(Journal Title) JIMD

(Journal type) Original Article categorized in Full Articles

Full Title (sentence case, 123/ characters)

Deficiency of 3-hydroxybutyrate dehydrogenase (BDH1) in mice causes low ketone body levels and fatty liver during fasting.

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39 Word counts for the summary: 195/250 words

40 Number of figures and tables: 3 /4 figures and tables

41

42 **Summary:**

43 D-3-hydroxy-n-butyrate dehydrogenase (BDH1; EC 1.1.1.30), encoded by *BDH1*, catalyzes
44 the reversible reduction of acetoacetate (AcAc) to 3-hydroxybutyrate (3HB). BDH1 is the
45 last enzyme of hepatic ketogenesis and the first enzyme of ketolysis. The hereditary
46 deficiency of BDH1 has not yet been described in humans. To define the features of BDH1
47 deficiency in a mammalian model, we generated *Bdh1*-deficient mice (*Bdh1* KO mice).
48 Under normal housing conditions, with unrestricted access to food, *Bdh1* KO mice showed
49 normal growth, appearance, behavior and fertility. In contrast, fasting produced marked
50 differences from controls. Although *Bdh1* KO survive fasting for at least 48 hours, blood
51 3HB levels remained very low in *Bdh1* KO mice, and despite AcAc levels moderately

higher than in controls, total ketone body (TKB) levels in *Bdh1* KO mice were significantly lower than in wild-type (WT) mice after 16, 24 and 48 hours fasting. Hepatic fat content at 24 hours of fasting was greater in *Bdh1* KO than in WT mice. Systemic BDH1 deficiency was well tolerated under normal fed conditions but manifested during fasting with a marked increase in AcAc/3HB ratio and hepatic steatosis, indicating the importance of ketogenesis for lipid energy balance in the liver.

Synopsis: Pathophysiology of BDH1 deficiency in mice

Key words (up to 6): 3-hydroxybutyrate dehydrogenase, CRISPR, fatty liver, ketone body, knockout mouse

Abbreviations: 3HB, 3-hydroxybutyrate; AcAc, acetoacetate; AcCoA, acetyl-CoA; FFA, free fatty acid; HMGCL, HMG-CoA lyase; HMGCS2, mitochondrial HMG-CoA synthase; SCOT, succinyl-CoA:3-oxoacid CoA transferase; T-Chol, total cholesterol; TG, triglyceride; TKB, Total ketone body; WT, wild-type

Compliance with Ethics Guidelines

Conflict of Interest:

Toshiyuki Fukao has received Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan [grant numbers 26114708, 16K09962]; Health and Labour Science Research Grant for Research on Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan and a grant for the Practical Research Project for Rare/Intractable Diseases from the Japan Agency for Medical Research and Development (AMED).

Hiroki Otsuka, Takeshi Kimura, Yasuhiro Ago, Hideo Sasai, Mina Nakama, Yuka Aoyama, Elsayed Abdelkreem, Hideki Matsumoto, Hidenori Ohnishi, Masatake Osawa, Seiji Yamaguchi and Grant A Mitchell declare that they have no conflict of interest.

Informed Consent:

This study does not include any experiments with human subjects.

Animal rights:

85 This study was approved by the ethical committee for animal experiment and recombinant
86 DNA experiments of Gifu University. All institutional and national guidelines for the care
87 and use of laboratory animals were followed.
88
89 **Details of the contributions of individual authors:**
90 Toshiyuki Fukao initiated and supervised all parts of the study, reviewed and revised the
91 manuscript. Hideo Sasai is the guarantor of the manuscript. Masatake Osawa engineered a
92 *Bdh1* KO mouse by CRISPR/Cas9 system. Hiroki Otsuka, Takeshi Kimura, Yasuhiko Ago,
93 Hideo Sasai, Mina Nakama, Yuka Aoyama, Elsayed Abdelkreem, Hideki Matsumoto and
94 Hidenori Ohnishi performed laboratory analyses. Seiji Yamaguchi performed urinary
95 organic acid analysis and reviewed the manuscript. Hiroki Otsuka drafted the first version
96 of the manuscript. Grant Mitchell reviewed experimental results and discussed
97 experimental plans during the project, and reviewed and participated in writing the
98 manuscript. All authors have approved the final version as submitted and agree to be
99 accountable for all aspects of the work. All authors confirm the absence to their knowledge
100 of previous similar or simultaneous publications.
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Introduction (391/500words for full articles)

