruvate kinase isozyme M2 (PKM2), which is expressed by the embryonic kidney (23, 74). In adult kidneys, PKM2 is shown to be protective against acute injury (24). Our results demonstrated decreased expression of *Pkm2* in MAPK/ERK-deficient NPs and reduced levels of TCA intermediate citrate. Analysis of the effects of different pyruvate concentrations in kidney cultures showed dose-dependent changes in the morphology of ureteric bud tips, which serve as an extrinsic niche for nephron progenitors (10). The experiment suggests that low pyruvate availability, for example due to diminished conversion of precursor PEP to pyruvate, supports ureteric bud tip expansion but reduces the number of tip niches, which are shown to have detrimental effects on NP maintenance (12). High pyruvate concentrations also reduce tip niche numbers but do not significantly affect tip morphology. Interestingly, forced pyruvate redirecting to mitochondrial acetyl-CoA production increases pluripotency while glycolytic Ac-CoA production drives histone acetylation in embryonic stem cells (22) suggesting that pyruvate availability critically contributes to the NP stemness.

We also identified down-regulation of *Pycr2* and *Pycr1* genes, which regulate the proline in cells and demonstrated reduced levels of proline metabolite in MAPK/ERK-deficient cells. Proline metabolism has versatile functions in cellular homeostasis (36) and its role as a stem cell regulator is of high relevance to our study. Proline supplementation to embryonic stem cells induces their proliferation and induction towards epiblast while maternal proline improves nutrient transport to better support pre-implantation embryo development (38, 49-51). Proline

deficiency causes several different inherited diseases that typically affect growth, central nervous system and renal functions (36). Our analysis of proline deficiency caused by simultaneous inactivation of *Pycr1* and *Pycr2* in developing kidney revealed its importance for NP maintenance in postnatal kidney. This is an important result not only because the exact functions of proline metabolism at organ and tissue level remained unknown, but also due to its therapeutic potential as a metabolite that is easy to administer.

MATERIALS AND METHODS

RNA isolation for sequencing, and data analysis

For sequencing, RNA was isolated from FAC-sorted nephron progenitors at E13.5 by a standard protocol for chloroform/isopropanol extraction, followed by DNase I treatment according to manufacturer's instructions (Thermo Fisher Scientific – 04/2016, rev. B.00). Total RNA quality and quantity were estimated by using the Bioanalyzer RNA Total Pico (Agilent Technologies, Inc.) and Nanodrop analysis. Library preparation was done using NuGen Ovation Solo and sequencing was performed with NextSeq at BIDGEN DNA Sequencing and Genomics Laboratory (University of Helsinki). bcl2fastq2 Conversion Software was used to convert BCL files to FASTQ file format and demultiplex samples. Sequenced reads were trimmed for adaptor sequence and masked for low-complexity or low-quality sequence using Trimmomatic. Trimmed reads were mapped to GENCODE Mus musculus Release M23 reference genome GRCm38 using STAR aligner (2.6.0c).

Raw gene counts were normalized using DESeq2 R-package (v.3.6.2) and sample clustering was visualized by PCA-plots. Multiple testing adjustment of p-values was done with Benjamini-Hochberg procedure to compare gene expressions in three control and three nephron-specific MAPK/ERK-deficient samples (*Six2-TGC;Mek1^{fl/fl};Mek2^{-/-}*).

Gene Ontology and Pathway Analysis

To further analyze how MAPK/ERK-deficient nephron progenitor (NP) populations have different metabolic characteristics, we utilized our recent whole-genome RNA-Seq data (Kurtzeborn et al, manuscript in revision). To interpret differentially expressed genes in NPs, we performed the pathway analysis using Cytoscape plug-in ClueGO to create a functionally organized gene ontology (GO). The GO_Cellular Component and KEGG ontology were used for GO analysis and we performed the GO analysis using total DEGs and up-regulated and down-regulated DEGs separately.

Metabolomics sample preparation

To investigate metabolic changes in NPs incurred by MEK inhibition, we analyzed metabolic changes of mK4 cell line, derived from embryonic kidney mesenchyme, through a LC/MS-based metabolomics approach. Prior to cell harvest for metabolite extraction, mK4 cells were incubated with DMSO (0.1% v/v), MEK inhibitor U0126 (15 μ M), or without any treatment. After 24 hours incubation, cells were harvested using 0.25% Trypsin/EDTA treatment and washed with cold PBS. The metabolites were extracted by the same method applied in our current RNA-Seq research (Kurtzeborn et al, manuscript in revision). In brief, harvested cells were dissolved with 600 μ l of methanol/chloroform solvent (2:1 v/v ratio), quick frozen twice with liquid nitrogen and thawed at room temperature. Then, 200 μ l of each of chloroform and distilled water were sequentially added, and metabolites were separated by following centrifugation at 15,000 g for 20 min at 4 °C. Finally, water soluble metabolites (upper phase after centrifugation) were dried using centrifugal vacuum evaporator. The middle phase (protein aggregate) after centrifugation was isolated and dissolved in 6M urea buffer and total protein concentration were measured to utilize for normalization.

