Developmental Defects of Caenorhabditis elegans Lacking Branched-chain $\alpha$-Ketoacid Dehydrogenase Are Mainly Caused by Monomethyl Branched-chain Fatty Acid Deficiency*

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Branched-chain $\alpha$-ketoacid dehydrogenase (BCKDH) catalyzes the critical step in the branched-chain amino acid (BCAA) catabolic pathway and has been the focus of extensive studies. Mutations in the complex disrupt many fundamental metabolic pathways and cause multiple human diseases including maple syrup urine disease (MSUD), autism, and other related neurological disorders. BCKDH may also be required for the synthesis of monomethyl branched-chain fatty acids (mmBCFAs) from BCAAs. The pathology of MSUD has been attributed mainly to BCAA accumulation, but the role of mmBCFA has not been evaluated. Here we show that disrupting BCKDH in Caenorhabditis elegans causes mmBCFA deficiency, in addition to BCAA accumulation. Worms with deficiency in BCKDH function manifest larval arrest and embryonic lethal phenotypes, and mmBCFA supplementation suppressed both without correcting BCAA levels. The majority of developmental defects caused by BCKDH deficiency may thus be attributed to lacking mmBCFAs in worms. Tissue-specific analysis shows that restoration of BCKDH function in multiple tissues can rescue the defects, but is especially effective in neurons. Taken together, we conclude that mmBCFA deficiency is largely responsible for the developmental defects in the worm and conceivably might also be a critical contributor to the pathology of human MSUD.

Branched-chain amino acids (BCAAs), leucine, isoleucine, and valine, are essential amino acids that are not only building blocks for protein synthesis but also play important physiological roles (1). Their catabolism is controlled by branched-chain $\alpha$-ketoacid dehydrogenase (BCKDH), a mitochondrial multisubunit enzyme complex (see Fig. 1A). BCKDH is composed of three subunits, E1, E2 and E3, of which E1 and E2 are unique to this complex. The E1 subunit contains two components, E1$\alpha$ and E1$\beta$, which in humans are encoded by BCKDHA and BCKDHB, respectively (2, 3). The E2 subunit is encoded by DBT (4). Autosomal recessive mutation in any of these genes results in BCKDH deficiency and causes maple syrup urine disease (MSUD, Online Mendelian Inheritance in Man (OMIM) 248600). Classic MSUD patients have less than 2% BCKDH activity, which results in elevated BCAAs and branched-chain $\alpha$-ketoacids in tissues and plasma. If untreated, patients can develop life-threatening cerebral edema within 10 days of life. MSUD also has a chronic effect on the central nervous system, resulting in dysmyelination and mental retardation in young patients (5, 6).

The neurotoxicity of MSUD has been attributed mainly to increased plasma leucine and its derivative, $\alpha$-ketoisocaproic acid (5). They compete with other large neutral amino acids for transportation across the blood-brain barrier by the LAT1 amino acid transporter, causing decreased levels of large neutral amino acids that are precursors for key neuronal factors such as dopamine and serotonin, in the brain in humans (5), classic MSUD mice (7), and rats (8). Increased $\alpha$-ketoisocaproic acid depletes glutamate, affecting transamination in the brain and causing dehydrating stress on astrocytes (5, 9). In addition, elevated BCAAs and branched-chain $\alpha$-ketoacids induce mitochondrial dysfunction and oxidative stresses, which could contribute to the neurological damage of MSUD (10–12). In accordance with this, MSUD is managed with lifelong dietary intervention (5, 13). Alternatively, liver transplantation partially restores BCKDH activity, normalizes BCAA levels, and eliminates the need for long-term dietary restriction (14). However, even with good dietary control or successful liver transplantation, controlled MSUD patients still show chronic neuropsychological problems (5), manifest an impairment of visuospatial abilities (15, 16), and have lower IQ scores (17) and poor social outcomes (18). It is possible that early cerebral amino acid misregulation, especially during developmental bursts of brain growth, may have chronic effects in MSUD patients (5). Alternatively, other pathways that are affected by BCKDH deficiency may contribute to the neuropsychological impairment in MSUD patients in addition to BCAA intoxication.

Monomethyl branched-chain fatty acids (mmBCFAs) in an “iso” or “anteiso” form are branched-chain fatty acids with a single methyl group located at the $\omega$-1 or $\omega$-2 position, respectively. mmBCFAs are widely present in various organisms including mammals (19–23). mmBCFAs are abundant in food (24) and have been detected in several tissues in healthy...
human (21, 25–32). Previous studies in our lab have shown that in Caenorhabditis elegans, mmBCFAs play critical roles in regulating postembryonic growth, development, and foraging behavior (22, 33–36). However, the developmental and physiological functions of mmBCFAs in mammals is essentially unknown.