The ketone bodies acetoacetate (AcAc) and 3-hydroxybutyrate (3HB) are important sources of energy, especially if glucose is in short supply (Mitchell et al 1995; Mitchell 2001; Cahill 2006; Sass 2012; Hori et al 2015). Ketone body energy metabolism consists of ketogenesis in the liver and ketolysis in extrahepatic tissues. In ketogenesis, mitochondrial HMG-CoA synthase (HMGCS2) and HMG-CoA lyase (HMGCL) produce AcAc. In ketolysis, succinyl-CoA:3-oxoacid CoA transferase (SCOT) and mitochondrial acetoacetyl-CoA thiolase (T2) use AcAc to produce acetyl-CoA (Fukao et al 2014; Fukao et al 2018). Deficiencies of each of these enzymes are reported in humans.

The enzyme D-3-hydroxy-n-butyrate dehydrogenase (BDH1; EC 1.1.1.30; gene symbol *BDH1*) catalyzes the reversible, NADH-dependent reduction of AcAc to D-3-hydroxy-n-butyrate (3HB) during ketogenesis in the liver and the opposite reaction during ketolysis in extrahepatic tissues (Mitchell et al 1995; Mitchell 2001). Of note, BDH1 is enantioselective for D-3-hydroxybutyrate, and D-3-hydroxybutyrate is the only product of ketogenesis (reviewed in Puchalska and Crawford 2017). Although L-3-hydroxybutyrate occurs in

118 tissues and comprises a small fraction of total circulating 3-hydroxybutyrate (Hsu et al
119 2011), it is not a substrate of BDH1 and is not considered further in this article.

120 BDH1 can therefore be considered both as the last enzyme of ketogenesis and the
121 first enzyme of ketolysis. Because AcAc can also be released by the liver into the
122 circulation and taken up by ketolytic tissues, the BDH1 reaction is not obligatory for
123 ketogenesis and ketolysis. Physiologically, in periods of ketosis, 3HB is generally more
124 abundant than AcAc in the circulation, lending support to the notion that BDH1 may be
125 important for normal ketone body flux and energy metabolism.

126 Human heart BDH1 consists of 297 amino acids (Marks et al 1992). It has 99.4 %
127 identity to its chimpanzee (*Pan troglodytes*) orthologue and 86 % identity with that of the
128 house mouse (*Mus musculus*) (Homologene. Available from:
129 https://www.ncbi.nlm.nih.gov/homologene). BDH1 is an integral mitochondrial inner
130 membrane protein that requires phosphatidylcholine for catalysis (Marks et al 1992). 3HBD
131 activity is greatest in liver, the main ketogenic tissue, about tenfold lower in kidney, heart
132 and adrenal glands, and twentyfold lower in brain (Lehninger et al 1960; Williamson et al
133 1971).

BDH1 deficiency has not been reported in humans. We generated mice with systemic BDH1 deficiency, using the CRISPR/Cas9 system, and studied the phenotype of *Bdh1* knockout (KO) mice under conditions of suppressed and active ketogenesis.

Materials and methods

Animals

All animal experiments were performed in accordance with a protocol approved by the Animal Care and Research Committee of Gifu University (Protocol number: 27−71). C57BL/6J, B6D2F1, and ICR mice were purchased from Japan SLC, Inc (Shizuoka, Japan). Mice were bred and crossed in the animal facility of the Life Sciences Research Center of Gifu University. To exclude any influences as a result of aggression, which frequently occurs between C57BL/6J males placed in the same cage, all animal experiments reported here were performed using female mice.

Generation of *Bdh1* KO mice

To generate *Bdh1* mutant mice, we employed CRISPR/Cas9-mediated targeted mutagenesis in mouse embryos, as previously described with slight modifications (Nakagawa et al 2016;

