Comparative Proteome Research in a Zebrafish Model for Vanishing White Matter Disease

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Abstract: Vanishing white matter (VWM) disease is a genetic leukodystrophy leading to severe neurological disease and early death. VWM is caused by bi-allelic mutations in any of the five genes encoding the subunits of the eukaryotic translation factor 2B (EIF2B). Previous studies have attempted to investigate the molecular mechanism of VWN by constructing models for each subunit of EIF2B that causes VWM disease. The underlying molecular mechanisms of the way in which mutations in EIF2B3 result in VWM are largely unknown. Based on our recent results, we generated an eif2b3 knockout (eif2b3−/−) zebrafish model and performed quantitative proteomic analysis between the wild-type (WT) and eif2b3−/− zebrafish, and identified 25 differentially expressed proteins. Four proteins were significantly upregulated, and 21 proteins were significantly downregulated in eif2b3−/− zebrafish compared to WT. Lon protease and the neutral amino acid transporter SLC1A4 were significantly increased in eif2b3−/− zebrafish, and crystallin proteins were significantly decreased. The differential expression of proteins was confirmed by the evaluation of mRNA levels in eif2b3−/− zebrafish, using whole-mount in situ hybridization analysis. This study identified proteins which candidates as key regulators of the progression of VWN disease, using quantitative proteomic analysis in the first EIF2B3 animal model of VWN disease.

Keywords: Vanishing White Matter disease; EIF2B; comparative proteomics; SLC1A4

1. Introduction

Childhood Ataxia with Central Nervous System Hypomyelination, also called Vanishing White Matter (VWM) disease, is one of the most prevalent inherited childhood leukoencephalopathies. VWM is a grave disease, and 50% of patients die within two years of diagnosis. It is a fatal, stress-sensitive leukodystrophy that mainly affects children, and for which there is currently no treatment [1]. VWM is characterized by ataxia, spasticity, and variable optic atrophy [2]. Over the past 20 years, various phenotypes of the disease have been identified, and it has been established that it can appear at all ages, not just in childhood [2,3]. VWM is a neurological disease caused by mutations in the eukaryotic translation initiation factor 2B (EIF2B) that produce bi-allelic pathogenic variants in one of five genes: EIF2B1, EIF2B2, EIF2B3, EIF2B4, or EIF2B5 [4].

EIF2B is indispensable for the initiation of mRNA translation and regulation of protein synthesis under different conditions, including cell stress [1,5]. Protein synthesis, an important component of which is protein synthesis and balancing of biological processes,
is a key step in the expression of a cell’s genetic information. EIF2B controls the first major stage of mRNA translation, initiation, which involves the recruitment of the ribosome to the mRNA and the identification of the correct start codon to commence translation. The molecular mechanisms controlling the pathological symptoms of VWM can be investigated by identifying changes in protein expression caused by mutations in EIF2B. In some previous studies, proteomics approaches were used to investigate the molecular mechanisms affecting VWM [6–8]. Although previous studies have identified mitochondrial dysfunction due to altered proteasomal activity, and impaired balance between protein synthesis and degradation, these results arise from limited investigations of effects at the cellular level, that are not related to the pathogenesis of VWM.

To understand the pathology of VWM, experimental models have been generated. In 2010, the first animal model was developed in mice, by introducing a point mutation into the mouse Eif2b5 gene locus A [9]. However, mouse knockouts of the Eif2b genes were lethal, precluding their use for further study of VWM pathogenicity. Recently, an in vitro model was constructed by differentiating gray and white matter astrocytes obtained from humans and mice into induced pluripotent stem (iPS) cells, as a model for differentiation, after the transformation of patient-derived fibroblast into iPS cell was achieved [10,11]. However, the cell model cannot be applied to the phenotyping of individuals, due to the mutation, and only limited molecular changes can be studied.

