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Drosophila Model in the Study Role of UCH-L1

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Abstract

UCH-L1 (ubiquitin carboxyl-terminal hydrolase L1) is a protein, which plays an important role in ubiquitin-proteasome system. Many previous reports showed the relation between UCH-L1 and neurodegenerative diseases, diabetes, as well as cancer. However, the mechanism still remains unclear. In the aim to investigate the functions and regulatory mechanism of UCH-L1 in living organism, *Drosophila melanogaster* model was utilized to examine the role of UCH-L1. This chapter provides a summary on recent findings related to the roles of UCH-L1 based on the model. First, abnormal expression of *Drosophila* ubiquitin carboxyl-terminal hydrolase (dUCH) leads to the defects on fly tissue development and function. Gain function of dUCH in the eye imaginal discs induced a rough eye phenotype in the adult, partly resulting from induction of caspase-dependent apoptosis, upset of photoreceptor cell distribution and ommatidium apical mispatterning. Interestingly, the dUCH overexpression of induced rough eye phenotype was completely rescued by co-expression either Sevenless or Draf of the mitogen-activated protein kinase pathway. Besides, knockdown dUCH in dopaminergic neurons resulted in some Parkinson's disease-like phenotypes in fly. Taken together, those findings in *Drosophila* model contributed a significant dUCH in tissue development and function.

Keywords: *Drosophila melanogaster*, UCH-L1, human diseases, eye development, anti-dUCH antibody

1. Introduction

Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1), a protein of 223 amino acids (aa), weighs about 24,824 Da, a period lasting for more than 48 half-hour. UCH-L1 is an abundant protein in neurons, accounting for 1–2% of the total protein in the human brain [1]. In addition to the brain, UCH-L1 is also expressed strongly in the peripheral nervous system,

including sensory and nervous system activity. UCH-L1 belongs to remove the tagged enzyme (deubiquitinating enzyme (DUB)), an important protein in ubiquitin proteasome system (UPS). UCH-L1 hydrolases the peptide bond between ubiquitins and also plays a function as a ligase when it be in dimer form [2, 3]. UCH-L1 is an enzyme which binds to the polyubiquitin chains and released the single ubiquitin in the ubiquitin proteasome system. However, when UCH-L1 is in binary form, UCH-L1 leads to the formation of a polyubiquitin chain linked through lysine 63 (K63). Although the main activity of UCH-L1 is still unclear, UCH-L1 has been believed to play its role through maintaining a pool of free monomeric ubiquitin which is important for the function of ubiquitin proteasome system [4]. Abnormal function of UCH-L1 leads to the reduction of protein degradation, followed by the accumulation of ubiquitinated protein [5–7]. UCH-L1, therefore, may relate to many biological processes which dependent to ubiquitination including DNA repair, cell signaling, trafficking, endocytosis and degradation.

In 1998, a missense mutation of UCH-L1 (I93M) was first identified in a German family with Parkinson's disease (PD) [8]. By contrast, another variant of UCH-L1 (S18Y) was discovered as a factor in the risk reduction of PD [9]. Other studies also found that UCH-L1 was related to abnormal accumulation and aggregation of α -synuclein which leads to formation of Lewy bodies [3]. Furthermore, gracile axonal dystrophy (GAD) mouse which carries a deletion within UCH-L1 gene manifested motor ataxia, axonal degeneration and a reduction in the monoubiquitin level in neurons [10–12].

On the other hand, many studies indicated that UCH-L1 involved too many types of human cancer [4]. High expression of UCH-L1 was found in many types of cancers such as breast cancer, non-small cell lung cancer [13, 14]. UCH-L1 expression can be self-upregulated via oncogenic β -catenin/TCF activation. The UCH-L1 upregulates oncogenic β -catenin by which feedback regulates the expression of *uch-l1* gene [15]. UCH-L1 may also promote cancer metastasis via β -catenin-induced epithelial-to-mesenchymal transition [16, 17]. High levels of UCH-L1 may promote oncogenic transformation, invasion and metastasis, and the function of UCH-L1 might due to the enhancement of Akt signalling in vitro and in vivo [16, 18, 19].

