Genetic Characterization of the Drosophila Birt-Hogg-Dubé Syndrome Gene

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Abstract

Folliculin (FLCN) is a conserved tumor suppressor gene whose loss is associated with the human Birt-Hogg-Dubé (BHD) syndrome. However, its molecular functions remain largely unknown. In this work, we generated a Drosophila BHD model through genomic deletion of the FLCN gene (DBHD−). The DBHD mutant larvae grew slowly and stopped development before pupation, displaying various characteristics of malnutrition. We found the growth delay was sensitive to the nutrient supplies. It became more severe upon restrictions of the dietary yeast; while high levels of yeast significantly restored the normal growth, but not viability. We further demonstrated that leucine was able to substitute for yeast to provide similar rescues. Moreover, the human FLCN could partially rescue the DBHD− phenotypes, indicating the two genes are involved in certain common mechanisms. Our work provides a new animal model of the BHD syndrome and suggests that modulation of the local nutrient condition might be a potential treatment of the BHD lesions.

Introduction

The BHD syndrome is a rare genetic disorder that is clinically characterized by frequent lung cysts, benign hair follicle tumors, and a high risk to develop kidney cancers. Inactivation of the folliculin (FLCN) gene is the genetic basis of BHD syndrome. FLCN is present in a wide range of organisms, from the single-cell yeast to human, indicating it may regulate certain basic cellular processes. However, its biological functions are still not clear [1–3].

Several FLCN mutant cell lines and animal models have been developed to unravel its functions. One discovery from these works is the intriguing relations between FLCN and the mechanistic target of rapamycin (mTOR), a highly conserved nutrient sensor among eukaryotes whose mutations have been found in certain human diseases including cancer [4]. The first clue of interactions between FLCN and mTOR came from biochemical works. Through purification of a FLCN interacting protein (FNIP), people found FLCN was a potential target of the 5′AMP-activated protein kinase (AMPK) and mTOR [5]. This was later realized to be a consequence of possible feedback mechanism, as the same group observed overactivated mTOR in the hyperplastic kidneys from the FLCN knock-out mice (FLCN−/−) [6,7]. Surprisingly, other researchers observed mTOR was both up- and down-regulated in certain FLCN mutant cell lines and the FLCN−/− mouse tissues [8,9]. At present, how FLCN interacts with mTOR is still not determined. Another solid observation is that the FLCN−/− mice stopped development at very early embryonic stages with severely disorganized structures [6–9]. The cause of the failed embryogenesis is not known yet and it has not been linked with other abnormalities in adults. Recently, people characterized an anti-apoptosis function of FLCN through the TGF-β pathway, which was proposed to be a new mechanism to account for its tumor suppressor roles [10,11].

Drosophila provides an ideal model system to study the genotype-phenotype relationships. Its genome contains a single FLCN homologue gene (DBHD). Using an RNAi-mediated gene knockdown assay, people uncovered a role of DBHD in the male germline stem cell maintenance and suggested the dysregulated stem cell homeostasis might be a potential mechanism of the BHD tumorigenesis [12]. The RNAi is a method to partly suppress gene functions, while most genetic lesions of the reported BHD cases are FLCN loss [3]. Therefore, it is better to use null mutant to model the pathological conditions. For this reason, we generated a DBHD knockout allele (DBHD−). The DBHD− mutant larvae displayed various features of malnutrition, including growth retardation, small body size and larval lethality. The growth defects, but not the lethality, could be significantly rescued by dietary yeast or the branched-chain amino acid of leucine. We further demonstrated that the rescue effect is likely a consequence of elevated dTOR signaling, because a specific dTOR signaling suppressor, rapamycin, could reverse the rescues of DBHD− mutants by yeast or leucine. Moreover, the human FLCN could partially rescue the DBHD− mutants, indicating at least some molecular functions of the two homologous genes are conserved. Our work provides a novel animal model of the BHD syndrome...
and suggests that modulation of the local nutrient conditions deserves further investigations for treatment of BHD.

