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Quantitative Proteomics Suggests Metabolic Reprogramming during ETHE1 Deficiency

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Abstract

Deficiency of mitochondrial sulfur dioxygenase (ETHE1) causes the severe metabolic disorder ethylmalonic encephalopathy, which is characterized by early-onset encephalopathy and defective cytochrome C oxidase because of hydrogen sulfide accumulation. Although the severe systemic consequences of the disorder are becoming clear, the molecular effects are not well defined. Therefore, for further elucidating the effects of ETHE1-deficiency, we performed a large scale quantitative proteomics study on liver tissue from ETHE1-deficient mice. Our results demonstrated a clear link between ETHE1-deficiency and redox active proteins, as reflected by down-regulation of several proteins related to oxidation-reduction, such as different dehydrogenases and cytochrome P450 (CYP450) members. Furthermore, the protein data indicated impact of the ETHE1-deficiency on metabolic reprogramming through up-regulation of glycolytic enzymes and by altering several heterogeneous ribonucleoproteins (hnRNPs), indicating novel link between ETHE1 and gene expression regulation. We also found increase in total protein acetylation level, pointing out the link between ETHE1 and acetylation, which is likely controlled by both redox state and cellular metabolites. These findings are relevant for understanding the complexity of the disease and may shed light on important functions influenced by ETHE1 deficiency and by the concomitant increase in the gaseous mediator hydrogen sulfide.

Key words: *ethylmalonic encephalopathy, CYP 450, heterogeneous ribonucleoprotein (hnRNP), acetylation, sulfide, Heat shock protein 90 (HSP90), metal, magnesium, iron, zinc, ICP-MS*

Statement of Significance of the Study

The protein ETHE1 is known to have persulfide dioxygenase activity, and it's homozygous or compound heterozygous deficiency causes the severe ethylmalonic encephalopathy. ETHE1 is also known to be a regulator of the signaling gas sulfide. However, the functional context of ETHE1 is still sparsely understood and we here apply large scale proteomics to show the influence of ETHE1 on several pathways not previously described. Whereas the phenotype during ETHE1 deficiency mainly is neurodevelopmental, the liver proteomics data of this study capture metabolic reprogramming towards glycolysis with concomitant overall increase in protein acetylation. Pronounced decreases of the levels of oxido-reductases indicated altered redox regulation. This together with alterations of several heterogeneous ribonucleoproteins (hnRNPs), important for gene regulation, indicated that ETHE1 has wider impact on the cellular and biochemical constitution than previously thought.

Introduction

Deficiency of mitochondrial protein ETHE1 causes ethylmalonic encephalopathy (EE) – a severe, inherited metabolic disorder- characterized by encephalopathy, chronic diarrhea and lactic acidosis. ETHE1 has persulfide dioxygenase activity, and when deficient it causes accumulation of hydrogen sulfide (H_2S), which can result in a multitude of biochemical/biological effects [1, 2], making ETHE1 biology a challenging topic to study. Two well-known characteristics of ETHE1 deficiency, linked to the toxic levels of sulfide, are inhibition of cytochrome c oxidase (COX) of mitochondrial respiration [3, 4], and short-chain acyl CoA dehydrogenase (SCAD) in fatty acid oxidation pathway [1]. The latter leads to butyryl-CoA accumulation, which after conversion is excreted as ethylmalonic acid (EMA) [5]. The biochemical profile of patients with the EE disorder is also characterized by high lactate, and C4- and C5-acylcarnitines in blood [1]. ETHE1 belongs to the metallo- β -lactamase superfamily [6] and has sequence homology to glyoxylase II, but unlike glyoxylase II it has a mononuclear iron in the active site [7]. Sulfide is a gasotransmitter [8] with several biological functions, for example vasorelaxation [9], although at high concentration it is toxic to cells [10]. This can explain the clinical findings of vascular symptoms in EE patients, such as petechiae and acrocyanosis. Sulfide is produced endogenously by cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE) by using L-cysteine as a substrate [11, 12]. ETHE1 takes part in the main pathway for sulfide catabolism, which resides in the mitochondrial matrix and consists of an oxidation reaction catalyzed mainly by sulfide quinone reductase (SQR) and ETHE1 [13, 14]. ETHE1 is a sulfur dioxygenase oxidizing persulfide (e.g. glutathione persulfide) to sulfite (SO_3^{2-}) while consuming molecular oxygen and water [14, 15]. It has been shown that O_2 and sulfide have low degree of coexistence in tissues [16]. Loss of ETHE1 function causes sulfide accumulation and thus hypoxic condition, and as a

1 vicious cycle hypoxia can raise sulfide levels as oxygen dependent catabolism of sulfide
2 slows down. This close interaction implies the role of sulfide in oxygen sensing [17].
3 Sulfide is a known redox regulator [15], and our research group have previously described
4 that oxidoreductases show altered levels in fibroblast cells from EE patients [18]. The clinical
5 symptoms of EE and the reported effects of ETHE1 deficiency on mitochondrial fatty acid β
6 oxidation, cytoskeleton and branched chain amino acid catabolism [19], exemplify that the
7 ETHE1 deficiency affects several different pathways within the cell. Still further elucidation
8 is necessary to elucidate the interplay between these different molecular mechanisms. These
9 severe systematic consequences of the disease encouraged us to perform large scale
10 proteomics studies on liver tissue from ETHE1-deficient and control mice. Our data confirm
11 the link between ETHE1-deficiency and redox related proteins [18], based on lowered level
12 of several dehydrogenases and of several members of cytochrome P450 (cyp450) family.
13 Metabolic reprogramming towards glycolysis was seen, and a novel link between ETHE1
14 deficiency and heterogeneous ribonucleoproteins (hnRNPs) was established.