By contrast, UCH-L1 had been also reported as a tumor suppressor in many other studies. The downregulation of UCH-L1 was observed in various types of cancer such as esophageal cancer, breast cancer, prostate cancer and pancreatic cancer [20–24]. Reduction in UCH-L1 expression leads to cell proliferation arrest and p53-mediated apoptosis [22, 25].

In humans, the gene coding for UCH-L1 is located in the short arm of chromosome 4 at position 14, from base pair 40,953,685 to 40,965,202, 11,518 base pairs long [26]. In *Drosophila melanogaster*, ubiquitin carboxyl-terminal hydrolase (dUCH) encoded by CG4265 gene is a homolog of human UCH-L1 (hUCH-L1). The identity and similarity between dUCH and hUCH-L1 are 44.5 and 75.7%, respectively. In this chapter, we provide a summary on recent findings related to the roles of UCH-L1 in living organisms by *Drosophila* models. Those findings indicated that dUCH (ortholog of human UCH-L1 in *Drosophila*) plays an important role tissue development and involves in Parkinson's disease.

2. *Drosophila* model in the study role of UCH-L1

2.1. Homolog of human ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) in *Drosophila melanogaster*

The survey of the *Drosophila* genome database allowed an identification of the CG4265 as a homolog of the human UCH-L1. The CG4265 gene, named as dUCH (*Drosophila* ubiquitin carboxyl-terminal hydrolase), encodes a 224-amino-acid protein that shows 44.5% identity and 75.7% similarity with human UCH-L1. The Cys residue at amino acid (aa) position 90 and the His residue at aa 161, both of which are essential for hydrolase activity of human UCH-L1 [27–29], are conserved in *Drosophila melanogaster* along with several other species including *Mus musculus* and *Caenorhabditis elegans* (Figure 1).

2.2. Generation of anti-dUCH antibody

Since *Drosophila melanogaster* has been shown to be a compatible model for studying human diseases, the UCH-L1 homologous protein in *Drosophila melanogaster* (dUCH) is utilized for analyzing the role of UCH-L1 in living system. Thereby, anti-dUCH antibody is essential for research and needs to be generated. The produced anti-dUCH antibody was shown to have high specificity and sensitivity to the dUCH protein. The affinity of the antibody is 1:320,000 at 7.81 ng/μl antigen concentration. The 1:40,000 dilution-produced antibodies can detect antigen at a low concentration 0.98 ng/μl [30]. Besides, the antibody showed a high specificity for

hUCH-L1	1	--MQLKPMEINPEMLNKVLSRLGVAGQWRFVDVLGLEEEESLGSVPAPACALLLLFPLTAQ	58
mUCH-L1	1	--MQLKPMEINPEMLNKVLAKLGVAGQWRFADVLGLEEEETLGSVPSPACALLLLFPLTAQ	58
dUCH	1	-MLTWTPLESNPEVLTKYIHKLGVS PAWSVTDVIGLEDDTLEWIPRPVKAFILLFPCSET	59
cUBH-1	1	MAAPWTPLESNPSVINPMIEKMGVSGVK--TVDVLFDDDESIGK---PQHAVILCFPEYKK	56
hUCH-L1	59	HENFRKKQIEELKGQEVSPKVYFMKQTIGNSCGTIGLIHAVANNQDKLGFEDGSVLKQFL	118
mUCH-L1	59	HENFRKKQIEELKGQEVSPKVYFMKQTIGNSCGTIGLIHAVANNQDKLEFEDGSVLKQFL	118
dUCH	60	HRAEEHDRIKEVEEQ--HPEDLFYMRQFTHNACGTVALIHSVANNKEV--DIDRGVLKDFL	116
cUBH-1	57	VDEIMKPIYEQ--AKAADDVFFMKQKISNACGTFAHFHSLANLEDRLNLGDSFA-KWL	113
hUCH-L1	119	SETEKMSPEDRAKCFEKNEAIQAADDAVAQEGQCRV--DDKVNHFILFNNVDGHLIELD	176
mUCH-L1	119	SETEKLSPEDRAKCFEKNEAIQAADDSVAQEGQCRV--DDKVNHFILFNNVDGHLIELD	176
dUCH	117	EKTASLSPEERGRALEKDEKFTADHEALAEQGTNAANHEKVIHFIALVNKEGTLYELD	176
cUBH-1	114	AEAKKVGIEERSDFLANNALAGIHAAAATDGTAP--SGDVEHFICFVGKNGILYEID	171
hUCH-L1	177	GRMPFPVNHGASSEDTLKDAKVCREFTEREQGEVRFSAVALCKAA--	223
mUCH-L1	177	GRMPFPVNHGASSEDLLQDAKVCREFTEREQGEVRFSAVALCKAA--	223
dUCH	177	GRKSFPKIHGPTSEETFVKDAKVCKEFMARDPNEVRFTVLALTAAQQ	224
cUBH-1	172	SRPFAREIGPTSDATLVKDAGAACQHLIEKLD-NVSFSAIAVVNQ--	216