Materials and Methods
Generating the DBHD Knockout Fly
To make the DBHD targeting construct, two genomic fragments from both sides of the DBHD locus, about 5 kb each, were amplified and inserted into the NotI and AscI cloning sites of the pW25 vector [13]. PCR amplification primers: TCTTTTTAGGGGCTTACACCTGGGCTCC and TGAACCAGGGGGCCCGCAATTCACTGAAATACGAG (with AscI site); CAATCGCGCGGCTTTCATCAGATAAAAACGAG and GACTATCGGCGCCGAATTCATGAGGCTG (with NotI site). The screening procedure was performed according the protocol described before [13].

Making the DBHD Rescue Constructs a DBHD Polyclonal Antibody
To make the DBHD-res, a genomic fragment covering the complete DBHD locus till the adjacent gene (CG14829) was amplified from the Drosophila genomic DNA. PCR amplification primers: GCACCTCTAGAGATCCAGAATGAACAG and GCTGTCTAGACTGGATTCGGCATC. EGFP tag was then amplified by fusion-PCR. The human FLCN cDNA (a kind gift of Laura S. Schmidt) was amplified by PCR and cloned into the pUAST vector to make the UAS-hFLCN transgene. To generate a DBHD rescue construct, two genomic fragments from both sides of the DBHD locus, about 5 kb each, were amplified by PCR and inserted into the expression vector pGEX-T1. This construct was transfected into bacteria BL21 for expressing GST-fusion protein. The purified GST-fusion protein was injected into rabbit to generate antiserum against DBHD. On SDS-PAGE, it recognizes a major band at about 55 kDa, the same size as the predicted DBHD protein.

Fly Stocks and Food Preparation
In the DBHD mutant screening experiment, we used the following stock: y w; 70FLP, 70S-Sco (BL#6934) and w1118; 70FLP (BL#6938). The following flies were used to generate mosaic clones: ksp-flp; RRT80B ubi-GFP/FRT80B DBHD+ and eyes-fly; FRT80B ubi-GFP/FRT80B DBHD+. We crossed the following flies to rescue DBHD+ with hFLCN: ksp-Gal4, DBHD+/TM3, Sb and UAS-hFLCN; DBHD+/TM3, Sb. The normal food recipe used in our lab: 8% sugar, 10% corn flour, 1.5% baker’s yeast, 1% agar, 0.4% Propionic Acid and 0.1% Nipagin. Chemicals used in the feeding experiments: 3-methyl adenine (Invitrogen); Rapamycin (LC Laboratories). Leucine, arginine, glutamine, tryptophan, cholesterol, and riboflavin were all bought from Sigma. For the free amino acids analysis, the third instar larvae were rinsed with 70% ethanol and allowed to be air-dried. More than 55 larvae were homogenized followed by sonication. Proteins were precipitated by sulfosalicyclic acid. After centrifugation, the supernatant were passed through 0.22 μm filter and analyzed using the Hitachi automatic amino acid analyzer L-8900.

Immunocytochemistry
The following primary antibodies were used: rabbit anti GFP (1:500, Invitrogen); rabbit anti PH3 (1:1000, Invitrogen). Mouse anti Armadillo (1:200) and Prospero (1:200) were from DSHB. Guinea pig anti-Dnp (1:1000) was a gift from Jim Skeath.
ApopTag-Red assay kit was from Millipore. The fluorescent secondary antibodies, LysoTracker, and Click-iT EdU assay kit were all bought from Invitrogen.