21 Materials and Methods

Experimental design

Liver samples from three replicates of both *Ethe1*^{-/-} and *Ethe1*^{+/+} mice were subjected to proteomics as described previously [20] and below. Due to the smaller size of the *Ethe1*^{-/-} mice, two livers from these were pooled to get each replicate. Two studies of iTRAQ based comparative proteomics were performed separately on soluble and membrane proteins, respectively, and the data from the two studies were merged prior to pathway analyses and biological interpretations.

ETHE1 deficient mice

Heterozygous *Ethe1*^{-/+} mice were mated to obtain homozygous *Ethe1*^{-/-} mice. For details on how the *Ethe1*^{-/+} mice were generated see Tiranti et al [1]. The mouse colony was maintained on a129Sv-C57BL/6 mixed background. The liver samples from six *Ethe1*^{-/-} mice and three *Ethe1*^{+/+} healthy controls, were taken for proteomics studies. Animal studies were approved by the Ethics Committee of the Foundation ‘Carlo Besta’ Neurological Institute, in accordance with guidelines of the Italian Law D.L. 116/1992 and the EU directive 63-2010.

Proteome fractionation

Mouse liver tissue was homogenized with a Potter-Elvehjem in cold (4 °C) lysis buffer (10 mmol/l Hepes, pH 7.2, with protease inhibitors (Complete, Roche Diagnostics), sonicated three times for 10 sec, and briefly centrifuged (400×g, 5 min, 4 °C) to remove cell debris. The supernatant from homogenization was ultracentrifuged (120,000×g, 60 min., 4°C) to separate the membranes from the new supernatant (S). The membrane pellet was washed in sodium

1 carbonate buffer to remove contaminant soluble proteins and thereafter ultracentrifuged
2 again. The new membrane pellet was dissolved on ice in approximately 1% Triton X-114.
3 After 15 min incubation at 30°C, a bottom phase of Triton-X114 micelles was formed and
4 collected by centrifugation (1300×g, 10 min). The Triton-X114 detergent phase, mostly
5 containing membrane proteins (M), and the S sample were purified by precipitation and
6 analyzed as previously described [21].
7

8 *Peptide labeling and fractionation*

9 Two iTRAQ-studies, on S and M samples respectively, were performed and three *EtheI*^{-/-}
10 and three *EtheI*^{+/+} samples were included in each. 100 µg peptide sample of each replicate
11 was labeled with a specific iTRAQ reagent in accordance with the manufacturer's
12 instructions (Applied Biosystems). The subsequent analytical steps were performed as
13 previously described [21]. Labeled peptides were mixed and purified on a Strong Cation
14 Exchange (SCX) chromatography column followed by elution with 5% ammonia and 30%
15 methanol. Pure peptides were dried and resuspended in denaturing buffer, and separated on
16 an Immobiline Drystrip gel (GE Healthcare, Uppsala, Sweden) using isoelectric focusing on a
17 Multiphor II unit (Pharmacia Biotech AB, Uppsala, Sweden). The pH range was 3.5-4.5 for S
18 peptides and 3-10 for M peptides. The gel strip was cut into twelve pieces, and the peptides
19 were extracted from the gel in two steps with 0.5% trifluoroacetic acid (TFA) in 5%
20 acetonitrile (ACN). Peptides were purified on PepClean C-18 Spin Columns (Pierce,
21 Rockford, IL, USA) according to manufacturer's protocol prior to nanoLC-MS/MS analysis.
22

23 *Nano-liquid chromatography and mass spectrometry (MS) analysis*

24 The peptide mixtures were separated by liquid chromatography (EASY nano LC from
25 Proxeon, Odense, Denmark) coupled to mass spectrometry (LTQ-Orbitrap, Thermo Fisher

Scientific, Waltham, USA), through a nano-electrospray source with stainless steel emitter (Proxeon). Each sample was LC-MS analysed twice. The peptides were separated on a reverse phase column, 75 μ m in diameter and 100 mm long, packed with 3.5 μ m Kromasil C18 particles (Eka Chemicals, Bohus, Sweden) at a flow of 300 nl/minute using a 100 minutes gradient of AcN in 0.4% acetic acid, starting with 5% and ending with 35% AcN. The mass spectrometry detection was full scan (m/z 400-2000) with Orbitrap detection at resolution $R=60,000$ (at m/z 400) followed by up to four data-dependent MS/MS scans, with linear ion trap (LTQ) detection of the most intense ions. Dynamic exclusion of 25 sec was employed as well as rejection of charge state +1. Pulsed Q dissociation (PQD) fragmentation was performed with activation time of 0.1 sec, two microscans, normalized collision energy of 33, and activation Q of 0.7.