Figure 1. Amino acid sequences of UCH-L1 protein between human (hUCH-L1), mouse (mUCH-L1), *Drosophila* (dUCH-L1) and *C. elegans* (cUBH-L1). The identity and similarity between human and *Drosophila* were 44.5 and 75.7%, respectively. Identical amino acids are shaded in dark grey, and similar amino acids are shaded in light grey. The red letters indicate the identical amino acids at active sites. Clustal Omega (1.2.4) multiple sequence alignment was applied.

Drosophila either in Western blot or in immunostaining. When the dUCH was overexpressed in fly eye imaginal discs using the GAL4/UAS system, the dUCH protein level was specifically recognized by the anti-dUCH antibody, and the antibody sensitivity showed different levels of the dUCH target protein in *Drosophila* tissues either in Western blot or in immunostaining (Figure 2). Success in producing dUCH antibody provides a good material for further experiments in the study role of UCH-L1 by *Drosophila* model.

2.3. *Drosophila* model for studying the UCH-L1 role in tissue development

Being a member of ubiquitin proteasome system (UPS), UCH-L1 is thought to be involved in many different processes in living organisms, such as cell proliferation and differentiation. In *Drosophila* model, tissue-specific knockdown of dUCH resulted in abnormal phenotype in adult flies. When dUCH was knocked down in posterior area of eye imaginal discs by the combination of GMR-Gal4 driver and UAS-duchIR cassette (GMR-Gal4 > UAS-duchIR), the *duch* knocked-down adult compound eye exhibited a rough eye phenotype, and ommatidium was bulged and stucked together, while the control fly showed a normal phenotype. Knockdown dUCH in the thorax by Pnr-Gal4 driver gave hair-deformed defection. The wing of the knocked-down dUCH flies also showed some extraordinary phenotype as the vein in the wing disappeared or deformed. When TH-Gal4 drives the synthesis of *duch* dsRNA

Figure 2. Generation of polyclonal anti-dUCH antibody for studying UCH-L1 function in *Drosophila melanogaster* model. (A) GAL4/UAS system is used for overexpressing dUCH protein in transgenic flies. Gal4 protein was expressed under GMR driving promoter in *Drosophila* posterior eye imaginal discs. Then, the expressed Gal4 bound to UAS element on the upstream of *duch* gene in transgenic *Drosophila* and caused the *duch* gene expression. (B) Western blot analysis of total protein from eye imaginal discs with polyclonal anti-dUCH antibody (above) and monoclonal anti-alpha tubulin antibody (below). GMR-Gal4: total protein from eye imaginal discs of transgenic fly, which showed dUCH endogenous protein. 12-4, 23-4, 50-5, 59: total protein from four different transgenic fly lines, which overexpresses dUCH protein under GMR-Gal4 driver. (C) Immunohistochemistry analysis on eye imaginal discs from the third instar *Drosophila melanogaster* larvae with polyclonal anti-dUCH antibody. GMR-Gal4: eye imaginal discs of transgenic fly, which showed dUCH endogenous protein. 23-4, 50-5: eye imaginal discs from two different transgenic fly lines, which overexpresses dUCH protein under GMR-Gal4 driver.

in *Drosophila* brain tissue, the third larval crawling ability was strongly defected (**Figure 3**). Emphatically, knockdown dUCH in whole bodies of the flies by Act5C-Gal4 resulted in pupal lethal effects. These observations strongly suggested that the dUCH plays an important role in maintaining normal *Drosophila* tissue development.