Results

Gene Targeting of DBHD

To genetically ablate the DBHD function, we used the homologous recombination strategy to delete the DBHD genomic sequence within the germ cells from living animals (“ends-out”, 13). The DBHD gene encodes a 460-amino-acid protein, spanning 1712 base pairs (bp) on the left arm of chromosome 3 with three exons. The targeting construct contains a 4.8 kb and a 5 kb of genomic fragments flanking the DBHD transcription unit (Figure 1A). It was firstly introduced into the fly genome through the standard P-element-mediated transformation. The targeting cassette was later released and linearized from the germ cell genome by two endogenously produced enzymes (Flipase and I-SceI, 13). Following homologous recombination, the complete exon1 of DBHD including sequences encoding the first 400 amino acids and the 5′ untranslated region (5′ UTR) will be replaced with a white marker gene. This should give rise to a DBHD null allele (Figure 1A). Because the BHD gene is conserved in a wide range of organisms and the BHD knockout mice die at very early embryonic stages, we suspected that DBHD was a vital gene. To this end, we screened about 500 gametes and uncovered a lethal allele on chromosome-3 where DBHD resides.

We performed several experiments to check the mutation. We first did PCR analysis and confirmed the targeting effects on both arms as predicted (Figure 1A, B). RT-PCR results further revealed the DBHD transcript was present at various developmental stages, but was absent in the homozygous mutants (Figure 1C). We also generated a DBHD polyclonal antibody. In the western blot experiment, it recognized a major band at about 55 kDa of the total larval extracts, which was missing in the mutant samples (arrow in Figure 1D). Finally, we made transgenic flies harboring an exogenous genomic fragment containing the complete DBHD exons and the upstream non-transcribed sequences till the adjacent gene (referred to as DBHD-res, see the later section for the structures). One copy of DBHD-res could rescue the mutants to healthy adults without any obvious abnormalities compared with the heterozygotes. These results revealed that we obtained a clean null allele of DBHD.

Figure 2. Mitosis and endoreplication are suppressed in DBHD−/− larvae. PH3 marks the mitotic cells. EdU marks the cells undergoing DNA synthesis. DAPI marks the nuclei. (A and B): Eye imaginal discs. (C, D, K, L): Brains. (E, F, I, J): Fat bodies. (G and H): Salivary glands. The sibling heterozygotes (−/+ ) were taken as the wild-type controls. Note all the DBHD−/− samples (−/− ) are reduced in size, the polyploidy are also reduced in cells from fat body (F) and salivary gland (H).

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DBHD is an Essential Gene

The homozygous DBHD mutants (hereafter referred to as DBHD\(^{-/-}\)) never survived to adults. To trace the developmental progresses, we combined the DBHD\(^{-/-}\) mutant allele with a GFP-marked balancer chromosome (referred to as \(-/\text{TM3, Kr::GFP}\)). Firstly, we wanted to check if the mutation had any dominant effects. We compared the developmental profiles of the heterozygotes (\(-/\text{TM3, Kr::GFP}\)) and another strain containing an isogenized wild-type third chromosome (recovered from single \(w^{1118}\) fly) with the same balancer chromosome (+/\text{TM3, Kr::GFP}). Embryos were collected within three hours and allowed to develop in stable environment (25°C, 60% humidity). Under such conditions, the heterozygotes behaved similarly with the wild-type discs. The brains were also reduced in size (DBHD\(^{-/-}\)/TM3, Kr::GFP) by developing into healthy adults at around the same time point (Figure 1E). Thus, DBHD\(^{-/-}\) is essentially a recessive gene.

Based on the statistical analysis and direct check under fluorescence microscope, we found that the DBHD\(^{-/-}\) embryos (negative of \(\text{Kr::GFP}\), hatched normally (Figure 1E, F). However, when most heterozygous larvae have entered into the third instar stage three days later, all DBHD\(^{-/-}\) larvae were much smaller than their sibling heterozygotes (Figure 1F). The mean weight of a heterozygous larva at third instar is 0.0013 g (\(n = 273\)). For DBHD\(^{-/-}\) this number is about 0.0003 g (\(n = 185\)). The DBHD\(^{-/-}\) larvae could survive for a prolonged period of time (up to three weeks). Eventually, all mutants died as small larvae. We separately cultured the mutants and the heterozygotes in different food vials. Each vial contained no more than ten newly hatched larvae. This should provide each individual with sufficient food and space, and minimize the potential toxicities brought by the crowds or heterozygotes. The DBHD\(^{-/-}\)/TM3, Kr::GFP larvae still showed the same growth retardation phenotype, which could rule out the weakness in intraspecific competition as a causal mechanism.

