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REGULAR ARTICLE

Overexpression of ubiquitin carboxyl terminal hydrolase impairs multiple pathways during eye development in *Drosophila melanogaster*

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Abstract UCH-L1 (ubiquitin carboxyl terminal hydrolase L1) is well known as an enzyme that hydrolyzes polyubiquitin at its C-terminal to release ubiquitin monomers. Although the overexpression of UCH-L1 inhibits proteasome activity in cultured cells, its biological significance in living organisms has not been clarified in detail. Here, we utilized *Drosophila* as a model system to examine the effects of the overexpression of dUCH, a *Drosophila* homologue of UCH-L1, on development. Overexpression in the eye imaginal discs induced a rough eye phenotype in the adult, at least partly resulting from the induction of caspase-dependent apoptosis followed by compensatory proliferation. Genetic crosses with enhancer trap lines marking the photoreceptor cells also revealed that the overexpression of dUCH specifically impaired R7 photoreceptor cell differentiation with a reduction in activated extracellular-signal-regulated kinase signals. Furthermore, the dUCH-induced rough eye phenotype was rescued by co-expression of the *sevenless* gene or the *Draf* gene, a downstream component of the mitogen-activated protein kinase (MAPK) cascade. These results indicate that the overexpression of dUCH impairs R7 photoreceptor cell differentiation by down-regulating the MAPK pathway. Interestingly, this process appears to be independent of its pro-apoptotic function.

Keywords UCH-L1 · Parkinson disease · MAPK pathway · Apoptosis · Proliferation · Differentiation · Drosophila melanogaster (Insecta)

Introduction

Ubiquitin carboxyl terminal hydrolase L1 (UCH-L1; PARK5/PGP9.5), one of the most abundant proteins in the brain, comprises up to 2 % of the total brain protein (Wilkinson et al. 1989, 1992). Belonging to the group of de-ubiquitinating enzymes, UCH-L1 recognizes and hydrolyzes peptide bonds at the C-terminal glycines of ubiquityl ester-amides, releasing ubiquitin (Larsen et al. 1998). UCH-L1 is reported to be associated with neurodegenerative diseases such as Huntington, Alzheimer and Parkinson disease (Choi et al. 2004; Xu et al. 2009). In Parkinson disease (PD) patients, UCH-L1 is oxidatively damaged and is also found in Lewy bodies (Nishikawa et al. 2003; Lowe et al. 1990). A mutation at amino acid residue 93 of UCH-L1 causing a 50 % reduction in hydrolase activity is linked to a familial form of the disease (Leroy et al. 1998). In addition, the S18Y polymorphism in UCH-L1 might be associated with decreased susceptibility to sporadic PD in a dose-dependent manner (Maraganore et al. 1999). Thus, the variability of

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UCH-L1 therefore appears of particular importance for the development of late-onset idiopathic PD.

On the other hand, many studies have reported that several kinds of cancer might be caused by the deregulation of UCH-L1, such as pancreatic cancer (Tezel et al. 2000), non-small cell lung cancer (Hibi et al. 1999), colorectal cancer (Yamazaki et al. 2002), osteosarcoma (Liu et al. 2009) and oesophageal cancer (Takase et al. 2003). In 1999, Hibi et al. observed that the PGP9.5 transcript was highly expressed in primary lung cancers and lung cancer cell lines but was not detectable in the normal lung; therefore, PGP9.5 might serve as a potential marker for the detection of lung cancer (Hibi et al. 1999). Moreover, tumour progression, size and invasiveness are affected by the overexpression of UCH-L1 (Yamazaki et al. 2002). UCH-L1 has been reported to be overexpressed because of the hypomethylation of its promoter in gallbladder cancer and the enhancement of UCH-L1 activity correlates with metastasis (Lee et al. 2006). However, the silencing of UCH-L1 has been described in the progression of squamous cell carcinoma, in gastric cancer and in pancreatic cancer cell lines because of the promoter hypermethylation that has been observed (Mandelker et al. 2005; Sato and Meltzer 2006; Kumagai et al. 2009); therefore, UCH-L1 is either up- or down-regulated because of promoter hypo- or hypermethylation depending on the type of malignant tissue. Although previous studies have suggested a putative role for UCH-L1 in various tumour types, the exact oncogenic mechanism remains unclear.