Database searches and statistics

The raw data files were processed using extract_msn.exe (Thermo Fisher Scientific, released 2/15/2009) to generate peak lists of the tandem spectra. To obtain protein identification and iTRAQ reporter quantification data the MS data were subjected to database searches in Proteome Discoverer 1.3 (Thermo) using the algorithm of Mascot (www.matrixscience.com) version 2.2.04 (Matrix Science, London, UK) for peptide and protein identification. In each study, the twelve different peptide fractions were MS-analysed in duplicate and all generated peak lists were merged. The merged files were searched against the mouse database, *Mus musculus* SwissProt_2012_3.fasta with 16520 sequences, using the MudPIT scoring algorithm of Mascot. Full scan tolerance was 5 ppm and MS/MS tolerance was 0.5 Da. Setting of trypsin digestion was cleavage at C-terminal of lysine and arginine except before proline, and up to two missed cleavages were accepted. Fixed modifications were those

originating from iTRAQ protocol: iTRAQ-8plex of lysine and N-terminal and methylthio modification of cysteines, whereas oxidation of methionine and iTRAQ-8plex of tyrosine were set as variable modifications. The iTRAQ labeling efficiency was estimated from database searches where the iTRAQ labeling of N-termini and lysine residues, and also lysine acetylation, were set as dynamic modifications. iTRAQ labeling was found to be present on more than 99% of the expected sites (calculated from more than 50,000 peptide scans), and was therefore subsequently set as a fixed modification. Less than 0.7% of the lysine residues were carrying acetylation instead of iTRAQ label. Deacetylase inhibitors were not used in sample preparation of the iTRAQ samples since loss of the lysine acetylations can be an advantage during the iTRAQ based quantification of the relative amounts of the proteins. The low degree of acetylation sites indicates negligible impact of the lysine acetylation on the accuracy of the iTRAQ-based protein quantitation in this study. The significance level of protein identifications was set to 0.001, which resulted in a false discovery rate (FDR) of less than 0.003 when searched in Mascot against the decoy database of random sequences. iTRAQ values were reported for proteins with five or more measured iTRAQ scan values from peptides with expectation values of 0.02 or below. The quantification data of each biological sample were normalized to protein median to compensate for possible variation in starting material. Only proteins detected from at least two peptides were included in the statistical calculations. Ratios for each protein were reported as significantly different from 1.0 if they passed two tests: 1) a fold change criterion (>1.25) based on two times the global standard error and 2) a two-tailed student's *t*-test ($p < 0.05$) for equal variance data. Proteins detected in both soluble and membrane fraction and with significance in the fraction with lowest abundance (as estimated from number of scans) were checked for coherency in fold change direction in the other fraction, and omitted from further data descriptions if found incoherent. Four such cases were found: Atp5a1, Dbt, Ganab and RPS14.

All the mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002741 and 10.6019/PXD002741 for the membrane proteome fraction (**Username:** reviewer73951@ebi.ac.uk, **Password:** 9LhL1Cr8) and dataset identifier PXD002742 and 10.6019/PXD002742 for the supernatant proteome fraction (**Username:** reviewer00023@ebi.ac.uk, **Password:** I40pJZR8) [22].

Data analysis

To analyze the protein lists derived from MS and extracting biological meaning from the lists we applied the DAVID bioinformatic resource and used the 967 proteins from the study as a background. Furthermore, to better understand what functional interactions these significantly changed proteins have we used STRING software.

Western blot analysis of protein acetylations

Western blot analysis was performed on proteins extracted from the liver samples in the presence of a cocktail of deacetylase inhibitors: 10 μ M trichostatin A (TSA), 10 mM sodium butyrate, 2 μ M suberoylanilide hydroxamic acid (SAHA, vorinostat) and 10 mM nicotinamide. The primary antibody was anti-acetyllysine antibody (Cell signaling), and the secondary was anti-rabbit-HRP antibody (Dako, Denmark). Detailed procedure can be found in Supplementary Materials and Methods.

Parallel Reaction Monitoring (PRM) MS

Based on the large-scale study, proteins were selected for validation by targeted MS analysis using Parallel Reaction Monitoring (PRM) on nanoLC coupled to Q-Exactive Plus MS

(Thermo). Protein extracts from livers of six *Ethel*^{-/-} and three *Ethel*^{+/+} mice were prepared in the same way as Western blot samples, and subsequently proteins were in-gel trypsin digested (Supplementary Materials and Methods). Proteins selected for PRM validation were imported to the software Skyline where tryptic *in silico* digestions were carried out. More details on the targeted proteins and peptides can be found in Supplementary Materials and Methods.