Liquid chromatography-mass spectrometry (LC-MS) analysis

The ZIC-HILIC column (3.5 μ m, 200 Å, 100 $\times$ 2.1 mm; Merck Millipore) and Acquity UPLC system (Waters, MA, USA) were used for liquid chromatography. The Q Exactive Focus

Orbitrap Mass Spectrometer (Thermo Fisher Scientific) equipped with HESI sources was used for metabolite identification. Chromatographic separation was performed on a ZIC-HILIC column (3.5 μ m, 200 Å, 100 $\times$ 2.1 mm; Merck Millipore) using an Acquity UPLC system (Waters, MA, USA). The column temperature was 35°C with a flow rate of 0.4 mL/min and the auto-sampler cooler temperature was set at 4°C with an injection volume of 2 μ L. Analytes were eluted with a mobile phase composed of 10 mM ammonium acetate in water with pH 6.8 (A) and 10 mM ammonium acetate in water:acetonitrile solution (1:3) with pH 6.8 (B). Gradient conditions were as follows: 0–1 min kept B as 100%, 1–7 min declined B from 100% to 20%, 7–11 min kept B as 20%, 11.1 min set B as 100% and kept the composition until 23 min. Mass spectrometry experiments were performed on a Q Exactive Focus Orbitrap Mass Spectrometer (Thermo Fisher Scientific) equipped with HESI sources. To detect as many ions as possible, both positive and negative detection modes were used in the LC/MS experiment. The inclusion list for parallel reaction method is shown in supplementary Table S2. The MS2 spectrum patterns comparison to the online MS database Metlin and high-resolution m/s from full MS experiment were applied to confirm target metabolites. All the measured data were normalized against total protein concentration which were measured using middle phase protein aggregate concentration.

Metabolomics analysis and systems biology approach

The multivariate statistical analysis was carried out using SIMCA-P version 11.0 program (Umetrics, Umeå, Sweden). To distinguish metabolic pattern differences from each experimental group, the supervised statistical method, partial least squares discriminant analysis (PLS-DA) analysis, was performed using the processed data from LC/MS positive and negative modes, and statistically contributing metabolites on group separation on PLS-DA were identified. To further analyze the identified metabolites, we performed Metabolite Set Enrichment Analysis (MSEA) thorough web-based analysis tool provided from

MetaboAnalyst (75), and the levels of individual contributing metabolites on MSEA result were visualized by heatmap analysis in R package.

Pyruvate effect on embryonic kidney culture

E11.5 control mouse kidneys were cultured under different concentrations of pyruvate supplementation for 72 hours. Cultured embryonic kidneys were analyzed via immunostaining with CALBINDIN, which labels ureteric bud epithelium and allows to determine the effect of pyruvate concentration on ureteric bud branching.

Hematoxylin-eosin staining, immunofluorescent detection of nephron progenitors and precursors, imaging and quantification

Postnatal day 1 (P1) kidneys from *Pycr1/2* mating were collected and fixed with 4% PFA, after which they were processed to paraffin and sectioned according to standard procedures. Hematoxylin-eosin and immunofluorescent staining, and imaging procedures were performed as previously described (20). Primary antibodies used are CALBINDIN (Santa Cruz Biotechnology, sc-7691) PAX2 (Invitrogen, 71-6000) and SIX2 (ProteinTech, 11562-1-AP). Alexa Fluor-conjugated secondary antibodies (1:400; Jackson Immuno Research Laboratories) were used as secondary antibodies. Quantification of nephron progenitor cells, ureteric tips and T-buds was performed blindly by counting 15 medullar sections in two wild type and two double knockout *Pycr1/2* kidneys by three independent researchers. Mean of three counts were used to calculate mean value of cortical nephron progenitors per each ureteric bud tip.

Author Contributions

H.N.K: Research design, experiment, analysis data, manuscript writing; K.K: Experiment, manuscript editing; X.J.: LC/MS experiment; S.P.: Manuscript editing; B.R., A.L and N.E.B mouse *Pycr1/2* knockouts, S.K.: Conceptualization of the research, manuscript writing and editing.