UCH-L1, whose half-life is more than 48 h, is mainly degraded by macroautophagy (Kabuta et al. 2008) with additional turnover by the proteasome (Ardley et al. 2004). Since UCH-L1 binds to ubiquitin and stabilizes ubiquitin pools (Osaka et al. 2003) its overexpression might result in undegradable K63-linked polyubiquitin chains with resistance to proteasome activity (Liu et al. 2002). However, because the overexpression studies so far have been mainly performed with cultured mammalian cells, effects in vivo remain unclear. In the present study, we have therefore utilized *Drosophila melanogaster* as a model system to examine the consequences of the overexpression of dUCH, a *Drosophila* homologue of UCH-L1. In eye imaginal discs, it induces a rough eye phenotype in the adult with impaired R7 photoreceptor cell differentiation attributable to the down-regulation of the mitogen-activated protein kinase (MAPK) pathway, apparently independently of its pro-apoptotic functions.

Materials and methods

Fly stocks

Fly stocks were maintained at 25°C on standard food containing 0.7 % agar, 5 % glucose and 7 % dry yeast. Canton S was used as the wild-type. The transgenic fly line carrying glass minimal response element (GMR)-GAL4 on the X chromosome (strain number 16) was as described previously (Hirose et al. 2001). The P82 (*deadpan*-LacZ)/CyO and B38 (*klingon*-LacZ) lines were kindly provided by Dr. Y. Hiromi. A strain carrying UAS-dUCHIR was obtained from the Vienna *Drosophila* RNAi Center (VDRC, Vienna, Austria). All other stocks used in this study were from the Bloomington *Drosophila* Stock Center (Bloomington, USA) or VDRC.

Establishment of transgenic flies and production of rabbit polyclonal antibodies

The detailed methods to establish the transgenic flies and to produce rabbit anti-dUCH polyclonal antibodies will be published elsewhere. In brief, the full-length cDNA of dUCH was cloned into pUAST, a P-element vector, and then injected into embryos to obtain stable transformant lines carrying UAS-dUCH. P-element-mediated germ-line transformation was carried out as described previously (Spradling and Rubin 1982) and F1 transformants were selected on the basis of white-eye-colour rescue (Robertson et al. 1988). We obtained essentially the same results with four independent lines established for pUAST-dUCH.

The full-length dUCH cDNA was also cloned into a plasmid pET-28a (Novagene, USA) to carry a C-terminal His tag fusion sequence. After confirmation of the nucleotide sequence, a transformant of *Escherichia coli*BL21 (DE3) was used to produce dUCH-His fusion proteins, which were affinity-purified with a Ni-NTA column (GE Healthcare Bioscience, HCMC, Vietnam) and injected into rabbits. The obtained antiserum was checked by Western blotting and enzyme-linked immunosorbent assay.

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 $MgCl_2$, 150 mM NaCl, 10 % glycerol, 0.1 % Triton X-100, 0.1 % NP-40, 1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 10 μg/ml each of aprotinin, leupeptin and pepstatin A and 1 μg/ml each of antipain, chymostatin and phosphoramidon. Homogenates were centrifuged and extracts (200 μg protein) were electrophoretically separated on SDS-polyacrylamide gels containing 10 % acrylamide and then transferred to polyvinylidene-difluoride membranes (BioRad, HCMC, Vietnam). The blotted membranes were blocked with TRIS-buffered saline/0.05 % Tween 20 containing 5 % skim milk for 1 h at 25°C, followed by incubation with rabbit polyclonal anti-dUCH at a 1:1000 dilution or mouse monoclonal anti-α-tubulin (Developmental Studies Hybridoma Bank, DSHB, Iowa, USA) at a 1:5000 dilution for 16 h at 4°C. After

being washed, the membranes were incubated with horseradish-peroxidase-conjugated secondary antibodies (GE Healthcare Bioscience, HCMC, Vietnam) at a 1:10,000 dilution for 1 h at 25°C. Detection was performed with ECL Western blotting detection reagents (GE Healthcare Bioscience, HCMC, Vietnam) and images were quantified with a Lumivision Pro HS II image analyser (Aisin Seiki, Kariya, Japan).