In mammals, mmBCFAs are synthesized from BCAAs (37, 38), which likely requires BCKDH activity because C21antelISO, the constitutive mmBCF in the hair, is missing from the hair of MSUD patients (see Fig. 1A) (21). Despite the observation that MSUD patients may have lost the ability to synthesize mmBCFAs, and that mmBCFAs are expected to play important physiological roles in mammals, the impact of mmBCF deficiency on MSUD-related developmental and physiological defects has not been reported. In this study, we use C. elegans as a model system to evaluate the essential role of BCKDH in mmBCFA biosynthesis and the contribution of mmBCFA deficiency to the developmental defects caused by a BCKDH mutation mimicking MSUD.

**Experimental Procedures**

**Strains and Maintenance—** C. elegans were maintained at 20 °C on NGM plates covered with bacterial food Escherichia coli OP50 (NGM/OP50). The wild-type N2 Bristol and dbt-1(ok3001) deletion strain VC2309 were obtained from the Caenorhabditis Genetics Center (CGC). The VC2309 strain was out-crossed three times before being used in this study and was maintained on C15ISO (high) plates (see “Results”).

**Plasmid Construction—** To make the transcriptional reporter construct dbt-1::Prom::gfp, a 5-kb region upstream of dbt-1 was fused with GFP and put into vector pBSIKS(−). To make the translational fusion construct dbt-1::gfp, the entire promoter and the genomic region before the stop codon were fused to GFP and cloned into vector pBSIKS(−). For tissue-specific analysis, the endogenous promoter in dbt-1::gfp was replaced with tissue-specific promoters. A 3-kb region upstream of myo-3 was used as a muscle-specific promoter; a 3-kb region upstream of rgef-1 was used as a neuronal promoter; a 1-kb region upstream of col-10 was used as a hypodermis-specific promoter; and a 3-kb region upstream of ges-1 was used as an intestinal promoter. Transgenic animals were made through microinjections.

**Dietary Supplementation—** mmBCFAs C13ISO, C15ISO, and d17SPA (custom synthesis; Larodan) (36) were prepared as 10 mM stocks in DMSO. Stock solutions were mixed with 400 μl of M9 solution and spread evenly onto each NGM/OP50 plate. The unit μmol/plate used in Fig. 3 and throughout the study indicates the total amount of mmBCFA added to each plate (e.g. 10 μmol/plate means 10 μmol of mmBCFA were added topically to each NGM/OP50 plate). Plates were then UV-treated for 45 min to eliminate metabolic interference from the live bacteria and used within 24 h. This supplementation method significantly reduces the degradation of C13ISO and C15ISO by bacteria food (33) and gives more consistent results.

**Hatching Rate Analysis—** 15–20 adult worms were transferred (39) to an NGM plate with a very thin OP50 lawn. After 3 h, adult worms were removed from the plate, and the number of eggs laid (>100) was counted. After 24 h, the number of hatched larvae was counted. The hatching rate is calculated as: number of larvae/number of eggs. Experiments were done in triplicate, and each experiment was repeated at least twice.

**RNAi Analysis by Feeding—** All RNAi HT115 E. coli strains were from the ORF-RNAi library (Open Biosystems). Feeding RNAi experiments were done as described previously (34).

**GC Analysis of Fatty Acid Profile—** GC analysis was performed as described previously (34). Briefly, a pellet of synchronized worms (L1s were used in Fig. 1C, and L4s were used in Fig. 4A) or bleached eggs (see Fig. 4C) was mixed with 100 μl of methanol and 900 μl of hexane and vortexed vigorously for 2 min. The mixture was then centrifuged at 13,000 rpm for 2 min, and the top hexane layer was collected for GC analysis. GC was performed on an HP6890N (Agilent Technologies) apparatus equipped with a DB-23 column (30 m × 250 μm × 0.25 μm) (Agilent Technologies) and flame ionization detector. The area value of each peak was calculated by the GC ChemStation software (Agilent), normalized to the combined signal of all peaks, and presented as “% of total fatty acids” (see Figs. 1C and 4, A and C). Each experiment was repeated at least three times.