151 Shinmyo et al 2016). Briefly, one-cell stage embryos were collected from the oviduct of
152 superovulated C57BL/6N females that had been treated with consecutive injections of
153 pregnant mare serum gonadotropin (PMSG: ASKA Animal Health Co., Tokyo, Japan) and
154 human chorionic gonadotropin (hCG: ASKA Animal Health Co., Tokyo, Japan) and then
155 mated overnight with B6D2F1 males. For zygotic microinjection, 0.3 μM Cas9 protein
156 (PNA Bio Inc., CA, USA), 0.75 μM cRNAs (Integrated DNA Technologies, Inc, IA, USA),
157 and 0.75 μM tracrRNA (Integrated DNA Technologies, Inc, IA, USA) were co-injected
158 into the cytoplasm of pronuclear-stage embryos using a Piezo microinjector (Prime Tech
159 Ltd., Tsuchiura, Japan). The injected embryos were cultured overnight in KSOM medium
160 (Merk KGaA, Darmstadt, Germany) and embryos that developed to the two-cell stage were
161 transferred to the oviduct of pseudopregnant ICR females. The sequences of the injected
162 crRNAs were as follows: Bdh1-crRNA1 5'-CACCGAGACGGGCAGCTAGCATCG-
163 3'and Bdh1-crRNA2 5'-TTTCTCTGTCACGGACACTT-3'.
164 Pups were genotyped and founder mice harboring frameshift mutations as a result of indel
165 formation were identified by Sanger sequencing. The founder mice were back-crossed to
166 wild-type C57BL/6J mice for at least 3 additional generations before performing detailed
167 phenotype assessments.

The ablation of Bdh1 protein expression was confirmed by performing immunoblotting of liver protein extracts. For this, liver samples from the mice were homogenized in RIPA buffer（20 mM Tris HCl, pH 7.4/ 150 mM NaCl/ 1 mM EDTA/ 1% Nonidet P-40/ 0.1% sodium deoxycholate/ 0.1% SDS）containing protease inhibitor cocktail® (Epigentek, Farmingdale, NY, USA) with five strokes of a Digital Homogenizer (Iuchiseieido, Osaka). Then it was sonicated three times on ice for one second each, at 3W and 28 kHz, with a Handy Sonic® (Tomy Seiko, Tokyo). After centrifugation of the liver homogenates at 15,000 g for 10 minutes at 4°C, the supernatants were collected as liver protein extracts. Protein concentration was determined by Lowry method. The protein extracts were subjected to SDS-PAGE and immunoblotting. For immunoblotting, a rabbit polyclonal antibody to BDH1 (Proteintech Group, Rosemont, USA) was used as a first antibody and an alkaline phosphatase-conjugated polyclonal antibody against rabbit IgG was used as a secondary antibody.

Fasting test

Fasting tests were performed with KO or wild-type (WT) mice at 8 weeks of age. Groups of mice were sacrificed at each of four fasting times (0, 16, 24, and 48 hours). For the test,

mice were moved to new cages, with free access to water but no food. At each fasting time, blood glucose level was measured (NIPRO STAT STRIP XP3®, Nova Biomedical, Waltham MA, USA). All samples were obtained by tail blood sampling, except the last, which was collected by cardiac puncture under anesthesia with tribromoethanol. If the mouse urinated during tail sampling, a sample was obtained as described below.

Blood samples were centrifuged at 1,200 g for 30 minutes at 4 °C after 30 minutes incubation at 25 °C and they were collected as serum. Total ketone bodies (TKB), AcAc, 3HB and free fatty acids (FFA) were measured in serum samples, by the Nagahama Life Science Laboratory, Oriental Yeast Co, Nagahama, Japan. TKB and 3HB were measured with "TKB Shiyaku Kainos" and "3HB Shiyaku Kainos" kits, respectively (KAINOS Laboratories, Inc., Tokyo, Japan), using enzymatic cycling methods, performed as recommended by the manufacturer. AcAc level was calculated as TKB minus 3HB. FFA level was measured with HR series NEFA-HR(2)® kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), as described (Dole and Meinertz 1960; Duncombe 1964).

Urine samples were prepared and analysed for organic acids as follows. A 20 x 30 mm filter paper (ADVANTEC 327, Advantec Toyo Roshi Kaisha, ltd., Tokyo, Japan) was used to capture the urine, then dried at room temperature. The filter paper was immersed in filter

1.2 ml of distilled water then centrifuged at 1,120 g for 5 minutes. About 0.8 ml of eluate was obtained (Fu et al 2001). The creatinine concentration of the eluate was determined by the Jaffe's method (Lustgarten and Wenk 1972). A volume of eluate containing 0.1 mg creatinine was used for organic acids analysis by GC/MS, performed using a Shimadzu GCMS QP2010 Plus.

For histopathology, liver sections were stained with Sudan III. Measurement of total cholesterol (T-Chol) and triglyceride (TG) are performed in liver extracts using the Folch method (Skylight Biotech, Akita, Japan).