Some Drosophila zygotic mutants can survive the embryogenesis because of the large amounts of maternal gene products deposited in the eggs. To exclude the potential maternal contributions, we generated DBHD\(^{-/-}\) germ line clones by removing the DBHD products at the beginning of egg formation [14]. Animals developed from the DBHD\(^{-/-}\) germ line clones showed no difference with the zygotic mutants in development: they successively passed through the embryo stage and died before pupation. We conclude that DBHD plays essential roles in the larval stages.

Mitosis and Endoreplication were Suppressed in DBHD\(^{-/-}\) Larvae

After hatching from eggs, the Drosophila larvae feed continuously to increase their body mass dramatically before the onset of pupation. The larval imaginal and endoreplicative cells make the main contributions to this change. The imaginal cells, including those in the imaginal discs, the gonads and the brain, go through active mitosis to increase the cell number. In contrast, the endoreplicative cells from the gut, salivary gland and fat body, increase the DNA polyploidy and cell volume without further cell divisions [15]. We dissected the 4-day-old larvae and checked both the imaginal and the endoreplicative tissues. Most DBHD\(^{-/-}\) larvae had tiny or even no visible imaginal discs. The brains were also reduced in size (Figure 2). These phenotypes could be caused by decreased cell division, increased cell death or both. We analyzed the apoptosis in larval brains (ApopTag, Invitrogen). No clear differences were observed between the larval brains of mutants and heterozygotes (unpublished observation). The Phospho-Histone H3 (PH3) is a reliable mitotic cell marker by labeling the condensed chromosomes during mitosis. We found the PH3-positive cells were dramatically declined in various imaginal tissues of the DBHD\(^{-/-}\)/TM3, Kr::GFP larvae (Figures 2A–D). Thus, cell division is suppressed in the DBHD mutants.

Another obvious discrepancy was that the mutant larvae were less opaque than their heterozygote siblings. The latter were normally filled with white fat body, a nutrient storage and sensing organ that is equivalent to the mammalian liver/adipose tissues. We found that the DBHD\(^{-/-}\) larvae had very thin fat bodies. In addition, the mutant cells were filled with large vacuoles and their nuclei seemed to be shrunken, which were in contrast with the large polyploid nuclei in heterozygotes (Figure 2E, F). Similarly, both the cell volumes and DNA contents of the salivary gland cells were also markedly reduced (Figure 2G, H). We used the EdU incorporation assay, a thymidine analogue, to label the DNA synthesis. In the early third instar heterozygotes, a large number of the imaginal cells and endoreplicative cells were undergoing DNA replication. In contrast, the reaction was rather quiescent in the DBHD\(^{-/-}\) tissues (Figures 2I–L). Taken together, a combined suppression of cell division and cell growth should account for the small body phenotype of the DBHD\(^{-/-}\) larvae.

Autophagy is Elevated in the DBHD\(^{-/-}\) Larvae

The above DBHD\(^{-/-}\) phenotypes were reminiscent of starvation or mutations that blocked the nutrient-sensing signaling pathways in Drosophila. It is known that upon nutrient restrictions,
especially the dietary protein, autophagy is induced in the fat bodies by degradation of the non-essential cell organelles to supply critical nutrients for survival [15]. We stained the freshly dissected fat body with a red-fluorescent dye to mark autophagy (LysoTracker, Invitrogen). Under normal feeding conditions, the LysoTracker signal is very faint in the heterozygotes. In contrast, it is greatly elevated in the DBHD+/− larvae (Figure 3A). The heterozygous larvae showed stimulated autophagy upon nutrient starvation for several hours (Figure 3B). To check if the strong autophagy in DBHD+/− larvae is responsible for their growth defects, we fed them with 3-Methyladenine, an autophagy inhibitor [16]. It efficiently suppressed autophagy (Figure 3C),