Immunostaining

For immunohistochemistry, larval eye imaginal discs were dissected and fixed in 4 % paraformaldehyde/phosphate-buffered saline (PBS) for 15 min at 25°C. After being washed with PBS containing 0.3 % Triton X-100, the samples were blocked with PBS containing 0.15 % Triton X-100 and 10 % normal goat serum for 30 min at 25°C and incubated with primary antibodies diluted in PBS containing 0.15 % Triton X-100 and 10 % normal goat serum for 16 h at 4°C. The following antibodies were used: anti-dUCH (1:500), anti-active caspase3 (1:500; BD Biosciences, Tokyo, Japan), anti-phospho-histone H3 (PH3; 1:500; Cell Signaling, Tokyo, Japan), mouse monoclonal anti-β-galactosidase (1:500, DSHB), anti-diphospho-extracellular-signal-regulated kinase (dpERK; 1:200; Cell Signaling). After extensive washes with PBS containing 0.3 % Triton X-100, samples were incubated with secondary antibodies labelled with either Alexa 594 or Alexa 488 (1:400; Invitrogen, Tokyo, Japan) for 2 h at 25°C. Following further washes with PBS containing 0.3 % Triton X-100 and with PBS, samples were mounted in Vectashield Mounting Medium (Vector Laboratories, Tokyo, Japan). Preparations were examined under a fluorescence BX-50 microscope (Olympus, Tokyo, Japan) equipped with a cooled charge-coupled device camera (ORCA-ER, Hamamatsu Photonics, Tokyo, Japan). The immunofluorescent signal intensities in several imaginal discs (obtained from three independent immunostaining experiments) with each antibody were quantified by using Image J software. The obtained values were statistically analysed by Welch's *t*-test.

Scanning electron microscopy

Adult flies were anaesthetized, mounted on stages and observed under a scanning electron microscope, the VE-7800 (Keyence, Tokyo, Japan) in the low-vacuum mode.

Results

Overexpression of dUCH in eye imaginal discs induces rough eye phenotype in adults

In an attempt to find clues to the functions of dUCH in living flies, we utilized the GAL4/UAS targeted expression system to examine the effects of overexpression on *Drosophila* development (Brand and Perrimon 1993). Among the GAL4 driver lines examined (to be published elsewhere), the most prominent phenotype was observed with the GMR-GAL4 driver. Therefore, in the subsequent experiments, we focused on the eye system with GMR-GAL4.

In comparison with control fly eyes carrying GMR-GAL4 alone (Fig. 1a), the compound eyes of flies heterozygous for UAS-dUCH (GMR-GAL4; UAS-dUCH/+) showed a mild-rough and blistered eye phenotype (Fig. 1b), whereas flies homozygous for UAS-dUCH (GMR-GAL4; UAS-dUCH) showed a severe rough eye phenotype with the fusion of ommatidia (Fig. 1c) suggesting dose-dependent abnormality. The aberrant morphology induced by overexpression of dUCH was effectively rescued by knockdown of dUCH (Fig. 1d).

To investigate whether this rough eye phenotype was truly caused by the overexpression of dUCH, we immunostained eye imaginal discs of third instar larvae with anti-dUCH antibodies. The intensity of the dUCH signal was quantified by ImageJ64 software (Fig. 1f). The eye discs of the control GMR-GAL4 line showed low but ubiquitous signals of endogenous dUCH (Fig. 1a', f). Notably, in the eye discs of GMR-GAL4>UAS-dUCH flies, strong dUCH signals were detected in the region from the morphogenetic furrow (MF) to the posterior end (Fig. 1b'). The dUCH signal increased depending on the UAS-dUCH gene dose (Fig. 1b', c', f). As expected, the signal intensity was reduced in GMR-GAL4>UAS-dUCH, dUCHIR flies to the level of control flies with GMR-GAL4 alone (Fig. 1d', f). These results confirmed that the observed rough eye phenotype was closely associated with a real increase of dUCH levels in eye imaginal discs. The results were further confirmed by Western immunoblot analysis with protein extracts from the heads of GMR-GAL4 control flies and GMR-GAL4>UAS-dUCH lines. The blots were probed with anti-dUCH antibody and anti-α-tubulin antibody as a loading control. The predicted size of the dUCH protein from the amino acid composition is 24.9 kDa; a single band of about 25 kDa was detected with the anti-dUCH antibody in extracts from the control GMR-GAL4 flies (Fig. 1e). This band was increased 3.3-fold (quantified with *LumiVision IMAGER*) in extracts from GMR-GAL4>UAS-dUCH flies (Fig. 1e).