Recently a model for, EIF2B5 in VWM disease was reproduced by the truncation of eif2b5 in zebrafish (Danio rerio), which produced impaired motor function, and led to further activation of the cellular integrated stress response [12]. We established the first eif2b3 mutant animal model for VWM disease that is useful for functional validation of human gene variants, including a novel variant identified in a Korean VWM patient [13]. Following defects in the expression of myelin genes and glial cell differentiation, we observed phenotypes resembling those of human patients in eif2b3 knockout (eif2b3−/−) zebrafish. Using the first EIF2B animal model, we used comparative quantitative proteomic analysis between WT and eif2b3−/− to understand the molecular mechanisms affecting VWM. Since the zebrafish has high genetic similarity to humans, the experimental results obtained in this study were applied to human data to identify the molecular mechanism of VWM.

2. Results
2.1. Establishment of a Vanishing White Matter Disease Model by eif2b3 Knockout

In our previous study, an eif2b3−/− model was generated in zebrafish based on the identification of a new variant in Korean patients. The suitability of the model was verified by confirming the phenotype, which was typical of that associated with VWM disease. The characteristic defects in the nervous system of the eif2b3−/− zebrafish were confirmed by visualization of myelination using in transgenic zebrafish, Tg(mbp:mGFP) (Figure 1A). A reduction of the expression of mbp was confirmed by whole-mount in situ hybridization (WISH) (Figure 1B). We also investigated the altered differentiation of cell fates in glial cell progenitors in eif2b3−/− zebrafish corresponding to VWM pathogenesis. While the expression of the mature glial cell marker olig2 was downregulated, the earlier neural cell marker nestin was upregulated (Figure 1B).

(A)

Figure 1.Cont.
Prior to comparative proteomic analysis, yolk was removed to prevent interference in our analysis. Deyolked embryos were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel staining after the removal of the yolk proteins, which showed a specific protein band at a molecular weight of 80–100 kDa (Figure S1) [14]. The protein expression between WT and eif2b3−/− larvae, among which 1351 proteins were quantified based on comparative proteomic analysis (Figure 2B). The total identified protein list is presented in Supplementary Table S1. Quantitative data were collected from triplicates of prepared samples, the reproducibility of which was validated by scatter plots, with protein intensity and Pearson correlation score showing values up to 0.9 (Figure 2C).

2.2. Comparative Proteome Analysis of eif2b3−/− Compared to Wild-Type

Among the proteins measured, were 25 differentially expressed proteins (DEPs) (Figure 2B). To identify significant DEPs, we used a fold-change cut-off of 2 for upregulated proteins and 0.5 for downregulated proteins, and p value of less than 0.05 in a volcano plot using a one-way t-test (Figure 3A). Four proteins were significantly upregulated, and 21 proteins were significantly downregulated in eif2b3−/− larvae compared to WT (Table 1). Four proteins were increased in eif2b3−/−. Three were mitochondrial proteins: Lon protease, 2-oxoglutarate dehydrogenase E1 component, and 4-aminobutyrate aminotransferase, all three of which were confirmed to be homologous with human proteins. Neutral amino acid transporter SLC1A4, encoding ASCT1, was also significantly increased in the eif2b3−/− model. In the functional annotation using Gene Ontology (GO) terms, three of the upregulated proteins were associated with mitochondria in the GO cellular component (GO CC) category (Figure 3B). According to previous studies, the functional change of mitochondria in VWM disease observed in the eif2b mutant is known, so the three proteins observed in this study may be related to the functional changes in mitochondria in VWM disease [15].
Figure 2. Workflow for comparative proteome analysis in an eif2b3 knockout. (A) Flowchart illustrating the experimental procedure for quantification of regulated proteins in the eif2b3<sup>−/−</sup> model. (B) The table showing the number of proteins identified in WT and eif2b3<sup>−/−</sup> model, respectively. (C) Highly correlated results of triplicate analyses.