Figure 4. Rescue of the DBHD+/− growth defects by nutrients. (A) DBHD+/− larvae were sensitive to yeast supply. NF: normal food; Star: starvation, normal food without yeast. All samples were picked at Day 8 after hatching from eggs. (B) The developmental profiles of flies cultured on two kinds of nutritious foods. yeast paste: the baker’s yeast powder was mixed with water and supplied on agar plate. leucine: normal food supplemented with 100 mM leucine. Fifty newly hatched larvae for each genotype were picked. Numbers of the heterozygotes (left) and DBHD+/− were separated by slashes. (C) Examples of four dead DBHD+/− pharates cultured on yeast pastes. (D) Rescue effects of leucine. Animals aged for 7 days after hatching were imaged. NF: normal food; leu: normal food with 100 mM leucine; rapa: normal food with 1 μM rapamycin; leu+rapa: normal food with 100 mM leucine and 1 μM rapamycin. (D) Free amino acids analysis of the larvae. The amino acids levels are displayed as milligram per gram of body weight (mg/g). The amounts of larvae for each experiment are listed in the parenthesis. doi:10.1371/journal.pone.0065869.g004
while it could not restore the normal growth or viability in the mutants. Thus, elevated autophagy alone is not sufficient to cause the growth defects in DBHD−/− larvae.

To check if the above starvation-like phenotypes are caused by foraging difficulties, we fed larvae with colored yeast paste. The DBHD−/− mutants behaved similarly with controls in swallowing and excretion. Both of them were attracted by this nutritious food and successively passed it into the midgut (Figure 3D). Once they were put back to the clean food, the colors within the guts were quickly cleaned out. Thus, the DBHD−/− larvae do not have obvious defects to obtain food and excrete the waste. All the above experiments were repeated with the DBHD−/− germline clones to remove maternal influences and similar results were obtained.

The Growth Delay of DBHD−/− Larvae was Significantly Rescued by Yeast-rich Food

Because the DBHD−/− larvae show certain starvation-like phenotypes, we sought to investigate their growth responses to different nutrient conditions. Dietary yeast is the major source of nutrients in fly food. Firstly, we cultured the newly hatched larvae on less nutrient food as described before (normal food recipe without yeast, [14]). The heterozygotes grew slowly on this kind of medium (Figure 4A). Interestingly, the mutants became even smaller than the mutants fed with normal food (Figure 4A), suggesting the DBHD−/− animals are sensitive to the yeast supplies.

Next, we checked their growth responses to nutrient food. We picked no more than twenty newly hatch larvae, put them on culture medium with different concentrations of yeast and analyzed their developmental profiles (Table 1). As expected, the heterozygotes took longer time for eclosion with diluted food or yeast-free food. Once the yeast concentration was above it of the normal food recipe, the heterozygotes showed similar growth rates. Surprisingly, the yeast-rich food significantly reversed the growth retardation phenotypes of the DBHD−/− larvae. The yeast-only food brought the best rescues, no matter it was living or dead (autoclaved).

On pure yeast paste, nearly all mutants could grow into fat third instar larvae and successively pupate at more or less the same time as heterozygotes (Table 1 and Figure 4B). Some mutant phenotypes, including the increased autophagy, suppressed mitosis and endoreplication of larval cells, were also mostly rescued. However no mutant eclosed, they mostly died at the pupal stage. Some dead pupae even developed discernable adult structures including bristles, legs, wings and eyes, suggesting DBHD had no gross influences on cell fate specifications (Figure 4C). Therefore, nutrient is an effective factor to rescue the growth defects in DBHD−/− larvae.