**Amino Acid Quantitation—** Amino acid quantitation was performed by HPLC and UV detection (SHIMADZU LC-20A and SPD-20A) using pre-column derivatization with o-phthalaldehyde (P378 Sigma) (40). Specifically, in a reaction vial with a conical insert, 30 μl of supernatant sample were mixed with 45 μl of borate buffer (200 mM sodium borate, pH 10.2) and 75 μl of o-phthalaldehyde derivatization reagent (7.45 mM sodium borate, pH 10.2) and 75 μl of o-phthalaldehyde derivatization reagent (7.45 mM o-phthalaldehyde, 11.4 mM 3-mercaptopropionic acid, made fresh). The solutions were mixed by pipetting and were kept at room temperature for 20 min. 10 μl of the mixture were injected onto the Gemini C18 column (ID Number 15992, Phenomenex). Mobile phase A was aqueous buffer (40 mM Na2HPO4/NaH2PO4, pH 7.8), and mobile phase B was methanol/acetonitrile/water (45:45:10, v/v/v). The elution was facilitated by a gradient program: 0–40.5 min 40% B, 40.5–41 min 61% B, 41–43 min 61% B, 43–44 min 82% B, 44–45.5 min 100% B, 45.5–46.5 min 100% B, 46.5–49 min 0% B. The temperature of the column oven was kept at 40 °C. The constant flow rate was 1 ml/min. Fluorescence detection and quantification were carried out at an excitation wavelength of 340 nm. The identification of sample peaks was done by comparison of retention time with reference substances.
The arrest phenotype was effectively suppressed by supplemental larval supplementation (0.4 mmol/plate for each supplementation, n = 3). Similar to the larval arrest phenotype, we found that dbt-1(lf) animals. As a comparison, WT worms laid eggs that almost all hatched (98.3 ± 1.1%), and the hatching rate was not affected by C13ISO (97.2 ± 1.0%), C15ISO (98.5 ± 0.3%), or C17ISO (97.0 ± 0.7%) supplementation (0.4 μmol/plate for each supplementation, n = 3). Similar to the larval arrest phenotype, we found that C15ISO was the most effective mmBCFA supplement. On the contrary, d17SPA was not able to rescue the embryonic lethal phenotype at any concentration tested (Fig. 3B), suggesting that different higher order, mmBCFA-containing lipids may mediate the role of mmBCFAs during embryogenesis.

Functional Study of BCKDH Complex in Worms

Results

Loss of Function of the BCKDH E2 Subunit Causes Accumulation of BCAAs and Reduction of mmBCFAs—To characterize the function of BCKDH in C. elegans, we focused on the gene ZK669.4, which encodes a homologue of human dihydrolipoyltransacetylase/DBT (E2 subunit). The ok3001 mutation deletes the entire eighth and part of the ninth exon of ZK669.4, which is expected to disrupt the catalytic domain of the encoded protein. ok3001 animals showed elevated levels of BCAAs (Fig. 1), resembling MSUD patients, and also had reduced C15ISO and C17ISO levels (Fig. 1C), which are the major mmBCFAs in C. elegans (22, 39, 41). Reduced mmBCFA levels were also found in F27D4.5/BCKDB (E1β) (Fig. 2A) and Y39E4A.3/BCKDA (E1α) (22) RNAi knockdown animals. In addition, introducing a WT copy of ZK669.4 rescued all the phenotypes caused by the ok3001 mutation (see below). These data indicate that ZK669.4 is an orthologue of DBT and that ok3001 is a loss-of-function (lf) allele. We thus name ZK669.4 dbt-1 and refer to dbt-1(ok3001) as dbt-1(lf). In addition, RNAi knockdown of dbt-1 or F27D4.5 produced a similar larval arrest phenotype in the second generation (Fig. 2, B–D), supporting the notion that they belong to the same enzyme complex.

mmBCFA Supplementation Suppresses Larval Arrest and Embryonic Lethality Caused by dbt-1(lf)—When synchronized dbt-1(lf) L1 larvae were cultured on plates with standard food (E. coli OP50) for 3 days, 100% of them remained as L1 or L2 larvae, whereas WT worms all grew to adulthood. This larval arrest phenotype was effectively suppressed by supplementation of mmBCFAs C13ISO, C15ISO, or C17ISO, with C15ISO being the most effective among them (Fig. 3A), suggesting that lack of mmBCFAs is likely a key cause of the BCKDH deficiency larval arrest phenotype. In addition, d17SPA, a sphingolipid containing C15ISO, was slightly more effective than C15ISO but worms on F27D4.5 (n = 200(C) or dbt-1 (n = 200(D)) RNAi plates remained as larvae. Experiments were done using rrf-3 worms, which are hypersensitive to RNAi. Synchronized L1s were put on RNAi plates, and the subsequent young adults of the same generation were analyzed by GC. RNAi of Y39E4A.3, which is a homologue of the E1α subunit of human BCKDH, has been shown previously to also cause a reduction in mmBCFA levels (22) and was therefore not tested here.