Statistical analysis

Body weights and biochemical data were analyzed using GraphPad Prism® version 7.00e for Mac, GraphPad Software (La Jolla California USA, www.graphpad.com).

Results

Engineering *Bdh1* KO mouse

To test for gene targeting and its effects, we sequenced around the target sites in *Bdh1* exon 2 and performed immunoblotting for liver Bdh1. The *Bdh1* sequence in the gene-targeted

mice revealed the expected single nucleotide deletion of the adenine of the initiation methionine codon and the c.58_63del(GTCCGT) deletion downstream. (Supplemental Fig.) In immunoblot analysis, Bdh1 protein was not detected even when 80 μg protein of KO liver were applied, although Bdh1 protein was clearly detected in 5 μg protein of WT liver (Supplemental Fig.1).

Growth & development

There was no detectable difference of growth, appearance or behavior between *Bdh1* KO mice and WT mice. *Bdh1* KO mice had normal fertility and there was no sex difference in offspring (the male: female ratio was 48: 54). The offspring of heterozygote crosses were born in the fractions consistent with Mendelian segregation and normal viability (25 WT: 41 heterozygote: 25 KO, i.e. 0.275: 0.450: 0.275). Body weights at 3 weeks were 7.84 ± 0.7 g for KO and 7.71 ± 0.5 g for WT mice, indicating that KO mice grow normally during the suckling period. The body weights of KO and WT mice were similar until the age of 8 weeks, showing no significant difference (Fig. 1A).

Ketone body metabolism in *Bdh1* KO mice

Under fed conditions, *Bdh1* KO and WT mice had similar levels of blood glucose and FFA (Fig. 1B, 1C). The mean value of 3HB was lower in *Bdh1* KO than in WT mice although this was not statistically significant under fed conditions (Fig. 1D).

To induce ketogenesis, mice were fasted. All WT mice and *Bdh1* KO mice tested survived fasting, for periods up to 48 hours, despite the loss of about 20% of body weight. Blood glucose levels reduced with fasting in both genotypes but tended to be higher in KO mice (Fig. 1B). This reached statistical significance after 24 hours ($p = 0.002$ and 0.001 at 24 and 48 hours respectively). The mean levels of FFA were greater in *Bdh1* KO than in WT mice at zero and 24 hours of fasting, but this did not reach significance.

3HB levels increased with fasting in WT mice, reaching 4,418 ± 360 μmol/L at 48 hours fasting, compared to 122 ± 15 in *Bdh1* KO mice at this time ($p < 0.001$). In contrast, at 48 hours fasting, mean levels of AcAc were higher in *Bdh1* KO mice (1261 ± 233 μmol/L) than in WT controls (842 ± 125 μmol/L). However, at all fasting times, total KB levels were lower in *Bdh1* KO than in WT mice because, despite the higher levels of AcAc in *Bdh1* KO mice with respect to controls, the difference in 3HB levels between *Bdh1* KO and control mice was much greater, resulting in a marked hypoketonemia in *Bdh1* KO mice that intensified during fasting (Fig. 1E, 1F) (***$p < 0.001$ at 16, 24 and 48 hours fasting).

Except for the low excretion of 3HB in *Bdh1* KO mice, there were no marked differences from WT mice in urinary organic acid patterns (Supplemental Fig. 2). Histological examination of liver sections obtained at 24 hours of fasting and stained with Sudan III showed substantially more fat accumulation in *Bdh1* KO than in WT mice. In contrast, in fed mice, little fat was detectable in livers of either *Bdh1* KO or WT (Fig. 2A). Additionally, assays of triglycerides in liver extracts showed significant accumulation of triglycerides in 24-hour-fasted *Bdh1* KO mice with respect to WT controls (**$p = 0.04$). Total cholesterol content did not differ significantly between WT and KO (Fig. 2B).

Discussion

The genetic deficiency of BDH1 has not yet been described in humans. The sequence of *BDH1* has been conserved during vertebrate evolution, suggesting that it plays a valuable physiological role. Mouse models of other inborn errors of ketone body metabolism successfully reproduce many features of affected humans (Cox et al 2001; Ibdah et al 2001; Ibdah et al 2005; Houten and Wanders 2010; Cotter et al 2011; Cotter et al 2014; Wang et al 2016; Knottnerus et al 2018; Sass et al 2018). We created gene-targeted

Bdh1 KO mice, which provide the first description of complete systemic BDH1 deficiency in a mammal.