Leucine is Able to Substitute for Yeast to Restore the Normal Growth of DBHD−/− Larvae

Yeast is the major source of three groups of nutrients in fly food, including cholesterol, vitamins (especially the B family) and amino acids. The larval growth will be delayed if any of these components is limited [17–20]. To find out the active gradient(s) in yeast

### Table 1. The developmental profiles of flies cultured on various yeast foods.

| Culture media | developmental profile |
|---------------|-----------------------|
| normal food (1.5% yeast) | eclosed, 8–9D, no pupation |
| diluted food (0.75% yeast) | eclosed, 14D, no pupation |
| yeast-free food | eclosed, >15D, no pupation |
| rich food-1 (10% yeast) | eclosed, 8–9D, pupated, 5–6D |
| rich food-2 (20% yeast) | eclosed, 8–9D, pupated, 5–6D |
| yeast paste | eclosed, 8–9D, pupated, 5–6D |
| yeast paste +5% sugar | eclosed, 8–9D, pupated, 4–5D |
| yeast paste on normal food | eclosed, 8–9D, pupated, 5–6D |

Note: The developmental profiles were displayed by listing the last stages that they survived and the time point when they started to enter (days after hatching). Pure yeast paste had the best rescue effects. Diluted food means the normal food recipe (1.5% yeast) with less nutrient food as described before (normal food recipe without yeast). In all tests, we picked the newly hatched larvae at the same time point. No more than twenty larvae were cultured within each food chamber. At least 100 larvae in total were counted for each experiment. 

### Table 2. Rescue of the DBHD−/− larvae by supplemented nutrients.

| Culture media | developmental profile |
|---------------|-----------------------|
| Normal food | no pupation |
| +Riboflavin (0.1 mg/ml) | no pupation |
| +cholesterol (40 mM) | no pupation |
| +Leucine | most died as pupae |
| +Tryptophan | no pupation |
| +Arginine (100 mM) | no pupation |
| +Glutamine (100 mM) | no pupation |

Note: All tested components were supplemented as additions in the normal food. Leucine and tryptophan were tested at various concentrations (10, 50, 100, and 500 mM). Supplementation of leucine provided consistent rescues at all tested concentrations. Excessive tryptophan further inhibited growth at high concentrations (>100 mM). At least 200 embryos collected within three hours were tested. 

![Figure 5. The human FLCN could partially rescue the DBHD−/− larvae.](image)

(A) Dorsal view of pupae. The heterozygote (−/+), reveals by Sb (marked with short and thick bristles on the notum, arrow), see materials and methods for the cross scheme. (B) The genotypes of the pupae were confirmed by PCR analysis of genomic DNA. The fly CG10414 gene was used as a positive control. 

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responsible for the rescues of $DBHD^{-/-}$ larvae, we cultured flies on normal food supplemented with single nutrient (Table 2). For all the components that we tested, only leucine provided comparable rescues with the yeast-rich food. With additional leucine, most $DBHD^{-/-}$ larvae successfully pupated and died during metamorphosis (Table 2 and Figure 4D). The rescue effects were consistent at all the concentrations we tested (10, 50, 100, and 500 mM). Other essential amino acids, including tryptophan, arginine or glutamine, did not show obvious rescues at high concentrations (100 mM). High levels of tryptophan were even toxic by suppressing the larval growth further (Table 2, [21]). We conclude that the leucine-mediated mechanism is responsible for the rescue of $DBHD^{-/-}$ larvae.