Overexpression of dUCH induces caspase3-dependent cell death in eye imaginal discs

To focus on the mechanism, we first examined whether apoptosis was induced by the overexpression of dUCH in eye imaginal discs, since it has sometimes been seen with a rough eye phenotype (Wolff et al. 1991; Hirose et al. 2001).

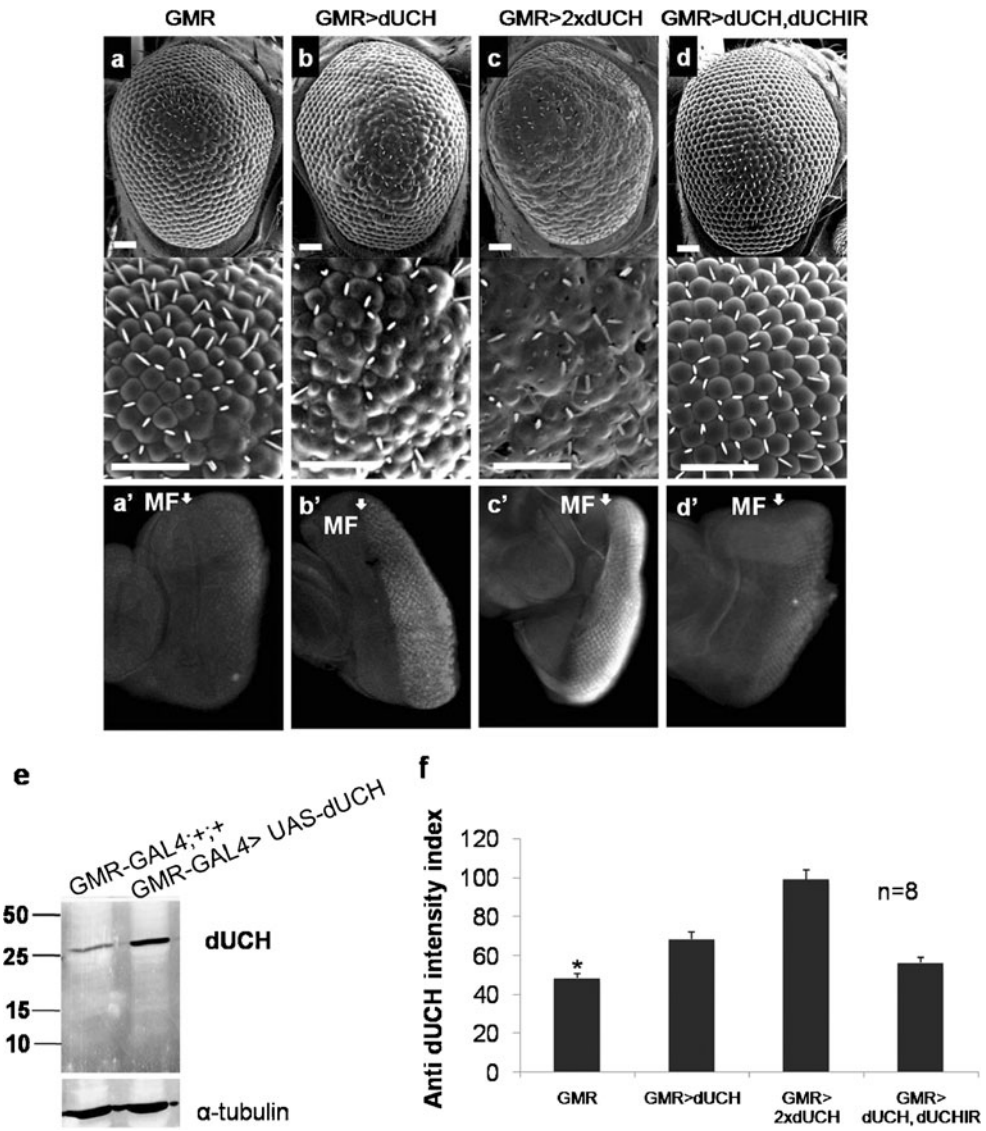

Fig. 1 Overexpression of dUCH in eye imaginal discs induces a rough eye phenotype in adults. **a-d** Scanning electron micrographs of adult compound eyes. Magnification ×200 (*top*), ×700 (*bottom*). **a'-d'** Immunostaining of eye imaginal discs with anti-dUCH antibodies. **a**, **a'** GMR-GAL4, **b**, **b'** GMR-GAL4;UAS-dUCH/+, **c**, **c'** GMR-GAL4; UAS-dUCH, **d**, **d'** GMR-GAL4;UAS-dUCH/+;UAS-dUCH IR/+ (*arrows*/*MF* morphogenetic furrow). *Bars* 50 μm. **e** Specificity of the anti-dUCH antibodies. Western immunoblot of *Drosophila* head protein extracts from GMR-GAL4 (*left lane*) and GMR-GAL4;UAS-dUCH (*right lane*) flies. The blots were probed with anti-dUCH and anti-α tubulin antibodies. The flies were reared at 28°C. **f** Quantification of dUCH intensity in eye imaginal discs. Mean intensities with standard deviation from eight discs are shown; **P*-value less than 0.005 by Welch's *t*-test

Immunostaining of eye imaginal discs with anti-active caspase3 antibody revealed that the overexpression of dUCH induced an 4.1-fold increase of apoptotic cells in the posterior part of the eye discs, especially in the region behind the MF (Fig. 2b'), as compared with the control line showing few active caspase3-positive cells (Fig. 2a'). The quantified data for caspase3-positive cells further confirmed that the results were statistically significant (Fig. S1).