Elemental analysis by ICP-MS

The concentrations of elements (iron, zinc, magnesium and sulfur) were determined by inductively coupled plasma mass spectrometry (ICP-MS) following micro-scaled pressurised digestion [23] Briefly, mouse liver tissue samples (10-20 mg) were digested in a microwave oven (Multiwave 3000, Anton Paar, Graz, Austria) in 5 ml glass vials using 250 µL concentrated nitric acid and 100 µL hydrogenperoxide (both Merck, Darmstadt, Germany). The digests were diluted to a volume of 5 mL with milliQ water and subsequently their content of elements was determined using external calibration by ICP-QQQ-MS (Agilent 8800, Agilent Technologies, Yokogawa, Japan) run in the helium (⁵⁷Fe, ⁶⁶Zn, ²⁴Mg) and oxygen modes (³⁴S (32->48), respectively).

Results and Discussion

To elucidate the impact of deficiency of the persulfide dioxygenase ETHE1, we performed large scale proteomics on liver tissue samples from ETHE1-deficient mice and control mice. The mass spectrometry based proteomics yielded identification and quantification data for 595 and 524 proteins in soluble (S) and membrane (M) protein fractions, respectively, with 152 proteins detected in both. Detection in both fractions can be due to cross-contamination of abundant proteins (eg albumin), however, the 152 proteins are mostly enzymes and are included in the statistical analysis since many enzymes are known to have partial or temporary membrane association with biological relevance. Table S1 lists the data of all the 967 unique proteins (1119 protein entries). 137 proteins passed the tests of statistical significance ($p < 0.05$ and fold change > 1.25 -fold change) (Table S2). These proteins were subjected to bioinformatics analyses.

Bioinformatic analysis of protein functionalities

DAVID and STRING software tools were applied to gain functional and biological enrichment of the proteins with significantly altered levels and to elucidate how these proteins are linked to each other, and to identify enriched functional annotation terms within the datasets. All 137 statistically significant proteins were subjected to DAVID with 1119 obtained proteins as background data set to identify enriched functional annotation (Table 1). There were 28 proteins with the functional annotation “Mitochondrion”, where ETHE1 is located. The other functional categories cover a variety of functionalities indicating wide biochemical and functional impact of ETHE1 deficiency, as described in sections below. The same data-set was applied to STRING in order to elucidate more details about protein-protein interactions and functional associations (Figure S1A, B).

Hydrogen sulfide and oxidation-reduction

1 Among the categories obtained in Table 1 two were particularly pronounced; oxidation-
2 reduction and metal ion binding proteins (Table 2). 12 proteins were common for these two
3 groups, and since all proteins were down-regulated in our study, there seems to be a link
4 between sulfide accumulation and proteins involved in oxidation-reduction. Of the proteins
5 which have metal as a cofactor, six depend on iron for activity including four members of the
6 Cytochrome P450 family, while the rest need zinc or other metals such as magnesium or
7 manganese.

8 Approximately 44% of oxidoreductases have been described as metalloenzymes and 81% of
9 them are iron-dependent [24]. Since 60% of the Oxidation-Reduction proteins (12 out of the
10 20) in Table 2 are metal-binding, there might be an interaction between sulfide and
11 metalloproteins, notwithstanding different kind of metals are represented. Furthermore,
12 according to the DAVID analyses, six proteins were solely in the metal binding cluster
13 category (Table 2). To better delineate whether it is the oxidoreductase function or the metal
14 binding protein property being more central we performed elemental analysis of metals.

16 **Elemental analysis of metals and sulfur**

17 Sulfide is able to precipitate iron and zinc ions at neutral pH to form metal sulfides [25],
18 which might lead to intracellular accumulation of both the metals and the element sulfur. We
19 therefore quantitated the total, bound and unbound, amount of zinc and iron together with
20 magnesium (also a co-factor in Table 2). The concentrations of zinc, iron and magnesium in
21 liver tissue of ETHE1-deficient and healthy control mice were measured by inductively
22 coupled plasma mass spectrometry (ICP-MS) in fully hydrolysed samples. The
23 concentrations (mean±standard deviation) for ETHE1 deficient (n=6) and controls (n=3),
24 respectively, were for zinc 45±13 vs 42±5, for iron 180±121 vs 222±79, and for magnesium
25 350±16 vs 372±36 µg/kg. No statistical significant changes were observed between the two

sample groups. Moreover, the total amounts of the element sulfur were also measured and found to be 3420 ± 415 and 3550 ± 268 $\mu\text{g/kg}$ in ETHE1 deficient and control livers, respectively. Since the concentrations of the measured metals and sulfur were unaltered it indicates that the observed proteomics alterations of the oxidation-reduction proteins instead are caused by the cellular redox status and specific biological regulation.