The Gross Absorption of Amino Acids is not Impaired in $DBHD^{-/-}$ Larvae

The foraging assay revealed that the $DBHD^{-/-}$ larvae fed normally. Next, we checked their abilities of food digestion and amino acid absorption. The third instar larvae were homogenized. The concentrations of 16 free amino acids from the whole extracts (including those in the haemolymph and cytoplasm) were measured using the automatic amino acid analyzer (Hitachi, L-8900). With either normal food or yeast paste, no significant differences were observed between the heterozygotes and the $DBHD^{-/-}$ larvae (6 amino acids are selectively shown in Figure 4E). This result further demonstrates that food digestion and the gross absorption of amino acids are not apparently impaired in the $DBHD^{-/-}$ larvae.
Rapamycin Suppressed the Rescue Effects of Leucine on DBHD\(^{-/-}\) Larvae

Amino acids are important stimulators of mTOR signaling, among which leucine is the most efficient [22,23]. We noticed that the DBHD\(^{-/-}\) phenotypes, including small body size, growth delay and larval lethality, were indeed similar to those of the dTOR mutants [24,25]. Therefore, we checked if dTOR signaling is responsible for the rescue of DBHD\(^{-/-}\) larvae.

Rapamycin is a specific inhibitor of the mTOR signaling. Flies fed with a low dose of rapamycin displayed the starvation-like phenotypes [24]. We used the same concentration of rapamycin in the food (1 \(\mu\)M) and found that it efficiently suppressed the rescue effects of leucine or yeast paste (Figure 4D and data not shown), all the DBHD\(^{-/-}\) larvae eventually died before pupation. We propose that active dTOR signaling is responsible for the rescue of growth defects in DBHD\(^{-/-}\) larvae.

The Human FLCN could Perform Partial DBHD Functions

To investigate if the roles of DBHD are conserved in mammals, we attempted to rescue the DBHD\(^{-/-}\) flies with human FLCN (hFLCN). We generated a UAS-hFLCN transgenic fly and expressed the human FLCN gene under the control of hip-Gal4 driver. The newly expressed larval cells grew with normal food were heated for 30 minutes at 37°C, twice a day, to ubiquitously induce the expression of hFLCN. No rescued DBHD\(^{-/-}\) flies eclosed. However, some DBHD\(^{-/-}\) larvae expressing hip/hFLCN (about one third) could develop into pupae with no clear defects of the cuticles (Figure 5). Because 46% of the amino acids of DBHD and hFLCN proteins are similar [12], and we used a ubiquitous expression driver, it is not so surprising that we obtained only partial rescues. Nevertheless, this result reveals that the hFLCN could at least perform partial DBHD functions, suggesting the two genes are involved in common mechanisms.

DBHD is Expressed Broadly

We made two DBHD rescue constructs in which the EGFP tag was fused in frame with either terminus of the DBHD transcription unit (Figure 6, together referred to as DBHD-res). The transcription were under the control of native DBHD promoter and the upstream sequence till the adjacent gene. Either transgene could fully rescue the DBHD\(^{-/-}\) animals into healthy adults. Therefore, the EGFP signal should be able to monitor the essential localizations of DBHD proteins. Both transgenes showed the same EGFP expression patterns, suggesting the DBHD proteins functioned in full length throughout their lifetime.

We used the rescued homozygous mutants (DBHD-res; DBHD\(^{-/-}\)) to check the expression of DBHD-res, so that there were no endogenous DBHD proteins. The EGFP is broadly present in larval phases, including the imaginal discs, fat bodies, central nervous system (CNS) and midguts (Figure 6). It is expressed in fat bodies, in both nuclei and cytoplasm (Figure 6A). It is also concentrated in the cytoplasm of the entire eye, wing and leg imaginal disc cells. Using a neuroblast marker of Deadpan (dpn) [26], we detected DBHD-res in most, if not all neuroblasts in the larval CNS (Figure 6E). It has specific patterns in the midguts throughout larval and adult stages (Figures 6G–I), where it is mainly in the cytoplasm of diploid cells, including intestinal stem cells (ISCs), enteroblast cells (EBs) and enteroendocrine cells (EEs). It is also apparently expressed in many, but not all polyplody enterocytes (ECs). In adults, it is also enriched in the tips of both testis and germarium, and the nutritive follicle cells of the eggs (Figures 6J–L).