On the other hand, overexpression of dUCH in *Drosophila melanogaster* showed an apoptosis induction in eye imaginal discs and resulted in rough eye phenotype in adult flies. The apoptosis induction was vanished by co-expression of P35, a vacuolar viral protein that inhibits downstream effector caspases. The apoptosis induction is followed by compensatory proliferation (**Figure 4**) [31].

Furthermore, dUCH overexpression also caused the upset in distribution of photoreceptor clusters in fly pupal retina (**Figure 5**).

In *Drosophila* pupal retinae, the ommatidia were arranged precisely. Different cell types appeared in typical shape and position. However, overexpression of dUCH in pupal retinae increased apical mispatterning. In many regions of dUCH-overexpressing retinae, ommatidia showed defects in alignment and orientation. Cone cell clusters are in different sizes and distorted. In addition, the morphology of pigment cells was aberrant. Defects in the shape and the number of primary pigment cells were detected. The shape of secondary and tertiary pigment cells (interommatidial pigment cells) was altered. In addition to the morphological

GAL4 line	Expression of GAL4	Phenotype		
		Control	Knockdown of dUCH	
Act5C	All tissues	Normal	Pupal lethal	
GMR	Cells behind the morpho-genetic furrow of eye discs			Rough eye
Pnr	Dorsal wing discs			Disappeared/deformed some hairs in thorax
MS1096	Dorsal wing discs			Disappeared/deformed some veins in wings
TH	Dopaminergic cells			Locomotor dysfunction in the third larvae

Figure 3. Tissue-specific knockdown of dUCH resulted in defects in adult flies.

Figure 4. Overexpression of dUCH induces caspase-dependent apoptosis in eye imaginal discs. (a–e) Scanning electron micrographs of adult compound eyes. (a'–e') Immunostaining of the eye imaginal discs with anti-active caspase-3 antibody. (a,a') GMR-GAL4; (b,b') GMR-GAL4;UAS-dUCH/+; (c,c') GMR-GAL4;UAS-dUCH/+;UAS-P35/+; (d,d') GMR-GAL4;UAS-dUCH/+;UAS-LacZ/+; (e,e') GMR-GAL4; UAS-P35/+. Note the increased number of caspase-3 positive cells (brackets) behind the morphogenetic furrow of eye discs overexpressing dUCH (b') and the lack of signals detected in eye discs co-expressing both dUCH and P35 (c'). The arrow indicates the morphogenetic furrow (MF). The bars are for 50 μ m.

changes, the alignment of these cells was confused. In many regions, adjacent ommatidia were separated by more than one layer of interommatidial pigment cells. As a consequence, ommatidia in abnormal region did not maintain hexagonal shape. Bristles were misplaced, possibly due to the aberrance of pigment cells (**Figure 6**).

Interestingly, co-expressing dUCH with Sevenless or Draf in eye imaginal discs could suppress the rough eye phenotype induced by overexpressing dUCH. It is therefore likely that overexpression of dUCH downregulates the MAPK pathway, resulting in impairment of eye development (**Figure 7**) [31].

2.4. *Drosophila* model for studying the UCH-L1 role in Parkinson's disease

UCH-L1 was first linked to PD when mutation UCH-L1I93M was found in two siblings from a family with autosomal dominant PD [8]. Transgenic mice that overexpression of UCH-L1I93M showed an accumulation of α -synuclein with ubiquitin in the brain [3]. UCH-L1-deficient

Figure 5. Immunostaining of retinae at 42 h after puparium formation (APF) with anti-chaoptin antibody. (A) Control retina and (B) dUCH-overexpressing retina. The bars indicate 10 μm.

mice showed neuronal loss in the spinal gracile tract and exhibit early development sensory and progressive motor ataxia [7]. However, another mutation UCH-L1S18Y is dedicated that decreased risk in PD by antioxidant and neuron-protective function [32]. Therefore, the mechanism of UCH-L1 still remains unclear. In *Drosophila* model, specific knockdown dUCH in dopaminergic neuron caused a degeneration of DA neurons and resulted in locomotor dysfunctions (Figures 8 and 9).