Figure 3. Functional annotation of differentially expressed proteins (DEP) in the WT and eif2b3 knockout. (A) Distribution of the 1352 quantified proteins (upregulated, red filled circles; downregulated, blue filled circles) in eif2b3<sup>−/−</sup> compared to the WT, according to one-sample t-tests and fold change (<i>p</i> < 0.05). (B) DAVID-generated GO enrichment and KEGG pathway analysis of DEPs. The log of Fisher’s exact test was used to represent the enrichment index. GOBP: Gene Ontology Biological Process, CC: cellular component, MF: molecular function, KEGG: Kyoto Encyclopedia of Genes and Genomes.
Table 1. Differentially expressed proteins.

| Protein Level | D. rerio | Human Homology |
|---------------|----------|----------------|
|               | Protein IDs | Protein Names | Gene Names | Ratio (KO vs. WT) | Protein IDs | Protein Names | Gene Names |
| Up            | A0A0R4HDF9 | Lon protease homolog, mitochondrial | lonp1 | 2.97 | P36776 | Lon protease homolog, mitochondrial | LONPI |
| Up            | Q5FRA2 | Probable 2-oxoglutarate dehydrogenase E1 component DHKTD1, mitochondrial | dhktd1 | 2.21 | Q6H4Y7 | Probable 2-oxoglutarate dehydrogenase E1 component DHKTD1, mitochondrial | DHKTD1 |
| Up            | I3IRW7 | 4-aminobutyrate aminotransferase | abat | 2.09 | P80404 | 4-aminobutyrate aminotransferase, mitochondrial | ABAT |
| Down          | D7RVS1 | Neutral amino acid transporter SLC1A4 | slc1a4 | 2.73 | P43007 | Neutral amino acid transporter A | SLC1A4 |
| Down          | B6IDE1 | Slow myosin heavy chain 2 | smyc2 | 0.37 | P12883 | Myosin-7 | MYH7 |
| Down          | F1R3Q3 | Septin 6 | sept6 | 0.46 | Q14141 | Septin-6 | SEPTIN6 |
| Down          | A7E2K7 | Crystallin, gamma M2d12 | crygm2d12 | 0.37 | P07316 | Gamma-crystallin B | CRYGB |
| Down          | Q4DH12 | Crystallin, gamma M2d13 | crygm2d13 | 0.36 | P07316 | Gamma-crystallin B | CRYGB |
| Down          | Q9W6A9 | Opsin-1, short-wave-sensitive 1 | opn1sw1 | 0.20 | P03999 | Short-wave-sensitive opsin 1 | OPN1SW |
| Down          | Q15B4 | Crystallin gamma EM2-5 | crygm2d5 | 0.25 | P07316 | Gamma-crystallin B | CRYGB |
| Down          | Q4DX6 | Crystallin gamma EM2-8 | crygm2d8 | 0.20 | P07316 | Gamma-crystallin B | CRYGB |
| Down          | Q4FX9 | BetaA2-2-crystallin | cryba2b | 0.47 | P53672 | Beta-crystallin A2 | CRYBA2 |
| Down          | A0A0R4HYV7 | Citrulline-aspartate ligase | N/A | 0.19 | P09666 | Argininosuccinate synthase | ASS1 |
| Down          | Q521J6 | Beta A1-2-crystallin | cryba1d1 | 0.46 | P05813 | Beta-crystallin A3 | CRYBA1 |
| Down          | Q6DGY4 | Crystallin, beta A1b | cryba1b | 0.44 | P05813 | Beta-crystallin A3 | CRYBA1 |
| Down          | U3JAV8 | Aspartate carbamoyltransferase | cad | 0.46 | P27708 | CAD protein | CAD |
| Down          | Q5XTN0 | Cysgmx protein | crygmx | 0.37 | P07316 | Gamma-crystallin B | CRYGB |
| Down          | B06M3 | Crystallin, gamma M2d1 | crygm2d1 | 0.25 | P07316 | Gamma-crystallin B | CRYGB |
| Down          | Q52JD7 | Beta A4-crystallin | cryba4 | 0.45 | P53673 | Beta-crystallin A4 | CRYBA4 |
| Down          | A5E4S8 | Crystallin, gamma M2d10 | crygm2d10 | 0.25 | P07316 | Gamma-crystallin B | CRYGB |
| Down          | Q6DGY7 | Gamma-crystallin N-B | crygmb | 0.47 | Q8W6F5 | Gamma-crystallin N | CRYGN |
| Down          | Q6QU2 | Beta A2-crystallin | cryba2a | 0.43 | P53672 | Beta-crystallin A2 | CRYBA2 |
| Down          | B0V191 | Crystallin, gamma M2d18 | crygm2d18 | 0.23 | P07316 | Gamma-crystallin B | CRYGB |
| Down          | E7HSM1 | Crystallin, beta B1-like 1 | crybh1d1 | 0.44 | P53674 | Beta-crystallin B1 | CRYBB1 |
| Down          | Q8WT1 | Beta B1-crystallin | crybb1 | 0.43 | P53674 | Beta-crystallin B1 | CRYBB1 |