DBHD is Not Required Cell-autonomously for the Growth of Larval Imaginal Disc Cells

The Drosophila larval imaginal discs are active proliferative tissues, which will develop into the adult appendages during morphogenesis. They provide excellent systems to study the mechanisms of cell proliferation and cell fate specifications. We generated DBHD\(^{-/-}\) clones in the larval imaginal discs. Surprisingly, the mutant clones were similar in size to the wild-type twin spots (Figures 6A, B). The cell numbers in the twin clones did not show clear differences (counted by DAPI signals). Therefore, the DBHD\(^{-/-}\) cells do not have growth advantages over their sibling wild-type cells. In addition, we did not find any morphological defects associated with the mutant cells in adults. We also used the eyeless-flipase to generate large DBHD\(^{-/-}\) clones in eye discs, again there were no obvious phenotypes in adult eyes and mitosis seemed normal within the mutant clones (Figures 7C, C′). These results suggest that DBHD is not required cell-autonomously in these tissues.

Discussion

In summary, we developed a new animal model of the BHD syndrome and demonstrated that some functions of FLCN are conserved in Drosophila and mammals. An interesting discovery from our fly model is that the growth defects of DBHD mutant larvae could be substantially rescued by nutrient. It is, therefore, of great interest to investigate if modulation of the local nutrient conditions is beneficial for the treatment of BHD lesions in mammalian systems.

It is not clear why nutrient (particularly leucine) could rescue the growth defects of DBHD\(^{-/-}\) animals. DBHD either functions in parallel with, or is directly involved in the leucine-mediated mechanisms. Because the normal food can support the growth of heterozygotes, but not DBHD\(^{-/-}\), we speculate that leucine must play roles other than protein synthesis. So far, the best known role of leucine as a signaling factor is to activate mTOR [21,22]. In consistent with this, inhibition of dTOR by rapamycin reversed the rescue effects of leucine or yeast.

If DBHD is involved in dTOR signaling, as it does in mammals, we propose here one mechanism that DBHD functions to sequester amino acids within cellular organelles to activate dTOR in some Drosophila tissues. DBHD does not encode a typical membrane protein and DBHD-res is mainly expressed in the cytoplasm of...
several cell types (Figure 6). The DBHD^{-/-} larvae could feed, and obtain sufficient nutrient from the yeast-rich foods, implying the food digestion and gross amino acids absorption are not severely impaired. Recently, it was found that mTOR needs to be translocated to the surfaces of lysosomes for activation [27]. It is thus possible that DBHD helps to accumulate leucine within the lysosomes to evoke dTOR. This locally enriched leucine could be alternatively achieved by saturation mechanism through increasing its supply in the food. Further experiments are definitely required to clarify the mechanism.

Unlike mice, DBHD is not required for the embryonic development. This is nevertheless consistent with a role of DBHD in sequestering amino acids from the environment. The Drosophila embryos rely on the nutrients deposited in the eggs entirely. It is not until the larval stage that they start to obtain nutrients from the food. The eggs might contain sufficient leucine to support the embryogenesis of DBHD^{-/-}, which takes about only 24 hours. After hatching, the DBHD^{-/-} larvae fed with nutritious food could pupate. However, as the leucine is gradually consumed during metamorphosis (about 96 hours), they eventually stop development before eclosion.

It is surprising that DBHD controls the growth of the imaginal disc cells in a non-cell-autonomous manner, which does not support a classical anti-tumor function. Our work suggests that at least in the Drosophila imaginal discs, DBHD controls growth through some neuronal/hormonal mechanisms. Consistently, DBHD-ros is expressed in various endocrine cells or tissues, including the gut epithelia, fat body, brain, and ring glands (Figure 6). The latter is an endocrine organ to secrete hormones including juvenile hormone (JH) and the Drosophila insulin-like peptides (DILPs). Flies missing these products will develop the starvation-like phenotypes. At present, we are analyzing the functions of DBHD in these places.

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