To explore further the involvement of caspase-dependent cell death in flies overexpressing dUCH, we investigated whether P35, a vacuolar viral protein that inhibits downstream effecter caspases (Bruce et al. 1994), could block cell death induced by the overexpression of dUCH. The transgenic flies carrying UAS-P35 have been used in a number of studies to inhibit caspase-dependent apoptosis (Bruce et al. 1994; Yamaguchi et al. 1999; Hirose et al. 2001; Umehara et al. 2010). Co-expression of P35 with dUCH strongly suppressed the rough eye phenotype (Fig. 2c) and cell death signals were no longer detected in the posterior region of eye imaginal discs of co-expressing flies (Fig. 2c'). However, suppression of the rough eye phenotype was not observed on co-expressing dUCH and LacZ flies (Fig. 2d) and ectopic induction of apoptosis was still observed (Fig. 2d'). Ectopic expression of P35 alone exerted no effect on the compound eye morphology (Fig. 2e). Taken together, these results indicate that dUCH overexpression can induce caspase-dependent apoptosis that can be blocked by P35 expression.

To counteract the induced apoptosis, compensatory proliferation might be activated in eye imaginal discs of dUCH-overexpressing flies

In the third larval stage, the MF sweeps from posterior to anterior in the eye imaginal disc. Ahead of the MF, cells proliferate asynchronously: at the MF, cells become synchronized in mitosis during the first mitotic wave and are