N/A; not available.

The direct effect of the eif2b3 mutant which is related to VWM disease pathology can be predicted through the analysis of proteins decreased in DEP. To acquire an in-depth understanding of the biological effect of eif2b3−/−, we used GO terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and Interpro enrichment analyses (Figure 3B). The downregulated proteins in eif2b3−/− were largely divided into two groups: those of visual and those of metabolic relevance. In the eye, downregulated proteins were involved in the structure of the lens and visual perception, according to GO molecular function (GO MF) and visual perception in the GO biological process (GO BP) categories. In Interpro, 16 downregulated proteins in eif2b3−/− were annotated as Beta/gamma crystallin and Gamma-crystallin-related, which corresponds to 76.2% of the downregulated proteins. Most of the downregulated proteins were crystallin-related proteins.

Other proteins in the downregulated group in eif2b3−/− were associated with metabolism. The downregulated proteins were annotated as participating in the arginine biosynthetic process and the urea cycle in the GO BP category, and alanine, aspartate and glutamate metabolism in the KEGG pathways (Figure 3B). Citrulline–aspartate ligase and aspartate carbamoyl transferase were included among 21 downregulated proteins.
2.4. Validation of Differentially Expressed Proteins Using Whole-Mount In Situ Hybridization

In order to confirm the alteration of protein expression identified by comparative proteome analysis in \( \text{eif2b}^{-/-} \) compared to WT zebrafish, the mRNA expression of genes related to DEPs was performed using whole-mount in situ hybridization (WISH) analysis. The gene related to four significantly upregulated proteins is shown in Figure 4A. The mRNAs of \( \text{lonp1} \), \( \text{slc1a4} \) and \( \text{abat} \) were increased in \( \text{eif2b}^{-/-} \). However, the expression of \( \text{dhtkd1} \) was not significantly changed in the \( \text{eif2b}^{-/-} \) mutant (Figure S2A). The mRNA expression of the DEPs increased by at least 1.5-fold in quantified proteins, and four DEPs increased by two-fold compared with WT zebrafish (Table S1). The mRNA expression of \( \text{psat1} \) and \( \text{xbp1} \) was upregulated in \( \text{eif2b}^{-/-} \). The mRNA expression of the unfolded protein response pathway genes \( \text{xbp1} \), \( \text{atf4} \), and \( \text{atf6} \) were upregulated in \( \text{eif2b}^{-/-} \) zebrafish, as early as 2 dpf, preceding those of \( \text{slc1a4} \), \( \text{lonp1} \), and \( \text{psat1} \), especially in the midbrain-hindbrain boundary (MHB) (Figures S2B, C and S3). The mRNA expression of downregulated proteins was also confirmed. The mRNA expression of \( \text{ass1} \), \( \text{cad} \) and \( \text{sept6} \) were downregulated in \( \text{eif2b}^{-/-} \) zebrafish, especially in the MHB region where \( \text{lonp1} \) and \( \text{slc1a4} \) genes are up-regulated (Figure 4B). The MHB is a secondary organizer region that develops at the junction of the midbrain and hindbrain. Although the lens size was reduced, the mRNA expression of \( \text{crybb1} \) was not significantly changed in the \( \text{eif2b}^{-/-} \) mutant (Figure S4).