Cytochrome P450 and oxidative stress

Of the 20 oxidation-reduction proteins significantly down-regulated in ETHE1-deficient mice, there were four cytochrome P450 (CYP) proteins. Cytochrome P450 is an endoplasmic reticulum-anchored family of heme proteins [26] that are important for various kinds of detoxifications reactions. CYPs can produce superoxide anion and hydrogen peroxide [27], which in turn may release 'catalytic' iron and generate potent oxidants. Four members of the large CYP family showed clearly lowered levels in the ETHE1-deficient mice: CYP1A2, CYP2C29, CYP2C70 and CYP2D10 (Figure 1A), and CYPs of these CYP1, CYP2 and CYP4 families typically have activities in eicosanoid metabolism [28]. CYP1A2 has previously been shown to be down-regulated under acute sulfide poisoning [29]. It has also been shown that CYP450 inhibition decreases hydrogen sulfide induced oxidative stress [30]. Thus the observed down-regulation of four CYP proteins may be a way for the cells to alleviate sulfide-induced stress.

Four proteins with relation to oxidative stress or glutathione were significantly down-regulated (Figure 1B); catalase, glutathione s transferase 2 and 3 (GSTM2, GSTM3), and methionine sulfoxide reductase (MSR). Superoxide dismutase (SOD2) was previously found down-regulated in mitochondria from ETHE1 deficient human fibroblasts [18], but was not significantly altered in our mouse liver study. However other oxidative stress related proteins

1 detected in our study showed a general tendency to be down-regulated (13 out of 18 were
2 below 1.0, with a median ratio of 0.8) (Figure 1B). These observations together, indicate that
3 redox balance and antioxidant activity were affected in ETHE1-deficient mice.

4 5 **Metabolic reprogramming in ETHE1 deficiency**

6 Our data clarified a clear link between ETHE1 and energy status of cell, through the
7 significant up-regulation of several metabolic pathways (Figure 1C and 1D), which likely can
8 compensate for the lack of energy caused by the established dysfunction of mitochondrial
9 metabolism, including lactic acidosis and SCAD inhibition. Two important proteins in
10 glycolysis were found to have increased levels: fructose biphosphate aldolase (FBA) and
11 pyruvate kinase (PK). FBA catalyzes the reversible reaction of Fructose 1,6 biphosphate to
12 Glyceraldehyde 3-phosphate in glycolysis. PK is the last enzyme in glycolysis, which is
13 responsible for producing pyruvate from phosphoenolpyruvate [31]. Lactate dehydrogenase
14 using pyruvate as substrate was also up-regulated. Apart from lactate dehydrogenase and the
15 glycolytic FBA and PK with significant increases, six other enzymes of glycolysis were
16 successfully quantitated, however not found significant and with levels ranging from 0.82 to
17 1.17 (Figure S2). Increased levels of glycolytic enzymes (FBA and PK) in combination with
18 increased LDH can lead to increased production of lactate, which is in line with the fact that
19 patients with mutation in ETHE1 show lactic acidosis [32].

20 Pyruvate carboxylase, which is responsible for pyruvate carboxylation and converting it to
21 oxaloacetate, was up-regulated in our study [33]. Oxaloacetate is an important intermediate in
22 metabolism, linking carbohydrate, lipid, amino acid and nucleotide metabolism [34], and can
23 either enter Krebs cycle or gluconeogenesis pathway. Another sign of the shift from
24 mitochondrial respiration towards glycolysis was calcium-binding mitochondrial carrier

1 protein (citrin) up-regulation. Citrin is the hepatic isoform of the mitochondrial aspartate–
2 glutamate carrier [35], and is known to participate in gluconeogenesis [36], which keeps
3 glycolysis reactions active. Up-regulation of citrin in our study further support the notion that
4 ETHE1-deficient cells favored glycolysis rather than mitochondrial respiration, probably
5 driven by compromised COX and thus respiratory chain.

6 Enzymes of fatty acid oxidation (FAO) and branched chain amino acid (BCAA) metabolism
7 were found up-regulated in ETHE1-deficient mice, namely medium-chain acyl-CoA
8 dehydrogenase (ACADM), very-long-acyl-CoA dehydrogenase (ACADVL), enoyl-CoA
9 hydratase, propionyl-CoA carboxylase, and dihydrolipoyl dehydrogenase (DLD).

11 **ETHE1 deficiency and gene expression via hnRNP**

12 The heterogeneous nuclear ribonucleoprotein (hnRNP) contains RNA-binding proteins which
13 associate with heterogeneous nuclear RNAs and are responsible for regulating gene
14 expression in response to stresses such as hypoxia [37]. In our study six different hnRNP
15 were found to have elevated level in the ETHE1-deficient mouse liver (Figure 1E), which is
16 consistent with data describing link between redox homeostasis and hnRNP proteins [38]. As
17 discussed above glycolysis is favored during ETHE1-deficiency, indicating metabolic
18 reprogramming, known as the Warburg effect [39]. It has been shown that pyruvate kinases
19 are regulated by heterogeneous nuclear ribonucleoprotein (hnRNP), and that the isoform
20 PKM2 is increased by hnRNPA1 and hnRNPA2 [40]. Interestingly, our data showed
21 increased level of pyruvate kinase and hnRNP proteins (eg. hnRNPA1), which indicate a
22 regulatory function of hnRNPs in cells with mitochondrial dysfunctions due to ETHE1
23 deficiency. Furthermore, hnRNP A1 has splicing activity on the ACADM (MCAD) altered in

1 this study [41]. Recently hnRNP A1 was found to be over-expressed in cancer [42], and
2 Warburg effect is present in cancer and also in inborn errors of metabolism (IEM) [43]. The
3 differentially altered hnRNPs in our study contain 6 to 18 acetylated lysine residues [44],
4 which suggests that acetylation has impact on hnRNP regulated splicing and gene regulation.
5 Acetylation has recently been described to increase stability of hnRNPs, e.g. hnRNP A1, I
6 and L [45]. A growing amount of data obtained through different approaches indicates the
7 importance of splicing activity of hnRNPs in response to metabolic state of the cells,
8 including protein acetylation status.