mmBCFA supplementation (0.4 mmol/plate for each supplementation, n = 3).
Functional Study of BCKDH Complex in Worms

In addition to C15ISO, supplementation with a short-chain mmBCFA, C5ISO, could also partially rescue the larval arrest and embryonic lethal phenotypes (Table 1). C5ISO is a product of leucine degradation, and is the precursor of C15ISO, C15ISO, and C17ISO (Fig. 1A). Consistent with our model, supplementation with C4ISO or C5anteISO, which are the degradation products of valine and isoleucine, respectively, and are the precursors of other mmBCFAs, was not able to rescue dbt-1(lf) (Fig. 1A) (Table 1).

Because C15ISO was able to rescue both phenotypes of dbt-1(lf), we further analyzed the dosage effect of C15ISO supplementation. Specifically, at a concentration of 0.05 μmol/plate, more than 90% of dbt-1(lf) L1 larvae grew to adult but laid 100% dead eggs. We define this treatment as dbt-1[C15ISO (low)]. At a concentration of 0.4 μmol/plate, almost all supplemented dbt-1(lf) L1 larvae grew to adults, and about 56% of the resulting eggs would hatch. We define this treatment as dbt-1[C15ISO (high)] (Fig. 3).

C15ISO Supplementation Restores mmBCFA Levels without Alleviating BCAA Accumulations—Because the levels of both BCAAs and mmBCFAs are affected by dbt-1(lf), albeit in opposing ways, we asked whether C15ISO supplementation could affect the level of either or both. dbt-1[C15ISO (low)] worms had partially restored levels of both C15ISO and C17ISO, and the levels were further elevated in dbt-1[C15ISO (high)] worms (Fig. 4, A and C). On the contrary, neither supplementation suppressed the abnormal accumulation of BCAAs in dbt-1(lf) worms. dbt-1[C15ISO (high)] had even further increased BCAAs in both L4 larvae and eggs (Fig. 4, B and D), which were partially viable (Fig. 3B). These results suggest that C. elegans development can tolerate elevated BCAA levels and, in contrast to what has been shown in studies on MSUD patients, mmBCFA deficiency is the major cause of the developmental phenotypes when BCKDH function is disrupted.

It is interesting to note that although dbt-1[C15ISO (low)] eggs had a comparable C15ISO level relative to WT (Fig. 4C), it failed to rescue the embryonic lethality (Fig. 3B). There are two possible explanations: (a) mmBCFA is required in specific tissues to suppress the phenotypes (such as in neurons as suggested by our tissue-specific rescue data (see below)), and although the total level of C15ISO in supplemented worms is normal, the actual level in key tissues may remain low; and/or (b) mmBCFA is required in specific higher order lipids to suppress the phenotypes. Fatty acids in living organisms, including worms, are incorporated into higher order lipids (e.g. triacylglycerols, phospholipids, sphingolipids). Supplemented worms with a normal level of C15ISO fatty acid may therefore have a very different distribution of C15ISO among these higher order lipids (e.g. an enrichment in triacylglycerols but reduction in phospholipids). Therefore, a normal level of C15ISO does not mean a WT distribution of higher order lipids, or the presence of these fatty acids/lipids in the necessary tissue, and these differences may contribute to the insufficiency of our C15ISO supplementation.

Neuronal Expression of dbt-1 Significantly Represses the Developmental Defects—Transcriptional reporter lines (Ex[dbt-1Prom:gfp]) showed that dbt-1 was expressed ubiquitously in the worm (Fig. 5A). We also constructed a fusion protein containing the promoter, 5’-UTR, and the coding region of dbt-1 fused with GFP to the 3’ end (dbt-1::gfp). Injection of this construct into dbt-1(lf) partially rescued both the larval and the embryonic phenotypes, confirming that it is functional (Fig. 5, L and M). Consistent with the transcriptional result, in dbt-1(lf);Ex[dbt-1::gfp] worms, the GFP signal was detected in major tissues of the worm (Fig. 5B), including muscle, hypodermis, intestine, and head neurons (Fig. 5, B–I). In these tissues, GFP signals were located in puncta and sheets in the cytosol (Fig. 5, D, F, and H), which is consistent with the mitochondrial localization of BCKDH (42–44). GFP signals were also detected in the neuronal projections, forming patches in the dendrites and with strong expression in the cilia (Fig. 5, G–K).