Bdh1 deficiency was well tolerated in the fed state, with normal growth, behavior and fertility. The only known roles of BDH1 are in ketogenesis and ketolysis, and is likely that 3HBD is inactive in well fed animals, because both ketogenesis and ketolysis are suppressed by normal feeding.

In contrast, during fasting, striking biochemical differences appeared between *Bdh1* KO and WT mice, and intensified with increasing fasting time. *Bdh1* KO mice survived fasting for up to 48 hours. However, in *Bdh1* KO mice, fasting produced a biochemical pattern consisting of markedly low levels of 3HB, a modest increase of AcAc and fatty liver, with a smaller reduction of blood glucose than in controls when fasted for 24 hours or more. *Bdh1* KO mice thus provide a direct physiological demonstration that Bdh1 is the major enzyme of 3HB production.

The increase of plasma AcAc level with fasting was greater in KO than in WT mice, but this was nearly 10-fold less than the differences in the opposite direction of plasma 3HB levels, which were much higher in fasting control mice than in *Bdh1* KO mice. Plasma levels of AcAc and 3HB cannot be assumed to precisely reflect flux, and the two

286 compounds are not identical in chemical stability, physiological distribution or elimination.
287 Nonetheless, taken together, the fasting ketone body levels predict that ketone body release
288 from liver is much less in *Bdh1* KO than in wild type mice. AcAc, the substrate of Bdh1, is
289 a free organic acid, not esterified to coenzyme A, and can cross the mitochondrial and cell
290 membranes. A marked increase of AcAc was expected with fasting in *Bdh1* KO mice,
291 which would compensate for the lack of 3HB. However, this does not occur. The reasons
292 why AcAc increases only mildly are unknown and define a subject for future research.
293 *Bdh1* KO mice will also provide a tool for the study of the extrahepatic and
294 regulatory roles of 3HB. Mice with heart-specific BDH1 deficiency (Horton et al 2019) can
295 synthesize adequate amounts of 3HB but cannot use 3HB for energy production in
296 cardiomyocytes. The results support the notion that 3HB is an important substrate for the
297 failing heart (Horton et al 2019). 3HB is also attracting interest as a signalling molecule and
298 epigenetic modifier (Shimazu et al 2013; Youm et al 2015), roles that are distinct from that
299 as an energy substrate (Puchalska and Crawford 2017).
300 The pathway of hepatic ketogenesis (Fig. 3) suggests two major consequences of
301 BDH1 deficiency and the resulting low level of ketone body production by the liver: an
302 energy rich, reduced state in the mitochondrial matrix, with a high ratio of $NADH/NAD^+$,

303 and accumulation of acetyl-CoA, possibly with a corresponding reduction of other acyl-
304 CoA pools as in diseases of acyl-CoA metabolism (Yang et al 2019). Figure 3 shows how
305 this combination might divert carbon flux from acetyl-CoA towards the synthesis of
306 triglycerides for storage or export, and carbon from pyruvate, towards gluconeogenesis.
307 Blood glucose and liver TG indeed tend to be higher in fasting *Bdh1* KO mice than in WT
308 controls.

309 BDH1 deficiency has not yet been described in humans. How might such
310 individuals present? From basic considerations, they would be predicted to be unable to
311 produce or to utilize 3HB. The pathways of ketone body metabolism are conserved in mice
312 and humans but there are major differences between the two species because of their
313 evolutionary distance and different diets and body sizes (Kummitha et al 2014). *Bdh1*-
314 deficient mice offer the best available model of systemic BDH1 deficiency, but their
315 phenotype may therefore differ from that of human BDH1 deficiency. Because of the lesser
316 rate of energy metabolism characteristic of larger mammals (Kummitha et al 2014), people
317 with BDH1 deficiency may have milder signs than Bdh1 KO mice. They might even be
318 asymptomatic or have entirely different clinical signs. While keeping these possibilities in
319 mind, basic consideration of the central roles of ketogenesis and ketolysis in energy

metabolism and the phenotype of Bdh1 KO mice are both consistent with the notion that humans with BDH1 deficiency may be asymptomatic in the fed state, but that during fasting, hypoketosis, a high AcAc/3HB ratio and hepatic steatosis may emerge.