9 **Protein acetylation**

10 Our results showed a clear link between ETHE1 and altered energy metabolism, which in
11 turn is known to be influenced by the post-translational modification (PTM), such as protein
12 acetylation [46]. Based on recent proteomics studies, a majority of acetylation events occur in
13 the mitochondrial proteome and seems to be the most prevalent mitochondrial PTM;
14 regulating signaling networks through metabolic intermediates in glycolysis, Krebs cycle and
15 fatty acid β -oxidation [47, 48]. We decided to analyse the total level of protein acetylation, in
16 order to investigate how it was affected in ETHE1-deficient mice compared to healthy mice.
17 The level of acetylated proteins was visualized by western blotting, using an antibody against
18 acetylated lysine residues (Figure 2). Total protein staining with Sypro Ruby was used to
19 verify equal loading (Figure S3). Total protein acetylation pattern exhibited increased
20 intensities in ETHE1-deficient mice (p -value: 0.07), and since a high number of bands had
21 increased intensities it indicates a broad cellular alteration. Histones are well-characterized
22 highly acetylated small proteins [49]. Interestingly, we did not detect clear changes in 14-16
23 kDa area where histones normally migrate as dominating proteins (Figure 2). Acetylation is
24 catalyzed by lysine acetyltransferases (KATs), using acetyl-CoA as a cofactor, and can be

removed by lysine deacetylases (KDACs), consisting of a large family of histone deacetylases (HDAC) and Sirtuins [50]. Besides enzymatic reaction, acetylation occurs non-enzymatically, via direct contact of proteins with acetyl-CoA. Several non-acetyl metabolites such as NAD^+ have been shown to be key regulators of deacetylases. The NAD^+/NADH ratio, which reflects the redox state of the cell, regulates the activities of the sirtuins, and thus influences global acetylation levels [51]. The glycolysis product pyruvate is known as inhibitor of several histone deacetylases such as HDAC1 and HDAC3 [52]. So, shifting metabolism from mitochondria toward glycolysis in ETHE1-deficient mice could be correlated with the generally increased protein acetylation. Butyrate is also known as HDAC inhibitor metabolite [53], and SCAD inhibition in ETHE1 deficiency leads to butyrate accumulation that in turns can have inhibitory impact on HDACs. These findings point to a link between ETHE1 and acetylation, where both redox state and cellular metabolites can influence acetylation and deacetylation activity.

Hydrogen sulfide and HSP90

HSP90 is an abundant stress protein, mostly located in the cytoplasm, playing an important role in various cellular processes such as protein folding, protein degradation and signal transduction cascades [54]. In ETHE1-deficient mice HSP90 was down-regulated (Figure 1F). It has previously been shown that HSP90 interacts with TPR domain proteins (tetratricopeptide repeat), such as proteins of the CYP family [55], and our study showed simultaneous down-regulation of both. Since HSP90 has been shown to be up-regulated in oxidative stress [54, 56], down-regulation of HSP90 in our study suggests a reductive, instead of an oxidative, condition during ETHE1 deficiency.

Cytoskeleton proteins and Epithelial-Mesenchymal Transition (EMT)

Vimentin, a major constituent of the Intermediate Filament (IF) family of proteins was found up-regulated. Vimentin is central for maintaining cellular integrity in eg mesenchymal cells [57] and is a marker for Epithelial-Mesenchymal Transition (EMT) which is an important mechanism contributing to cancer [58, 59]. Our results show that besides vimentin, other cytoskeleton proteins were also affected by ETHE1 deficiency (Figure 1G).

Comparison with previous studies

Our results on cytoskeletal proteins are in line with elevated levels of cytoskeletal proteins previously found in muscle and brain tissues of ETHE1-deficient mice [19]. Furthermore, Hildebrandt *et al* also found a bioinformatic overrepresentation of acetylated proteins, and here we experimentally described the presence of increased protein acetylation. They performed 2D-PAGE based proteomics and identified 16 differentially altered proteins which we also detected, and out of those we could also get clear significance for 6 proteins (ATP5A1, ECHS1, FKPB4, PRDX1, RGN and SUOX), despite the very different procedures of the two studies.

Validation by targeted MS protein analysis

Accurate and precise quantification of proteins can be done by targeted proteomics via selected reaction monitoring (SRM), or the similar Parallel Reaction monitoring (PRM) on high mass accuracy instruments [60]. We applied PRM on unfractionated samples with addition of heavy labeled peptide internal standards.