![Figure 4](image-url)

Figure 4. Visualization of differently expressed proteins in WT and \( \text{eif2b}^{-/-} \) knockout zebrafish. (A) Whole-mount in situ hybridization of WT and \( \text{eif2b}^{-/-} \) probed for \( \text{lonp1} \), \( \text{slc1a4} \), and \( \text{abat} \) upregulated genes. (B) Whole-mount in situ hybridization of WT and \( \text{eif2b}^{-/-} \) probed for \( \text{ass1} \), \( \text{cad} \), and \( \text{sept6} \) downregulated genes. * The asterisk and arrow indicate the midbrain-hindbrain boundary (MHB).

3. Discussion

VWM disease is an autosomal recessive genetic leukodystrophy associated with mutation in one of the five subunits of EIF2B as EIF2B1-5 in humans [16]. In previous studies, a relationship with VWM was reported, due to the mutation of each subunit.
EIF2B2 mutations were found to cause complex instability by interfering with holocomplex formation, and EIF2B5 mutations are responsible for a decrease in the functional units present in the cells of VWM disease patients [17,18]. In order to understand the pathological mechanisms of each mutation, an animal model reproducing the phenotype by induced mutations was developed. Models due to point mutations of Eif2b4 and Eif2b5 in mice were reported, which expressed the characteristic phenotype of VWM disease [9,19,20].

Previous studies have undertaken proteomic profiling of the VWM disease model, based on the Eif2b5 mutant model [6–8]. In astrocytes of WT and Eif2b5R132H/R132H mice, 80 proteins were changed, 50% of which were related to secretory pathways [6]. Protein profiling of embryonic fibroblasts (MEFs) isolated from Eif2b5R132H/R132H mice revealed unbalanced stoichiometry of proteins involved in oxidative phosphorylation and the components of the mitochondrial translation machinery [7]. Changes in brain proteins in Eif2b5R132H/R132H mice showed that dysregulation of mitochondrial functions, altered proteasomal activity, and impaired balance between protein synthesis and degradation play a role in VWM pathology [8]. These results show that the disease characteristics of VWM disease are closely related to changes in proteins in the Eif2b mutant model.

In the present study, we identified four significantly increased proteins DEPs in eif2b3−/− larvae: the neutral amino acid transporter SLC1A4 (slc1a4), Lon protease (lonp1), 4-aminobutyrate aminotransferase (abat), and probable 2-oxoglutarate dehydrogenase E1 component DHKTD1 (dhtkd1) based on comparative proteomics analysis. The DHKTD1 was increased in the proteome results, however, it was not changed at the mRNA level. Understanding the effect of upregulation of proteins in the eif2b3 mutant will lead to increased understanding of the pathology of VWM disease.

The increase of the neutral amino acid transporter SLC1A4 (the alanine, serine, cysteine, and threonine transport, ASCT1) in eif2b3−/− larvae was also validated using WISH (Figure 4A). The neutral amino acid transporter SLC1A4 is a major d-serine uptake system in astrocytes, and contributes significantly to the uptake of l-serine in primary neurons [21]. SLC1A4 appears to be regulated to meet metabolic demands by differentiating and mature neurons through the transport of glial- and blood-borne small neutral amino acids [22]. The mis-regulation as SLC1A4 mutation in the brain can change the levels of neutral amino acids like l-serine, d-serine, l-alanine, l-threonine, and glycine, which can affect brain functions in ways such as decreased volume of different brain regions, altered gene expression, and motor and learning deficits [23]. l-Serine plays an essential role in neuronal development and function. l-Serine biosynthesis is largely confined to astrocytes, and it is shuttled to the neuronal cells by SLC1A4. SLC1A4 was revealed as physiological regulator of d-serine metabolism, a coagonist of NMDA receptors [23].