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Table 1. Metabolite marker identification

Table 2. Metabolic set enrichment analysis (MSEA) of identified metabolites

Table 3. The correlations between identified metabolites and differentially expressed genes

FIGURES AND THEIR LEGENDS

Figure 1.

Figure 1. ClueGO analysis of differentially expressed transcripts in MAPK/ERK-deficient nephron progenitors

Overview pie chart of cellular component and KEGG pathway analyses of the (A) total and (B) down-regulated differentially expressed genes (DEGs) between control and MAPK/ERK-deficient NPs. The percentage of genes per term is shown in each group. GO analysis was performed by Cytoscape plug-in application ClueGO. The pie chart shows the enriched signaling pathway categories based on the kappa coefficient of 0.5.

Figure 2.

Figure 2. Partial least squares discriminant analysis (PLS-DA) on positive and negative mode metabolites identified in mK4 cell upon MEK inhibition by LC/MS

PLS-DA score plot for m/z values of (A) positive ($R^2X = 0.92$; $R^2Y = 0.991$; $Q^2Y = 0.968$; $n = 1 + 4$ components) and (B) negative ($R^2X = 0.817$; $R^2Y = 0.999$; $Q^2Y = 0.992$; $n = 1 + 1$ components) detection mode of LC/MS data. Each symbol represents the metabolic profile of an individual sample on each group ($n=4$). Black box indicates the non-treated group, red triangle indicates the DMSO treated group, and blue rhombus indicates the MEK inhibitor UO126 treated group. In both detection modes, MEK-inhibited cells, where MAPK/ERK activation is abolished share the most similar metabolic profile.

Figure 3.

Figure 3. Metabolic Set Enrichment Analysis (MSEA) and their heatmap analyses.

(A) All 46 MAPK/ERK-dependent metabolites identified by LC/MS were applied to MSEA to distinguish the most affected metabolic pathways. The horizontal bar plots are ranked by p value. The color gradient is an indicator of how strongly a metabolic pathway changes, with

the strongest changes being red and lower changes white. **(B)** The heatmap analysis of the mRNA changes contributing to the differential pathways identified by MSEA.

Figure 4.

Figure 4. Cross linkage of identified metabolites and transcriptional changes caused by MAPK/ERK-deficiency.

Quantitative metabolites and gene expression levels from metabolomics and RNAseq analysis were used to connect metabolite-gene expression correlations. Metabolite levels were measured by LC/MS from 4 independent control (DMSO treated) and four MEK-inhibited (UO126 treated) mK4 cell samples. Gene expression level changes were retrieved from RNAseq performed in isolated populations of three control and three *Mek1/2* double knockout NPs (Kurtzeborn et al, in revision).

Actual quantitated metabolites and enzymes are represented with magenta (down-regulated) and green (up-regulated). Dotted boxes represent metabolic pathways including metabolites and genes. RPE; Ribulose-phosphate 3-epimerase, TKT; Transketolase, GAPDH; Glyceraldehyde-3-phosphate dehydrogenase, PGK1; Phosphoglycerate kinase 1, PHGDH; D-3-phosphoglycerate dehydrogenase, PGAM1; Phosphoglycerate mutase 1, ENO3; Beta-enolase, PCK2; Phosphoenolpyruvate carboxykinase [GTP], PKM; Pyruvate kinase, ADSL;

Adenylosuccinate lyase, CKB; Creatine kinase B-type, ODC1; Ornithine decarboxylase, PARS2; Probable proline--tRNA ligase, PYCR1; pyrroline-5-carboxylate reductase 1, PYCRL; Pyrroline-5-carboxylate reductase, GLS; Glutaminase, CLYBL; Citramalyl-CoA lyase, ACO1; Aconitate hydratase 1, CPT1; Carnitine palmitoyltransferase 1, CPT2; Carnitine palmitoyltransferase 2, FASN; Fatty Acid Synthase, GCLC; Glutamate--cysteine ligase catalytic subunit, GCLM; Glutamate--cysteine ligase regulatory subunit, SARDH; Sarcosine dehydrogenase, SHMT2; Serine hydroxymethyltransferase 2, GSS; Glutathione synthetase, GPx; Glutathione peroxidase, ASNS; Asparagine synthetase, GOT2; Aspartate aminotransferase. Magenta represents down-regulated, green represents up-regulated.

Figure 6.

Figure 6. Functional characterization of identified metabolite effects on nephron progenitor biology.