1 A group of peptides were selected to measure VLCAD, HSP90, APOE and CYP1A2, chosen
2 as four representative proteins significant in the iTRAQ study (Table 3). All but APOE
3 supported the data from the main study. VLCAD level was 1.45 fold up-regulated, and
4 HSP90 and CYP1A2 were down-regulated 0.66 fold and 0.45 fold respectively. VLCAD,
5 HSP90 and CYP1A2 are examples of three proteins clearly regulated during the ETHE1
6 deficient condition and they illustrate the wide impact the ETHE1 can have on the cell
7 pathophysiology.

8 *Mitochondrial proteins*

9 ETHE1 is localized to mitochondria and elicit effects there, eg the negative effects of ETHE1
10 deficiency on mitochondrial cytochrome C oxidase. Several mitochondrial proteins had
11 elevated levels, eg citrin, and enzymes of fatty acid oxidation and branched chain amino acid
12 metabolism. To test whether these proteins were specifically altered or it was due to a general
13 increase in mitochondrial amounts five reference proteins, chosen to represent stably
14 expressed proteins of both mitochondria (VDAC, HSP60) and cytosol (PGM, RL7A, and
15 RL19), were assayed by PRM. The reference proteins were detected in all samples and at a
16 rather constant level. By calculating the ratio of VDAC and HSP60 versus the PGM, RL7A
17 and RL19 an estimate of mitochondrial content was obtained which showed close to same
18 level in ETHE1 deficient and control samples (ratio: 0.93), indicating that the group of
19 mitochondrial proteins found significant were specifically altered. The level of SCAD and
20 ECHD1 proteins were assayed since the first is known to be inhibited by ETHE1 deficiency
21 and the second can catalyze the reaction ethylmanoyl-CoA to butanoyl-CoA, with relation
22 EMA [61]. None of them were found to have altered level in line with the data of the main
23 iTRAQ study.

Concluding remarks:

In conclusion, by performing large scale quantitative proteomics on ETHE1-deficient mice liver tissues a clear link between ETHE1-deficiency and cellular redox state was observed, since several oxidoreductases and dehydrogenases showed decreased levels, similar to what has been shown on cultivated fibroblasts [18]. In addition to confirming previous results on ETHE1 impact on BCAA and FA catabolism [19], we report metabolic reprogramming and shifting metabolism toward glycolysis. The current data also demonstrate increased protein acetylation and novel links between ETHE1 and gene expression through hnRNPs.

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Conflicts of Interest

The authors have no conflict of interest to declare

Legends:

Figure 1: A) The levels of CYP450 members in ETHE1 deficient mice compared with healthy controls. The majority of the CYPs members had values below 1.0. B) Proteomics data for redox regulating and oxidative stress response proteins. C) Protein description and fold change for significantly altered proteins involved in glycolysis and gluconeogenesis. D) Significantly altered proteins participating in branched chained amino acid catabolism and fatty acid oxidation. E) Proteomics data for heterogeneous ribonucleoproteins (hnRNPs). F) Class A and class B member of heat shock protein 90 with another heat shock protein 8 were

significantly down-regulated. CDC37 is HSP90 co-chaperone which promotes interactions of kinases and HSP90, resulting in stabilization and promotion of HSP90 activity. HSP90 co-chaperone was also down-regulated. G) Structural and cytoskeleton proteins up-regulated in ETHE1-deficient mice liver. Error bars represent $\pm$ SEM. Dark and light grey bars depict significant and non-significant differential protein expression, respectively. Statistical significance levels are illustrated as $p < 0.05$ with *, $p < 0.01$ with **, $p < 0.001$ with ***.

Figure 2: Western blot analysis of acetylated proteins in liver tissue of *Ethe1*^{+/+} and *Ethe1*^{-/-} mice with anti-acetyl lysine antibody.

Figure S1. STRING analysis of protein-protein interaction. Protein-protein interaction of 137 significantly altered proteins, A) up-regulated and B) down-regulated were analysed by applying STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) version 10 with the confidence cut off 0.5. Proteins are represented as nodes with connecting lines. Stronger associations are represented by thicker lines. Disconnected nodes were removed.

Figure S2. The levels of glycolytic enzymes, and lactate dehydrogenase, detected and quantified in the iTRAQ studies. All data are from the S fraction since all proteins were detected there with higher number of quantitative scans than in the M fraction. Hexokinase and phosphofructokinase escaped detection and are not depicted. Fold change was calculated as the ratio of the mean ETHE1 deficient divided by mean of control mice. Error bars represent $\pm$ SEM. Dark and light grey bars depict significant and non-significant differential protein expression, respectively. Statistical significance levels are illustrated as $p < 0.05$ with *, $p < 0.01$ with **, $p < 0.001$ with ***.

Figure S3. SYPRO ruby total protein stain of the Western blot membrane that subsequently was probed for protein acetylation.