2.5. Materials and methods

2.5.1. Fly stocks

Fly stocks were maintained at 25°C on standard food containing 0.7% agar, 5% glucose and 7% dry yeast. Wild-type strain Canton-S was obtained from the Bloomington *Drosophila* Stock Center (BDSC). RNAi lines carrying UAS-dUCH-IR fusion (GD#26468) for knockdown *Drosophila* ubiquitin carboxyl-terminal hydrolase (dUCH, CG4265) were received from the Vienna *Drosophila* Resource Center (VDRC). GAL4 drivers were used to perform the targeted knockdown of dUCH in various tissues of *D. melanogaster*: Act5C-GAL4 (BDSC#3954), GMR-GAL4 (line #16), MS1096-GAL4 (BDSC#8860), pnr-GAL4 (BDSC#3039) and TH-GAL4 (BDSC#8848).

2.5.2. Western immunoblot analysis

Wild-type and transgenic adult flies carrying GMR-GAL4 > UAS-dUCH were frozen in liquid nitrogen and homogenized in a solution containing 50 mM Tris-HCl (pH 7.5); 5 mM MgCl₂; 150 mM NaCl; 10% glycerol; 0.1% Triton X-100; 0.1% NP-40; 1 mM phenylmethylsulfonyl fluoride; 5 mM β-mercaptoethanol; 10 g/ml each of aprotinin, leupeptin and pepstatin A; and 1 g/ml each of antipain, chymostatin and phosphoramidon. Homogenates were centrifuged, and extracts (200 g of protein) were electrophoretically separated on SDS-polyacrylamide gels containing 10%

Figure 6. Overexpression of dUCH-induced apical mispatterning of 42 h APF retinas. (A) Normal adult *Drosophila* eye schematically representing orientation of the ommatidia with the green line representing the equator and schematically representing cross-sectional structure of a pupal ommatidium at the apical level with a, anterior cone cell; p, posterior cone cell; pl, polar cone cell; eq, equatorial cone cell. Red arrow marks equatorial-polar axis. (B-C) Immunostaining of retinas at 42 h APF with anti-Dlg antibody, (B) control retina and (C) dUCH-overexpressing retina. (D-E) Diagrams show orientation of the ommatidia in control fly (D) and dUCH-overexpressing fly (E). Black segments represent apical orientation of the ommatidia, black circles represent unclear cases and grey lines represent the anterior-posterior axis of the retinae. (F-G) The magnification of the ommatidia in control fly (F) and dUCH-overexpressing fly (G). Bars in all figures indicate 10 μ m.

acrylamide and then transferred to polyvinylidene difluoride membranes (Bio-Rad). The blotted membranes were blocked with TBS/0.05% Tween-20 containing 5% skim milk for 1 h at 25°C, followed by incubation with rabbit polyclonal anti-dUCH at 1:1000 dilution or mouse monoclonal anti- α tubulin (Developmental Studies Hybridoma Bank (DSHB)) at 1:5000 dilution for 16 h at 4°C. After washing, the membranes were incubated with HRP-conjugated secondary antibodies (GE Healthcare Bioscience) at 1:10,000 dilution for 1 h at 25°C. Detection was performed with ECL Western blotting detection reagents (GE Healthcare Bioscience), and images were analyzed with a Lumivision Pro HSII image analyzer (Aisin Seiki).

2.5.3. Immunostaining

Larval and adult brains were dissected in cold phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde at 25°C for 15 min. After washing with 0.3% PBS-T (PBS containing 0.3% Triton-X100) twice, the samples were blocked in blocking solution (0.15% PBS-T containing

Figure 7. Suppression of the dUCH-induced rough eye phenotype by co-expression of sev or DraF. (a) GMR-GAL4/+; (b) GMR-GAL4;UAS-d;CH/+; (c) GMR-GAL4;UAS-dUCH/+;hsp-DraF/+; (d) GMR-GAL4/hsp-sev;UAS-dUCH/+; (e) GMR-GAL4/+;UAS-LacZ/+. Magnifications are 200× for the upper and 700× for the lower panels. Flies were reared at 28°C. The bars indicate 50 μm.

10% normal goat serum) at 25°C for 20 min. Samples were then incubated with the following primary antibodies diluted in blocking solution: rabbit anti-*Drosophila* ubiquitin carboxyl-terminal hydrolase (anti-dUCH; 1:500) at 4°C for 16 h or rabbit anti-tyrosine hydroxylase (anti-TH; 1:250; Millipore, AB152) at 4°C for 20 h. After washing with 0.3% PBS-T, samples were incubated with secondary antibodies conjugated with Alexa 488 or FITC (1500, Invitrogen) at 25°C for 2 h and then washed and mounted in VECTASHILED Antifade Mounting Medium (Vector Laboratories, Japan). Finally, the samples were inspected by a confocal laser scanning microscope (Olympus FluoView FV10i or Olympus BX41 Microscope).