(A) Embryonic kidneys at E11.5 were cultured for 72 hours under different concentration of pyruvate supplementation, and immunostained against Calbindin, which visualizes the entire ureteric bud epithelium. Normal kidney culture medium contains 1mM pyruvate, which serves as a control for other concentrations. (B) Postnatal day 1 control (wild type) and *Pycr1/2* double knockout (dko) kidneys were immunostained for PAX2 (green, marker of nephron progenitors and precursors) or SIX2 (green, marker of nephron progenitors) in combination with Calbindin (red, marker of ureteric bud epithelium). White arrows indicate the regions where PAX2- and SIX2-positive NP cells should be located but are significantly diminished in the absence of *Pycr1/2*. White asterisks point to the differentiating nephron precursors, which show only very weak SIX2 signal in control kidneys but to where SIX2 almost exclusively localizes in *Pycr1/2* dko kidneys. (C) Quantification of ureteric tip numbers (total tip) and cortical nephron progenitors (C-NPC) per each tip in the cortex of wild type and *Pycr1/2* dko kidneys (n=2 kidney/ genotype, total of 15 randomly selected medullar sections were counted).

A

% terms per group

B

% terms per group

A

Metabolite Sets Enrichment Analysis

B

Non essential AA
Essential AA

A **B** **C**

Table 1

Name	DMSO	UO126	Fold change (%)	p value	Detection mode
N-Acetylputrescine	517,92	86,02	-83,39	0,0452	Positive
L-beta-aspartyl-L-leucine (dipeptide)	2300,03	718,95	-68,74	0,0001	Positive
aspartylleucine	578,74	195,51	-66,22	0,0208	Negative
ATP	1181506,7	400539,49	-66,10	0,00641087	Targeted
Glutamylleucine (dipeptide)	2401,49	832,05	-65,35	0,0002	Positive
ADP	15665432	5552987,4	-64,55	0,00725915	Targeted
Leucyl-Glutamate	904,59	327,07	-63,84	0,0442	Negative
Phosphoenolpyruvate	413249,67	166906,85	-59,61	0,00473705	Targeted
Glutamylthreonine	137,95	56,98	-58,70	0,0208	Negative
5-Amino-6-(5'-phosphoribitylamino)uracil	2261,14	937,08	-58,56	0,0075	Negative
D-Ribose	529,23	237,75	-55,07	0,0442	Negative
Uracil	1104,46	530,84	-51,94	0,0442	Negative
Ribose-5-Phosphate	2967069,6	1471881,7	-50,39	0,02669101	Targeted
N-Acetylglutamine	964,50	541,66	-43,84	0,0367	Negative
Pyroglutamine	7049,93	3981,30	-43,53	0,0001	Positive
Dihydrothymine	214,58	124,68	-41,90	0,0442	Negative
Ribothymidine	1604,28	974,24	-39,27	0,0442	Negative
3-Phosphoglyceric acid	2212051,3	1387916,3	-37,26	0,03299034	Targeted
L-Aspartic acid	2187,44	1384,33	-36,71	0,0083	Positive
Palmitic acid	5091,69	3291,31	-35,36	0,0049	Negative
Asparaginyllhistidine	410,91	269,75	-34,35	0,0485	Negative
5'-Methylthioadenosine	3815,56	2511,14	-34,19	0,0416	Positive

L-Tryptophan	2996,42	2001,86	-33,19	0,0026	Positive
Pyroglutamic acid	12625,70	8646,29	-31,52	0,0012	Positive
L-Leucine	49239,70	34975,68	-28,97	0,0083	Positive
Glycerophosphocholine	28559,80	20717,28	-27,46	0,0109	Positive
Citrate	6338539,8	4614380,6	-27,20	0,01201926	Targeted
Creatinine	65685,88	49458,70	-24,70	0,0001	Positive
Glucose	275261529	207639706	-24,57	0,01153043	Targeted
L-Threonine	8307,13	6406,17	-22,88	0,0002	Positive
L-Glutamine	29074,85	22457,10	-22,76	0,0009	Positive
Glucose-6-Phosphate	11482576	8901531,4	-22,48	0,02441322	Targeted
L-Glutamic acid	25101,95	20310,43	-19,09	0,0021	Positive
L-Phenylalanine	15609,50	12703,10	-18,62	0,0067	Positive
L-Acetylcarnitine	12948,78	10641,95	-17,82	0,0220	Positive
Creatine	64756,45	53431,98	-17,49	0,0039	Positive
Glycine	5983,78	5026,74	-15,99	0,0305	Positive
L-Tyrosine	16526,33	13884,15	-15,99	0,0020	Positive
L-Proline	34675,13	29584,80	-14,68	0,0050	Positive
Ornithine	5425,29	4683,48	-13,67	0,0345	Positive
L-Methionine	12073,33	10733,00	-11,10	0,0224	Positive
L-Carnitine	44474,68	39587,95	-10,99	0,0277	Positive
Oxidized glutathione	4685,70	6290,85	34,26	0,0005	Positive
Xanthine	361,89	546,31	50,96	0,0058	Negative
Methylglutamic acid	244,15	552,14	126,15	0,0266	Negative
Acetylasparagine	188,84	498,36	163,91	0,0049	Negative