Table S1. Identification and quantification data of 1119 protein entries (967 unique proteins) obtained from nLC MS/MS analyses of the mice liver tissue. The data were obtained from raw files from nLC MS/MS subjected to Proteome Discover. Min n denotes the number of peptide scans, and Fraction stands for two phase protein separation (M=Membrane and S=Supernatant), where 1 and 2 denotes the fraction with highest and lowest number of scans (Min N), respectively, when the protein was detected in both fractions.

Table S2. 137 significant proteins quantified in *Ethel*^{-/-} and *Ethel*^{+/+} mice liver tissue by nLC-MS/MS analysis. Ratio denotes iTRAQ quantitative data from *Ethel*^{-/-} divided by *Ethel*^{+/+}.

Table 1: Enriched Protein Functional annotation terms (top 5) among the 137 differentially regulated proteins in ETHE1 deficient mice compared with wild type was obtained through analysis in DAVID. Count signifies number of proteins in the cluster. In our study the significant functional annotations were selected using DAVID with the thresholds of enrichment score > 1.3 and count > 5.

Annotation cluster	Functional	Enrichment	count	P-value	Benjamini
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	annotation	score			
GOTERM_CC_FAT	Ribonucleoprotein complex	2.99	9	2.9E-1	9.6E-1
INTERPRO	RNA recognition motif	2.99	11	7.1E-6	1.6E-3
GOTERM_BP_FAT	Oxidation Reduction	2.11	20	8.0E-3	9.7E-1
GOTERM_MF_FAT	Transition metal ion binding	2.11	15	2.9E-2	9.2E-1
SP_PIR_KEYWORDS	Metal-binding	1.68	18	4.8E-3	1.8E-1
GOTERM_CC_FAT	Mitochondrion	1.3	26	1.4E-3	7.8E-2

Table 2: Proteins of the DAVID cluster “Oxidation –Reduction” and Metal binding proteins not present in the cluster of oxidoreductases. All were down-regulated in ETHE1-deficient mice. Cofactor information was obtained from Uniprot.

	Protein Name	Gene Name	Uniprot IDs	Cofactor
Oxidation-reduction cluster without metal cofactor	4-trimethylaminobutyraldehyde dehydrogenase	Aldh9a1	Q9JLJ2	
	cytidine monophospho-N-acetylneuraminic acid hydroxylase	Cmah	Q61419	
	cytosolic 10-formyltetrahydrofolate dehydrogenase	Aldh1l1	Q8R0Y6	
	estradiol 17 beta-dehydrogenase	Akr1c6	P70694	
	glycine dehydrogenase	Gldc	Q91W43	
	gulonolactone (L-) oxidase	Gulo	P58710	
	methionine sulfoxide reductase A	Msra	Q9D6Y7	
	peroxisomal multifunctional enzyme type 2	Hsd17b4	P51660	

Oxidation-reduction cluster with metal cofactor	3-Hydroxyanthranilate 3,4-Dioxygenase	Haa0	Q78JT3	iron
	alcohol dehydrogenase 1 (class I)	Adh1	P00329	zinc
	catalase	Cat	P24270	iron-sulfur
	cytochrome P450, family 1, subfamily a, polypeptide 2	Cyp1a2	P00186	iron
	cytochrome P450, family 2, subfamily c, polypeptide 29	Cyp2c29	Q64458	iron
	cytochrome P450, family 2, subfamily c, polypeptide 70	Cyp2c70	Q91W64	iron
	cytochrome P450, family 2, subfamily D, polypeptide 10	Cyp2d10	Q9EP75	iron
	isocitrate dehydrogenase 1 (NADP+), soluble	Idh1	O88844	magnesium or manganese
	NADP-dependent malic enzyme	Me1	P06801	magnesium or manganese
	ribosyldihydronicotinamide dehydrogenase	Nqo2	Q9JI75	zinc
	squalene synthase	Fdft1	P53798	magnesium
	sulfite oxidase	Suox	Q8R086	molybdenum-molybdopterin
	cytosol aminopeptidase	Lap3	Q9CPY7	zinc
Solely metal binding	delta-aminolevulinic acid dehydratase	Alad	P10518	zinc
	dipeptidyl peptidase 3	Dpp3	Q99KK7	zinc
	fructose biphosphatase 1	Fbp1	Q9QXD6	magnesium
	GTP-binding protein	Sar1b	Q9CQC9	magnesium
	hydroxymethylglutaryl-CoA lyase	Hmgcl	P38060	manganese magnesium

Table 3: Targeted proteomics data validation by PRM and comparison with iTRAQ proteomics data. VLCAD, HSP90 and CYP1A2 as representing proteins are regulated in same direction in both assays, while ApoE was not significant in PRM data.

PRM validation			iTRAQ proteomics data	
Protein	Fold change	<i>p</i> value	Fold change	<i>p</i> value
VLCAD	1.45	0.07	1.91	0.005
HSP90	0.66	0.0001	0.57	2.21E-05
CYP1A2	0.45	0.1	0.55	0.0003
APOE	1.12	0.68	1.64	0.02
ECHD1	0.75	0.19	1.0	0.99
ACADS	1.01	0.94	1.15	0.12