2.5.4. Crawling assay

Male larvae in the early third instar stage were collected randomly and washed with PBS to discard food traces. After that, larvae were transferred to agar plates containing 2% agar with a density of 2–4 larvae per plate. The movement of larvae was recorded by a digital camera for 60 s. The recorded videos were then converted into AVI type by MOV to AVI converter (Pazera Jacek, Poland) and then analyzed by ImageJ (NIH, USA) with wrMTrck plugin (developed by Dr. Jesper Søndergaard Pedersen) to track larval movement and draw motion paths.

2.5.5. Climbing assay

Newly eclosed adult male flies were collected and transferred to conical tubes which have heights of 15 cm and diameters of 2 cm. After that, the tubes were tapped to collect the flies to the bottom, and the length of time to record the movement of flies was 30 s. The procedures were repeated five times and recorded by a digital camera. For all of the climbing experiments, the height which each fly climbed to was scored as follows: 0 (less than 2 cm),

Figure 8. Loss of DL1 dopaminergic (DA) neurons in dUCH knockdown brain lobe. DA neuron clusters in the third instar larval were stained by anti-tyrosine hydroxylase antibody (anti-TH (green)). (A) Whole brain lobe with DA clusters in dUCH knockdown fly: TH-GAL4/UAS-dUCH-IR (TH > dUCH-IR). (A1) The magnification of DL1 DA cluster in knockdown fly brain lobe. (B) Whole brain lobe with DA clusters in control fly: TH-GAL4/+. (B1) The magnification of DL1 DA cluster in control fly brain lobe.

1 (between 2 and 4 cm), 2 (between 4 and 6 cm), 3 (between 6 and 8 cm), 4 (between 8 and 10 cm) and 5 (more than 10 cm). The climbing assay was performed every 5 days until all flies lose their locomotor abilities.

2.5.6. Conclusion and perspective

UCH-L1 was known as a complex and unclear function protein. It has several irrelevant activities as hydrolase and ligase, which are also related to ubiquitin. Previous reports showed that abnormal UCH-L1 functioning, caused by mutations or change in levels of protein expression. Those reports also implied that UCH-L1 could have many negative effects, with impacts on cell proliferation, cell cycling and cell death through activation of many genes [33, 34]. In this chapter, some data compatibly demonstrated that overexpression of dUCH, a homolog of human UCH-L1 in *Drosophila melanogaster*-induced apoptosis, interfered eye development by upset distribution of photoreceptor cell distribution and caused

Figure 9. The dysfunction in locomotor in dopaminergic neuron-specific dUCH knockdown flies. (A) Motion paths of larvae: control and dUCH knockdown larvae (TH > dUCH-IR). Knockdown larvae exhibit shorter and disorder crawling paths (upper panel) compared to control (below panel). (B) Climbing assay for measurement of adult fly locomotor ability. (C) Crawling velocity of control (TH) and knockdown larvae (TH > dUCH-IR). Knockdown larvae showed the reduction in crawling pace and parametric unpaired t test with Welch's correction, ****p < 0.0001; error bars present SD. (D) Climbing ability of control (TH) and dUCH knockdown adult flies (TH > dUCH-IR). Knockdown flies start to exhibit the decline in climbing ability at 5 days after eclosion, repeatedly measuring two way ANOVA with Bonferroni's post hoc test, **p < 0.01; error bars present SEM.

apical mispatterning in ommatidium. The effects of dUCH overexpression may involve in mitogen-activated protein kinase pathway. On the other hand, knockdown dUCH resulted in defect of tissue development and function. Particularly, knockdown dUCH in dopaminergic neuron impaired fly locomotion and degenerated dopaminergic neurons. Besides the *Drosophila* model's benefits, as well as the correlation between *Drosophila* UCH (dUCH) and human UCH (UCH-L1), these data strongly demonstrated that *Drosophila melanogaster* is an advantage model to investigate the functions and regulatory mechanism of UCH-L1 in living organism.

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