Table 2

<u>Metabolic changes</u>	<u>Total Comp</u>	<u>Hits</u>	<u>Raw p</u>	<u>Contributing metabolites</u>
Glycine and Serine Metabolism	59	8	0,0007233	3PG, ADP, Glycine, Methionine, Glutamate, Threonine, ADP, Ornithinine
Warburg Effect	58	7	0,0001962	3PG, R5P, G6P, Citrate, ADP, Glutamate, Glutamine
Purine Metabolism	74	7	0,0007233	Glutamine, ADP, R5P, Xanthine
Arginine and Proline Metabolism	53	6	0,0010469	Proline, Creatine, N-Acetyl putrescine
Glutamate Metabolism	49	6	0,0010469	Aspartate, Glycine, ADP, Glutamine, Glutamate, Oxidized Glutathione
Glutathione Metabolism	21	5	0,0010464	Pyroglutamic acid, ADP, Glutamate, Glycine, Oxidized Glutathione
Urea Cycle	29	5	0,0010482	Glutamine, Glutamate, ADP, Ornithine, Aspartate
Ammonia Recycling	32	5	0,0010486	Glutamine, Glutamate, ADP, Aspartate, Glycine
Pentose Phosphate Pathway	29	4	2,31E-05	D-ribose, R5P, G6P, ADP
Pyrimidine Metabolism	59	4	0,0010478	Glutamine, ADP, Uracil, Dihydrothymine

Table 3

Metabolites				Genes				
	<i>T-test</i>	<i>FC %</i>	<i>Up / Down</i>	<i>Gene Symbol</i>	<i>Name</i>	<i>p</i> _{adj}	<i>log</i> ₂ <i>FC</i>	<i>Up / Down</i>
ATP	0,0064	-66,1	down	CANT1	calcium activated nucleotidase 1	3,27,E-02	-1,07	down
				NME2	NME/NM23 nucleoside diphosphate kinase 2	3,81,E-06	-3,92	down
				NME4	NME/NM23 nucleoside diphosphate kinase 4	2,40,E-02	-1,14	down
				NME6	NME/NM23 nucleoside diphosphate kinase 6	2,53,E-06	-3,05	down
				TCIRG1	T cell, immune regulator 1, ATPase, H ⁺ transporting, lysosomal V0 protein A3	1,15,E-03	-1,90	down
				ATP1A1	ATPase, Na ⁺ /K ⁺ transporting, alpha 1 polypeptide	1,55,E-09	-1,35	down
				ATP1A2	ATPase, Na ⁺ /K ⁺ transporting, alpha 2 polypeptide	5,98,E-03	-1,55	down
				ATP2B1	ATPase, Ca ⁺⁺ transporting, plasma membrane 1	1,871E-14	1,46	up
				ATP2C1	ATPase, Ca ⁺⁺ -sequestering	8,252E-07	1,14	up
				ATP5A1	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, alpha subunit 1	4,09,E-07	-1,04	down
				ATP5B	ATP synthase, H ⁺ transporting mitochondrial F1 complex, beta subunit	2,325E-07	-1,14	down
				ATP5D	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, delta subunit	1,378E-11	-2,24	down
				ATP5G1	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit C1 (subunit 9)	5,12,E-03	-1,23	down
				ATP5G2	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit C2 (subunit 9)	1,815E-22	-3,2	down
				ATP5H	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit D	1,841E-15	-2,19	down
				ATP5J2	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit F2	1,29,E-06	-1,58	down
				ATP6AP1	ATPase, H ⁺ transporting, lysosomal accessory protein 1	5,20,E-03	-1,12	down
				ATP6V0B	ATPase, H ⁺ transporting, lysosomal V0 subunit B	2,76,E-04	-2,11	down
				ATP6V0C	ATPase, H ⁺ transporting, lysosomal V0 subunit C	4,70,E-05	-1,77	down