Recently, recessive mutations in SLC1A4 gene have been associated with hypomyelination, developmental delay, and microcephaly [24]. SLC1A4 mutations were identified in two affected siblings with severe intellectual disability (ID), microcephaly and spasticity, from an Ashkenazi Jewish consanguineous family [25]. Additional SLC1A4 deficiency was described in patients with neurodevelopmental disorders, presenting with thin corpus callosum, in addition to the phenotypes of developmental delay, microcephaly, and seizures [26–28]. Glutamate excitotoxicity is associated with neurodegenerative disorders, including demyelination. As one of the toxic demyelination models, cuprizone induces demyelination and axonal damage. Slc1a4 was upregulated in cuprizone-induced demyelination, especially in the corpus callosum in a region-specific manner [29]. These results suggest that the failure of proper SLC1A4 expression may be in part the consequence of astroglial dysfunction, glutamate signaling, demyelination, axonal damage, and thin corpus callosum, related to demyelinating neurological diseases.

Lon protease is a mitochondrial matrix protease, crucial for the maintenance of mitochondrial homeostasis, and Lon dysregulation is involved in cancer and in the cerebral, ocular, dental, auricular and skeletal syndrome [30]. In the VWM disease model, Eif2b mutant, functional abnormalities of mitochondria can be characterized, comprising the abnormal mitochondrial content phenotype of the mutant cells [7,15,31]. The increase in
Lon protease was verified in the zebrafish model through the WISH method (Figure 4A). The increased Lon protease in eif2b3−/− may be an important factor in the malfunction of mitochondria in VWM disease. Similarly, abnormal upregulation compared to WT in eif2b3−/− may be associated with pathological symptoms in the brain in VWM disease. In this study, new clues that can interpret the mechanism of pathology of VWM, by identifying the proteins changed in the mutant model, and their relevance needs to be clarified through further studies.

When a specific gene is reduced in the mutant model, its protein is likely to be a one whose expression is directly affected. In our study, crystallin proteins accounted for most of the reduced proteins which are annotated to structural constituents of the eye lens and visual perception categories in DAVID analysis with Opsin-1 (Figure 3B and Table 1). Although it was reported that α-crystalline B chains were significantly increased in astrocytes derived from VWM disease models in previous studies, the type of crystalline (β and γ, not α), were significantly decreased in eif2b3−/− [11,32]. γ-crystallin is particularly rich in the core region of the lens of the human eye [33]. From a genome-wide polysome-profiling strategy, it was found that a cohort of lens-associated crystallin isoform mRNAs is under the control of a specific mechanism of translation [34]. The optic atrophy, followed by blindness, in one of representative phenotypes in VWM disease patients, are possibly due to a significant decrease in β- and γ-crystalline, which plays an important role in the visual system.

Among the downregulated proteins, citrulline-aspartate ligase was reduced in mutant larvae as confirmed by WISH (Figure 4B). Citrulline-aspartate ligase in zebrafish has homology with the arginosuccinate synthase (ASS1) protein in humans (Table 1). Arginosuccinate synthase 1 is one of the enzymes of the urea cycle, and it catalyzes the formation of arginosuccinate from aspartate, citrulline and ATP and together with arginosuccinate lyase, and it is responsible for the biosynthesis of arginine in most body tissues. When arginosuccinate synthase 1 is deficient, the urea cycle does not work, causing hyperammonemina, in which the concentration of ammonia increases in blood. Ammonia is a neurotoxin involved in the pathogenesis of neurological conditions associated with hyperammonemina, including hepatic encephalopathy and brain edema [35,36]. Patients with neonatal-onset hyperammonemina should have early therapy to avoid severe brain damage [37]. Further studies should be conducted into the effect of the eif2b3−/− on the reduction of arginosuccinate synthase 1, but arginosuccinate synthase 1 may be suggested as a mechanism for the phenotype in the brain of VWM.