Ketone bodies are measured in many people and for diverse reasons, and these measurements may provide a clue. For instance, urine ketones are included in routine urinalyses. Measurements of ketone bodies in urine and blood are performed frequently in hospitalized patients with vomiting, diabetes, infections and other potentially ketogenic conditions. Increasingly, ketone body measurements are performed during ketogenic diets and diets involving intermittent fasting that are currently popular for weight loss, epilepsy and other possible benefits to health (de Cabo and Mattson 2019). The "ketones" of routine urinalysis are a measure of AcAc (Mitchell 2001), while "blood ketones", as measured in diabetes home monitoring, indicate the level of D-3-hydroxybutyrate. The observations in *Bdh1* KO mice provide support to basic metabolic reasoning; both suggest that BDH1 deficiency may result in a high ratio of AcAc to 3HB during ketogenic stress. Ideally, both AcAc and 3HB would be measured simultaneously in blood, but elevation of urinary organic acids is usually a reflection of high circulating concentrations during the interval since the bladder was last emptied. In clinical parlance, such individuals would therefore be

"urine ketones positive" despite having "negative blood ketones" in simultaneously obtained plasma and urine samples.

Acknowledgments

The authors thank Ms. Yoko Tezuka and Ms. Naomi Sakaguchi (Department of Pediatrics, Gifu University, Gifu, Japan) for expert technical assistance.

References (27/30 for full articles)

Cahill GF, Jr. (2006) Fuel metabolism in starvation. *Annu Rev Nutr* 26: 1-22.

Cotter DG, d'Avignon DA, Wentz AE, Weber ML, Crawford PA (2011) Obligate role for ketone body oxidation in neonatal metabolic homeostasis. *J Biol Chem* 286: 6902-6910.

Cotter DG, Ercal B, Huang X, et al (2014) Ketogenesis prevents diet-induced fatty liver injury and hyperglycemia. *J Clin Invest* 124: 5175-5190.

Cox KB, Hamm DA, Millington DS, et al (2001) Gestational, pathologic and biochemical differences between very long-chain acyl-CoA dehydrogenase deficiency and long-chain acyl-CoA dehydrogenase deficiency in the mouse. *Hum Mol Genet* 10: 2069-2077.

de Cabo R, Mattson MP (2019) Effects of Intermittent Fasting on Health, Aging, and Disease.

359 *N Engl J Med* 381: 2541-2551.
360 Dole VP, Meinertz H (1960) Microdetermination of long-chain fatty acids in plasma and
361 tissues. *J Biol Chem* 235: 2595-2599.
362 Duncombe WG (1964) THE COLORIMETRIC MICRO-DETERMINATION OF NON-
363 ESTERIFIED FATTY ACIDS IN PLASMA. *Clin Chim Acta* 9: 122-125.
364 Fu X, Kimura M, Iga M, Yamaguchi S (2001) Gas chromatographic-mass spectrometric
365 screening for organic acidemias using dried urine filter paper: determination of alpha-
366 ketoacids. *J Chromatogr B Biomed Sci Appl* 758: 87-94.
367 Fukao T, Mitchell G, Sass JO, Hori T, Orii K, Aoyama Y (2014) Ketone body metabolism
368 and its defects. *J Inherit Metab Dis* 37: 541-551.
369 Fukao T, Sasai H, Aoyama Y, et al (2018) Recent advances in understanding beta-ketothiolase
370 (mitochondrial acetoacetyl-CoA thiolase, T2) deficiency. *J Hum Genet*.
371 Hori T, Yamaguchi S, Shinkaku H, et al (2015) Inborn errors of ketone body utilization.
372 *Pediatr Int* 57: 41-48.
373 Horton JL, Davidson MT, Kurishima C, et al (2019) The failing heart utilizes 3-
374 hydroxybutyrate as a metabolic stress defense. *JCI Insight* 4.
375 Houten SM, Wanders RJ (2010) A general introduction to the biochemistry of mitochondrial
376 fatty acid beta-oxidation. *J Inherit Metab Dis* 33: 469-477.
377 Hsu WY, Kuo CY, Fukushima T, et al (2011) Enantioselective determination of 3-
378 hydroxybutyrate in the tissues of normal and streptozotocin-induced diabetic rats of
379 different ages. *J Chromatogr B Analyt Technol Biomed Life Sci* 879: 3331-3336.
380 Hugo SE, Cruz-Garcia L, Karanth S, Anderson RM, Stainier DY, Schlegel A (2012) A
381 monocarboxylate transporter required for hepatocyte secretion of ketone bodies
382 during fasting. *Genes Dev* 26: 282-293.
383 Ibdah JA, Paul H, Zhao Y, et al (2001) Lack of mitochondrial trifunctional protein in mice
384 causes neonatal hypoglycemia and sudden death. *J Clin Invest* 107: 1403-1409.
385 Ibdah JA, Perlegas P, Zhao Y, et al (2005) Mice heterozygous for a defect in mitochondrial
386 trifunctional protein develop hepatic steatosis and insulin resistance.
387 *Gastroenterology* 128: 1381-1390.
388 Knottnerus SJG, Bleeker JC, Wust RCI, et al (2018) Disorders of mitochondrial long-chain
389 fatty acid oxidation and the carnitine shuttle. *Rev Endocr Metab Disord*.
390 Kummitha CM, Kalhan SC, Saidel GM, Lai N (2014) Relating tissue/organ energy
391 expenditure to metabolic fluxes in mouse and human: experimental data integrated