				ATP6V0D1	ATPase, H ⁺ transporting, lysosomal V0 subunit D1	2,73,E-06	-1,82	down
				ATP6V0E2	ATPase, H ⁺ transporting, lysosomal V0 subunit E2	1,27,E-03	-1,80	down
				ATP6V1E1	ATPase, H ⁺ transporting, lysosomal V1 subunit E1	1,16,E-03	-1,08	down
				ATP9A	ATPase, class II, type 9A	0,02897	-2,04	down
				ATP11B	ATPase, class VI, type 11B	5,68E-15	1,63	up
				ATP11C	ATPase, class VI, type 11C	1,831E-13	1,48	up
				ADCY6	adenylate cyclase 6	2,68,E-06	-1,82	down
				POLR1A	polymerase (RNA) I polypeptide A	1,28,E-06	-1,18	down
				POLR1B	polymerase (RNA) I polypeptide B	1,75,E-02	-1,00	down
				POLR1C	polymerase (RNA) I polypeptide C	1,08,E-06	-2,01	down
				POLR1E	polymerase (RNA) I polypeptide E	1,05,E-03	-1,57	down
				POLR2C	polymerase (RNA) II (DNA directed) polypeptide C	1,46,E-03	-1,16	down
				POLR2E	polymerase (RNA) II (DNA directed) polypeptide E	2,63,E-06	-1,91	down
				POLR2F	polymerase (RNA) II (DNA directed) polypeptide F	7,20,E-04	-2,45	down
				POLR2I	polymerase (RNA) II (DNA directed) polypeptide I	3,39,E-08	-3,03	down
				POLR2J	polymerase (RNA) II (DNA directed) polypeptide J	1,19,E-03	-1,62	down
				POLR3C	polymerase (RNA) III (DNA directed) polypeptide C	5,90,E-04	-1,27	down
				POLR3D	polymerase (RNA) III (DNA directed) polypeptide D	5,22,E-04	-1,30	down
				POLRMT	polymerase (RNA) mitochondrial (DNA directed)	2,26,E-04	-1,94	down
				TAF9	TATA-box binding protein associated factor 9	6,17,E-03	2,17	up
				RRM2B	ribonucleotide reductase M2 B (TP53 inducible)	1,35,E-05	1,55	up
				CANT1	calcium activated nucleotidase 1	3,27,E-02	-1,07	down
				NME2	NME/NM23 nucleoside diphosphate kinase 2	3,81,E-06	-3,92	down
				NME4	NME/NM23 nucleoside diphosphate kinase 4	2,40,E-02	-1,14	down

ADP	0,0073	-64,55	down	ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	1,55,E-09	-1,35	down
				ATP1A2	ATPase, Na+/K+ transporting, alpha 2 polypeptide	5,98,E-03	-1,55	down
				ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	1,871E-14	1,46	up
				ATP2C1	ATPase, Ca++-sequestering	8,252E-07	1,14	up
				ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1	4,09,E-07	-1,04	down
				ATP5B	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit	2,325E-07	-1,14	down
				ATP5D	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	1,378E-11	-2,24	down
				ATP5G1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C1 (subunit 9)	5,12,E-03	-1,23	down
				ATP5G2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C2 (subunit 9)	1,815E-22	-3,2	down
				ATP5H	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit D	1,841E-15	-2,19	down
				ATP5J2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F2	1,29,E-06	-1,58	down
				ATP6AP1	ATPase, H+ transporting, lysosomal accessory protein 1	5,20,E-03	-1,12	down
				ATP6V0B	ATPase, H+ transporting, lysosomal V0 subunit B	2,76,E-04	-2,11	down
				ATP6V0C	ATPase, H+ transporting, lysosomal V0 subunit C	4,70,E-05	-1,77	down
				ATP6V0D1	ATPase, H+ transporting, lysosomal V0 subunit D1	2,73,E-06	-1,82	down
				ATP6V0E2	ATPase, H+ transporting, lysosomal V0 subunit E2	1,27,E-03	-1,80	down
				ATP6V1E1	ATPase, H+ transporting, lysosomal V1 subunit E1	1,16,E-03	-1,08	down
				ATP9A	ATPase, class II, type 9A	0,02897	-2,04	down
				ATP11B	ATPase, class VI, type 11B	5,68E-15	1,63	up
				ATP11C	ATPase, class VI, type 11C	1,831E-13	1,48	up