Using WISH analysis, we found a specific brain region in which both upregulation (lonp1 and slc1a4) and downregulation (ass1 and cad) of genes occurs. The MHB, also called the isthmic organizer, is a secondary organizer region that develops at the junction of the midbrain and hindbrain [38]. The MHB expresses signaling molecules that regulate the differentiation and patterning of the adjacent neuroepithelium. This organization allows for the development of the midbrain and hindbrain, as well as the specification of neuronal subtypes in these regions. Further studies focusing on the differential expression of these genes in MHB and their possible role in the pathogenesis of VWM disease, will be needed.

In conclusion, this study proposed candidate proteins as key regulators of the progression of VWN disease. We used quantitative proteomic analysis in the first EIF2B3 animal model of VWN disease. Abnormalities of myelin gene expression and glial cell differentiation were observed in the eif2b3 knockout zebrafish, and the DEPs were profiled by comparative quantitative proteomic analysis. Proteins related to the etiological phenomenon of VWM were identified, and the expression patterns were verified, thereby providing information on the molecular mechanisms underlying the symptoms of VWN disease.

4. Materials and Methods
4.1. Zebrafish Husbandry

Animal experiments were conducted according to approved guidelines and regulations of the Institutional Animal Care and Use Committee at the Animal Ethics Committee.
of Chungnam National University (Approval number: 202012A-CNU-170). Adult fish were reared under standard conditions with a 14 h/10 h light/dark cycle. We obtained embryos by natural mating, and zebrafish were reared in egg water at 28.5 °C. WT zebrafish were obtained from the Zebrafish Center for Disease Modeling.

4.2. Maintenance of eif2b3 Knockout Zebrafish

To understand the in vivo role of EIF2B3, we established a zebrafish knockout model utilizing the CRISPR/Cas9 system, as described previously [39]. PCR primers for genotyping of eif2b3−/− zebrafish were forward primer, 5’-TGTTCGGGATGGAGCTACAG-3’, and reverse primer, 5’-TTTGTTGCCCACAGGAAGCA-3’. PCR products (20 µL) were re-annealed in a thermal cycler under the following conditions: 95 °C for 2 min, 95–85 °C at 2 °C/s, 85–25 °C at 0.1 °C/s, then kept at 4 °C.

4.3. Protein Sample Preparation

The deyolked zebrafish embryos from each group were lysed with ice-cold radioimmunoprecipitation assay buffers (Thermo Fisher Scientific, Waltham, MA, USA) containing protease inhibitors (Thermo Fisher Scientific) using a homogenizer. Sonication was applied directly to all samples for 2 min (output 30%, 5 sec on and off intervals) on ice and centrifuged at 4 °C at 16,000 × g for 10 min [14]. The soluble fractions were moved into new sample tubes, and the protein concentration measured using BCA kits (Thermo Fisher Scientific). We placed 100 µg of the protein samples (triplicates) from each group (15 embryos) to new sample tubes and added 5 mM of dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 56 °C for 30 min, followed by treatment with 15 mM iodoacetamide (Sigma-Aldrich) in the dark for 30 min. Then, ice-cold acetone was added slowly to the samples for protein precipitation and kept for 18 hr in a −20 °C freezer. The samples were centrifuged at 4 °C at 16,000 × g for 10 min and supernatants were removed. The protein pellets were dissolved in 50 mM tetraethylammonium bromide (Sigma-Aldrich) and the protein concentration measured again. The pH was adjusted to 8 and trypsin (1:50) was directly added to the sample and left to digest for 18 hr at 37 °C. Finally, 1% trifluoroacetic acid (Sigma-Aldrich) added to complete the trypsin digestion. The peptides were dried in speed vac dryer at low temperature and directly dissolved in 50 mM tetraethylammonium bromide for 2-plex tandem mass tag (TMT) labeling (Thermo Fisher Scientific). After checking the peptide concentration using a Pierce™ Quantitative Colorimetric Peptide Assay Kit (Thermo Fisher Scientific), equal amounts of peptides from WT and eif2b3−/− group were labeled (n = 3) and placed in one sample tube. The pooled peptide samples were fractionated using a Pierce™ High-pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific).