392 with mathematical modeling. *Physiol Rep* 2.
393 Lehninger AL, Sudduth HC, Wise JB (1960) D-beta-Hydroxybutyric dehydrogenase of
394 muitochondria. *J Biol Chem* 235: 2450-2455.
395 Lustgarten JA, Wenk RE (1972) Simple, rapid, kinetic method for serum creatinine
396 measurement. *Clin Chem* 18: 1419-1422.
397 Marks AR, McIntyre JO, Duncan TM, Erdjument-Bromage H, Tempst P, Fleischer S (1992)
398 Molecular cloning and characterization of (R)-3-hydroxybutyrate dehydrogenase
399 from human heart. *J Biol Chem* 267: 15459-15463.
400 Mitchell GA, Fukao T (2001) Inborn errors of ketone body metabolism. In Scriver CR,
401 Beaudet AL, Sly WS, Valle D (eds). *The Metabolic & Molecular Bases of Inherited*
402 *Disease*: McGraw-Hill, New York, pp2327-2356.
403 Mitchell GA, Kassovska-Bratinova S, Boukaftane Y, et al (1995) Medical aspects of ketone
404 body metabolism. *Clin Invest Med* 18: 193-216.
405 Nakagawa Y, Sakuma T, Nishimichi N, et al (2016) Ultra-superovulation for the CRISPR-
406 Cas9-mediated production of gene-knockout, single-amino-acid-substituted, and
407 floxed mice. *Biol Open* 5: 1142-1148.
408 Puchalska P, Crawford PA (2017) Multi-dimensional Roles of Ketone Bodies in Fuel
409 Metabolism, Signaling, and Therapeutics. *Cell Metab* 25: 262-284.
410 Sass JO (2012) Inborn errors of ketogenesis and ketone body utilization. *J Inherit Metab Dis*
411 35: 23-28.
412 Sass JO, Fukao T, Mitchell GA (2018) Inborn Errors of Ketone Body Metabolism and
413 Transport. *Journal of Inborn Errors of Metabolism and Screening* 6: 1-7.
414 Shimazu T, Hirschey MD, Newman J, et al (2013) Suppression of oxidative stress by beta-
415 hydroxybutyrate, an endogenous histone deacetylase inhibitor. *Science* 339: 211-214.
416 Shinmyo Y, Tanaka S, Tsunoda S, Hosomichi K, Tajima A, Kawasaki H (2016)
417 CRISPR/Cas9-mediated gene knockout in the mouse brain using in utero
418 electroporation. *Sci Rep* 6: 20611.
419 van Hasselt PM, Ferdinandusse S, Monroe GR, et al (2014) Monocarboxylate transporter 1
420 deficiency and ketone utilization. *N Engl J Med* 371: 1900-1907.
421 Wang W, Palmfeldt J, Mohsen AW, Gregersen N, Vockley J (2016) Fasting induces prominent
422 proteomic changes in liver in very long chain Acyl-CoA dehydrogenase deficient
423 mice. *Biochem Biophys Rep* 8: 333-339.
424 Williamson DH, Bates MW, Page MA, Krebs HA (1971) Activities of enzymes involved in

acetoacetate utilization in adult mammalian tissues. *Biochem J* 121: 41-47.

Yang H, Zhao C, Tang MC, et al (2019) Inborn errors of mitochondrial acyl-coenzyme a metabolism: acyl-CoA biology meets the clinic. *Mol Genet Metab* 128: 30-44.