				ENO1	Enolase 1	1,01,E-20	-2,10	down
				NME6	NME/NM23 nucleoside diphosphate kinase 6	2,53,E-06	-3,05	down
				TCIRG1	T cell, immune regulator 1, ATPase, H ⁺ transporting, lysosomal V0 protein A3	1,15,E-03	-1,90	down
Phosphoenolpyruvate	0,0047	-59,61	down	ENO3	Enolase 3	1,31,E-03	-1,70	down
				ENO1	Enolase 1	1,01,E-20	-2,10	down
				PCK2	phosphoenolpyruvate carboxykinase 2	4,96,E-05	-2,36	down
Uracil	0,0442	-51,94	down	UCK1	uridine-cytidine kinase 1	6,87,E-04	-2,12	down
Ribose-5-phosphate	0,0267	-50,39	down	Tkt	transketolase	1,61,E-26	-2,68	down
				RPE	ribulose-5-phosphate-3-epimerase	3,87,E-05	1,35	up
				PGD	phosphogluconate dehydrogenase	1,61,E-07	-1,67	down
3-Phosphoglycerate (3PG)	0,033	-37,26	down	ACYP1	acylphosphatase 1	1,95,E-06	2,79	up
				ACYP2	acylphosphatase 2	4,88,E-02	1,99	up
				PGK1	phosphoglycerate kinase 1	1,67,E-06	-1,14	down
				PHGDH	3-phosphoglycerate dehydrogenase	6,80,E-16	-2,87	down
				PGAM1	phosphoglycerate mutase 1	1,14,E-08	-2,62	down
L-Aspartic acid	0,0083	-36,71	down	ASPA	aspartoacylase (Canavan disease)	5,22,E-04	3,50	up
				ADSL	adenylosuccinate lyase	3,90,E-09	-1,7	down
				ACY3	aspartoacylase (aminocyclase) 3	1,41,E-02	-2,02	down
				CAD	carbamoyl-phosphate synthetase 2	2,00,E-11	-2,04	down
				AGA	aspartylglucosaminidase	3,94,E-02	1,13	up
				GOT2	glutamic-oxaloacetic transaminase 2	2,60,E-08	-1,52	down
				ASNS	asparagine synthetase	1,35,E-02	-1,04	down
Palmitic acid	0,0049	-35,36	down	ACOT7	acyl-CoA thioesterase 7	1,03,E-04	-2,05	down
				FASN	fatty acid synthase	2,77,E-33	-2,71	down
				MCAT	malonyl CoA:ACP acyltransferase	9,44,E-03	-1,85	down
5'-Methylthioadenosine (MTA)	0,0416	-34,19	down	SRM	spermidine synthase	2,00,E-05	-1,49	down
L-Tryptophan	0,0026	-33,19	down	WARS	tryptophanyl-tRNA synthetase	1,23,E-06	-2,10	down
L-Leucine	0,0083	-28,97	down	BCAT2	branched chain aminotransferase 2,	2,53,E-05	-2,40	down
				LARS	leucyl-tRNA synthetase	3,53,E-04	-1,03	down
Citrate	0,012	-27,2	down	ACO1	aconitase 1	2,74,E-05	-1,16	down