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**TABLE 1**

| Supplementation* | % of L1s that grew to adulthood |
|------------------|-------------------------------|
| 100 mM C4ISO     | 0                             |
| 100 mM C5ISO     | 0                             |
| 10 mM C5ISO      | 45.3 ± 6.2%b                  |
| 100 mM C5ISO     | 54.9 ± 1.1%c                  |

* C4ISO, C5ISO, and C5anteISO were prepared as 1 x stock in DMSO. Stock solution was mixed with OP50 to indicated concentrations and was spread on NGM plates. Plates were dried and used within 1 week.

b n = 3, error represents standard deviation.

c Represents % of eggs hatched.

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**FIGURE 3.** dbt-1(lf) causes larval arrest and embryonic lethal phenotypes that are both suppressed by mmBCFA supplementation. A, dose-response curves of C15ISO, C15ISO, C17ISO, and d17SPA in rescuing the larval arrest phenotype of dbt-1(lf). More than 100 worms were tested for each measurement. n = 3; error bars indicate standard deviation. B, hatching rate of eggs laid by dbt-1(lf) worms treated with different concentrations (Conc.) of C15ISO, C15ISO, C15ISO, or d17SPA. dbt-1[C15ISO (low)] and dbt-1[C15ISO (high)] (see "Results") are highlighted in bold. The open arrow points to dbt-1[C15ISO (low)], and the filled arrow points to dbt-1[C15ISO (high)], n = 3; error bars indicate standard deviation.

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We then made transgenes that expressed the fusion protein behind tissue-specific promoters in muscles (myo-3Prom), neurons (rgef-1Prom), hypodermis (col-10Prom), or intestine (ges-1Prom) in dbt-1(lf) worms (45–48). We found that both the larval and embryonic phenotypes of dbt-1(lf) were effectively rescued by expressing the gene in multiple tissues (Fig. 5, L and M), suggesting that dbt-1 function (BCAA catabolism and mmBCFA biosynthesis), especially in embryogenesis, can be cell non-autonomous. Interestingly, both phenotypes were effectively rescued by the neuronal expression of dbt-1::gfp, and dbt-1 expression is also detected in neurons (Fig. 5, G–K), suggesting critical roles of BCKDH and mmBCFAs in the nervous system. DBT-1-GFP in muscle and hypodermis rescued the embryonic phenotype well but the larval arrest phenotype poorly, whereas intestinal expression of the fusion protein showed the opposite effect (Fig. 5, L and M). These differences may again suggest that the two phenotypes have different underlying mechanisms.

Discussion

In conclusion, our results show that mutating BCKDH, which leads to MSUD and other neurological diseases in humans, disrupts both BCAA catabolism and mmBCFA biosynthesis and causes detrimental developmental defects in C. elegans. Strikingly, the majority of such defects may be attributed to lacking mmBCFAs but not BCAA accumulation. This is in contrast to human studies that have shown that BCAA accumulation is the main cause, but possibly not the sole cause, of the pathology of MSUD. It is worth mentioning that, although over-accumulation of BCAA may not critically con-tribute to the developmental defects in C. elegans, it could lead to altered neuronal behavior, which was not tested in the current study. Interestingly, DBT-1 is expressed in neurons, and expressing DBT-1 only in neurons suppresses the developmental defects of dbt-1(lf) more effectively than expressing the protein in other major tissues, suggesting important functions of
BCKDH in the neuronal system. In addition, recent work from our lab showed that mmBCFA deficiency also causes altered behaviors in worms (35). Given the substantial presence of mmBCFA in humans and the unsatisfactory results of current MSUD management, we propose that downstream products of BCKDH and their derivatives including mmBCFAs, which have been largely neglected in the study and management of MSUD, may potentially contribute to the pathology, especially the neurotoxicity, of MSUD. Future research in animal models and humans is thus needed to elucidate the contribution of mmBCFAs to human health and to help improve the treatment outcome of MSUD.

Author Contributions—F. J., M. C., and M. T. T. designed and performed the experiments. F. J. analyzed the results and wrote the paper. M. H. supervised the study and edited the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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