Youm YH, Nguyen KY, Grant RW, et al (2015) The ketone metabolite beta-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. *Nat Med* 21: 263-269.

Figure Legends

Fig. 1 Growth curves of WT and *Bdh1* KO, and results from fasting test of WT and *Bdh1* KO female mice at 8 weeks of age.

(A) Body masses of wild-type (WT) and *Bdh1* KO mice as a function of age. Means and standard errors are shown. Each time point shows data on 14 WT and 11 KO mice. (B) Blood glucose levels during fasting. WT and KO mice were studied in the fed state (0 h) and at 16, 24 and 48 hours of fasting. (C) Free fatty acids (FFA), (D) 3-Hydroxybutyrate (3HB). (E) Acetoacetate (AcAc) levels, or (F) Total ketone body (TKB) levels during fasting. N = 10 - 14 /group. **, $p<0.01$; ***, $p<0.001$ by 2-way ANOVA. Error bars indicate one standard error.

Fig. 2 Fat content in liver samples of WT and *Bdh1* KO mice at 0 and 24 hours of fasting.

444 (A) Histopathology. Liver sections of *Bdh1* KO and WT mice in the fed state (0 h) and at
445 24 hours fasting (24h), showing greater staining for neutral fat in fasted *Bdh1* KO mice than
446 in controls. Sudan III staining. Magnification, 400X. (B) Levels of triglycerides (TG) and
447 total cholesterol (T-Chol) in liver. Means and standard errors are shown. N=3. *P = 0.04 by
448 2-way ANOVA.
449
450 **Fig. 3 Pathophysiology of BDH1 deficiency, a hypothesis**.
451 This figure is based on measurements in *Bdh1* KO mice (thick green arrows) and known
452 pathways of energy metabolism, shown schematically, including FA oxidation/ketogenesis
453 and FA and TG synthesis (black), glycolysis/gluconeogenesis (orange), pyruvate
454 metabolism (grey), the citrate/aspartate shuttle (blue) and the Krebs cycle (purple). The
455 hepatocyte (left) normally produces ketone bodies when acetyl-CoA (AcCoA) accumulates
456 in the mitochondrial matrix. Ketogenesis normally reduces intramitochondrial AcCoA
457 level, liberates free CoA and decreases the NADH/NAD^+ ratio, with release of 3HB and
458 AcAc. Hepatic BDH1, the last enzyme of ketogenesis, is shown in red. Deficiency of
459 BDH1 is predicted to cause accumulations of NADH and AcCoA in the hepatocyte
460 mitochondrial matrix. The high level of NADH and the high NADH/NAD^+ ratio are

predicted to inhibit further NADH production, thus slowing the degradative pathways that produce NADH. Enzymes that produce NADH, depicted as red dots, are present in glycolysis (glyceraldehyde phosphate dehydrogenase), FA beta oxidation (the third enzyme of each cycle is a NADH-producing dehydrogenase), pyruvate dehydrogenase (PDH) and the Krebs cycle (isocitrate dehydrogenase 3, *ICD3*, and 2-oxoglutarate dehydrogenase). Combined, the increase of AcCoA in the mitochondrial matrix and the reduced redox environment will shift carbon flux towards the synthesis of glucose and triglycerides (TG), consistent with observations of higher blood glucose and higher liver TG in *Bdh1* KO mice than in controls. AcAc could also fuel cholesterol synthesis in the hepatocyte outside of mitochondria. To the right is shown a nonhepatic cell and the position of BDH1 in the ketolytic pathway.

Multistep pathways are shown schematically as dashed lines. Key transport systems are shown as grey boxes, from left to right, the carnitine shuttle for mitochondrial entry of acyl-coenzyme A molecules, including CPT1A, which is inhibited by malonyl-CoA, the citrate-aspartate carrier (encoded by *SLC25A11*), the mitochondrial dicarboxylate (malate) transporter (*SLC25A10*), pyruvate carrier (*MPC1* and *MPC2*), the presumed hepatocyte membrane monocarboxylate transporter (*SLC16A6*) (Hugo et al 2012) and

478 monocarboxylate transporter 1 (*SLC16A1*) (van Hasselt et al 2014). Abbreviations: Cyto,
479 cytoplasm; MIM, mitochondrial intermembrane space; Matrix, mitochondrial matrix; Mal-
480 CoA, malonyl-CoA; OXA, oxaloacetate; AcCoA; acetyl-CoA; AcAcCoA, acetoacetyl-CoA
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