Citrate	0,012	-27,2	down	CLYBL	citrate lyase beta like	3,70,E-02	-1,67	down
Glycerophosphocholine	0,0109	-27,46	down	Lypla1	lysophospholipase I	1,58,E-22	2,08	up
				Lypla2	lysophospholipase 2	1,61,E-09	-3,23	down
Glucose	0,0115	-24,57	down	Hk1	hexokinase 1	1,52,E-02	-1,07	down
				GAA	glucosidase, alpha, acid	2,30,E-02	-1,34	down
				GANAB	alpha glucosidase 2 alpha neutral subunit	2,57,E-05	-1,08	down
Glucose-6-phosphate (G6P)	0,0244	-22,48	down	PGM1	phosphoglucomutase 1	3,60,E-03	-1,82	down
				Hk1	hexokinase 1	1,52,E-02	-1,07	down
L-Glutamine	0,0009	-22,76	down	CAD	carbamoyl-phosphate synthetase 2	2,00,E-11	-2,04	down
				QARS	glutamyl-tRNA synthetase	1,09,E-05	-1,20	down
				GLS	glutaminase	3,15,E-22	2,08	up
				ASNS	asparagine synthetase	1,35,E-02	-1,04	down
L-Threonine	0,0002	-22,88	down	TARS	threonyl-tRNA synthetase	1,46,E-04	-1,05	down
				TARS2	threonyl-tRNA synthetase 2	7,37,E-03	-1,12	down
L-Glutamic acid	0,0021	-19,09	down	GLS	glutaminase	3,15,E-22	2,08	up
				GOT2	glutamatic-oxaloacetic transaminase 2	2,60,E-08	-1,52	down
				GCLC	glutamate-cysteine ligase	9,20,E-10	1,49	up
				GCLM	glutamate-cysteine ligase	5,38,E-05	1,38	up
				ALDH18A1	aldehyde dehydrogenase 18 family, member A1	4,53,E-03	-1,12	down
				ALDH4A1	aldehyde dehydrogenase 4 family, member A1	1,53,E-02	-1,29	down
L-Phenylalanine	0,0067	-18,62	down	GOT2	glutamatic-oxaloacetic transaminase 2	2,60,E-08	-1,52	down
				FARSA	phenylalanyl-tRNA synthetase, alpha subunit	7,57,E-04	-1,36	down
				PRDX6	peroxiredoxin 6	7,81,E-07	-1,14	down
				SPR	sepiapterin reductase	2,60,E-02	-1,70	down
Creatine	0,0039	-17,49	down	CKB	creatine kinase	1,28,E-07	-2,52	down
Glycine	0,0305	-15,99	down	SHMT2	serine hydroxymethyltransferase 2	8,62,E-09	-2,49	down
				SARDH	sarcosine dehydrogenase	6,67,E-06	-2,87	
				GSS	glutathione synthetase	5,44,E-03	-2,34	
				GCAT	glycine C-acetyltransferase	5,93,E-07	-2,82	
				GATM	glycine amidinotransferase	1,54,E-03	1,86	up
				GLDC	glycine decarboxylase	8,89,E-04	-1,36	down
				RARS	arginyl-tRNA synthetase	1,44,E-06	-1,08	down

				AMT	aminomethyltransferase	1,28,E-03	-1,89	down
L-Tyrosine	0,0020	-15,99	down	ACOT7	acyl-CoA thioesterase 7	1,03,E-04	-2,05	down
				ACAA2	acetyl-Coenzyme A acyltransferase 2	2,16,E-03	-1,29	down
				ECHS1	enoyl Coenzyme A hydratase, short chain, 1	1,80,E-07	-1,54	down
				HADHA	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit	2,09,E-04	-1,04	down
				YARS	tyrosyl-tRNA synthetase	2,26,E-06	-1,49	down
				GOT2	glutamatic-oxaloacetic transaminase 2	2,60,E-08	-1,52	down
				SPR	sepiapterin reductase	2,60,E-02	-1,70	down
L-Proline	0,0050	-14,68	down	PYCR1	pyrroline-5-carboxylate reductase-like	2,41,E-05	-2,19	down
				PYCR2	pyrroline-5-carboxylate reductase family, member 2	2,63,E-06	-2,49	
				PARS2	prolyl-tRNA synthetase	1,41,E-02	-1,89	
				ALDH18A1	aldehyde dehydrogenase 18 family, member A1	4,53,E-03	-1,12	
				P4HB	prolyl 4-hydroxylase, beta polypeptide	5,44,E-07	-1,31	
				LAP3	leucine aminopeptidase 3	2,07,E-05	1,00	up
L-Ornithine	0,0345	-13,67	down	SIRT6	sirtuin (silent mating type information regulation 2 homolog) 6	1,73,E-07	-3,10	down
				ODC1	ornithine decarboxylase, structural 1	8,26,E-09	-1,31	down
				ACY1	aminoacylase 1	1,47,E-02	-1,60	down
L-Methionine	0,0224	-11,1	down	ACY1	aminoacylase 1	1,47,E-02	-1,60	down
				MARS	methionine-tRNA synthetase	9,68,E-08	-1,74	down
L-Carnitine	0,0277	-10,99	down	CPT1C	carnitine palmitoyltransferase 1c	4,75,E-02	-1,53	down
				CPT2	carnitine palmitoyltransferase 2	1,52,E-02	-1,16	down
Acetylasparagine	0,0049	163,91	up	ASPA	aspartoacylase (Canavan disease)	5,22,E-04	3,50	up
				ACY3	aspartoacylase (aminocyclase) 3	1,41,E-02	-2,02	down
Oxidized glutathione	0,0005	34,26	up	GPX1	glutathione peroxidase 1	2,17,E-14	-2,46	down
				GPX3	glutathione peroxidase 3	7,76,E-04	-2,10	
				GPX4	glutathione peroxidase 4	2,62,E-10	-3,02	
				GPX7	glutathione peroxidase 7	3,70,E-06	-3,19	