4.4. Comparative Proteome Analysis

The fractionated samples were dissolved in 10 µL of 2% in 0.1% formic acid solution, and 500 ng of each fraction was loaded onto a nano-LC 1D plus system (Eksigent, Framingham, MA, USA) consisting of an in-house C18 resin (5 µm Proteo 100 Å; Phenomenex Inc., Torrance, CA, USA) and a capillary column (ID 75 µm, OD 150 µm; Molex, Lisle, IL, USA). Elution was conducted using a gradient liquid chromatography method (5–25% acetonitrile for 90 min) and analyzed with an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) in positive ion mode at the Mass Spectrometry Convergence Research Center. The m/z data collection range was set at m/z 300–1800, and higher-energy collisional dissociation collision mode was used for fragmentation. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD023933.

4.5. Bioinformatics

All mass spectra data were input to MaxQuant 1.5.1.0 to obtain bioinformatics information, and the zebrafish proteome database (updated 25 October 2018) was down-
All MS/MS spectra obtained were analyzed using MaxQuant software (https://www.maxquant.org/, accessed on 4 March 2021), allowing a maximum false-discovery rate of 1% for the protein and peptides. GO, InterPro, and KEGG pathways were analyzed using DAVID Functional Annotation Bioinformatics Microarray Analysis (https://david.ncifcrf.gov/, accessed on 4 March 2021). Perseus 1.6.0.7 was used for clustering protein groups depending on the protein regulation patterns. The STRING analytical tool (https://string-db.org/, accessed on 4 March 2021) was used to search specific protein networks according to regulation differences resulting from mutation of eif2b3.

4.6. Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization (WISH) was performed essentially as described previously [13]. WISH was performed using probes for mbp, olig2, nestin, lonp1, slc1a4, abat, ass1, cad, sept6, dhtkd1, psat1, xbp1, atf4, atf6, and crybb (Table S2). Briefly, staged embryos were fixed overnight in 4% PFA, then dehydrated in a methanol gradient. Embryos were then rehydrated in phosphate buffered saline containing 0.1% Tween-20 (PBST). Embryos were permeabilized by proteinase K digestion and then hybridized with digoxin-labeled probes overnight at 70 °C. The next day, embryos were washed in a preheated mixture of 50% saline sodium citrate containing 0.1% Tween-20 and 50% hybridization solution at 70 °C. Embryos were washed again at room temperature and incubated in staining solution in the dark until sufficient staining appeared. Embryos were mounted in glycerol and visualized using a Nikon AZ100 microscope (Nikon). Images were captured using a Nikon DIGITAL SIGHT DS-Fil1 digital camera (Nikon) and processed with NIS-Elements F 3.0 (Nikon).

Supplementary Materials: Supplementary Materials can be found at https://www.mdpi.com/1422-0067/22/5/2707/s1. Figure S1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis after yolk protein remove, Figure S2: Whole-mount in situ hybridization of WT and eif2b3−/− probed for dhtkd1, past1, and xbp1, Figure S3: Whole-mount in situ hybridization of WT and eif2b3−/− probed for atf4 and atf6. Figure S4: Whole-mount in situ hybridization of WT and eif2b3−/− probed for crybb1, Table S1: All detected protein lists. Table S2: Primer sequences used for in situ probe synthesis. Hybridization temperature for each probe was 70 °C.

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