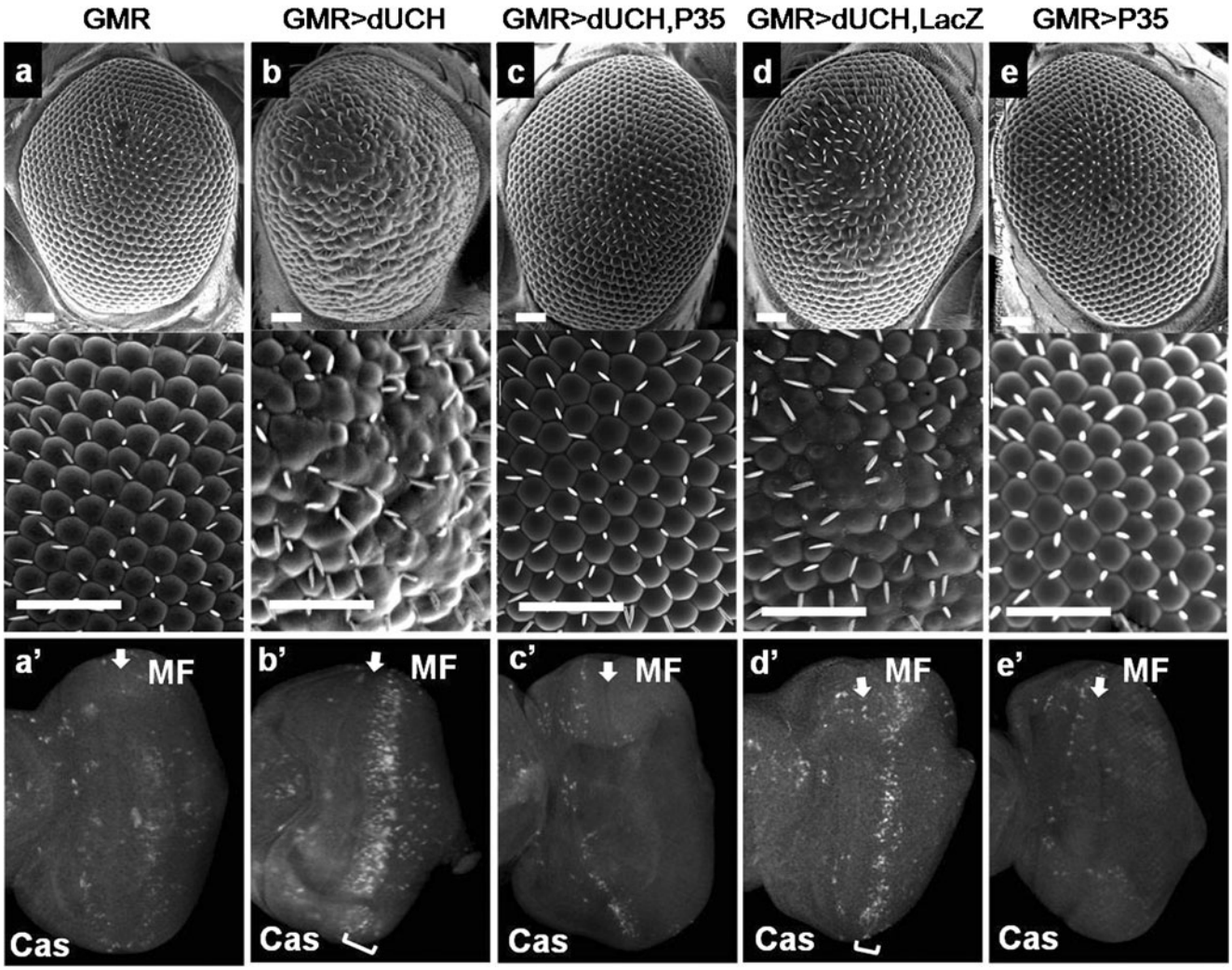

Fig. 2 Overexpression of dUCH induces caspase-dependent apoptosis in eye imaginal discs. **a-e** Scanning electron micrographs of adult compound eyes. **a'-e'** Immunostaining of the eye imaginal discs with anti-active caspase3 antibody. **a**, **a'** GMR-GAL4, **b**, **b'** GMR-GAL4; UAS-dUCH/+, **c**, **c'** GMR-GAL4;UAS-dUCH/+;UAS-P35/+, **d**, **d'** GMR-GAL4;UAS-dUCH/+;UAS-LacZ/+, **e**, **e'** GMR-GAL4;UAS-P35/+ (*arrows* morphogenetic furrow). Note the increased number of caspase3-positive cells (*brackets*) behind the morphogenetic furrow (*MF*) of eye discs overexpressing dUCH (**b'**) and the lack of signals detected in eye discs co-expressing both dUCH and P35 (**c'**). *Bars* 50 μm

then arrested in G1 in a non-proliferating region; behind the MF, cells either directly differentiate into photoreceptors or enter a final synchronous S phase, which is followed by a second mitotic wave and subsequent differentiation (Gonczy and Budirahardja 2009).

During development, achieving proper organ size requires a balance between proliferation and cell death. In eye discs of dUCH-overexpressing flies, even though a high level of apoptotic signal was detected behind the MF, sizes of the adult compound eyes were not significantly reduced (Figs. 1, 2), suggesting that after apoptosis compensatory proliferation was induced.

To elucidate this possibility, we immunostained the eye discs with anti-PH3 antibody to detect mitotic cells. As predicted, we found that the overexpression of dUCH caused an increase of PH3-positive cells in the region posterior to the synchronized M phase zone behind the MF (Fig. 3b) compared with that of the control line (Fig. 3a). Ectopic mitotic signals were reduced by co-expressing P35 (Fig. 3d). Quantification of the number of PH3-positive cells in the region posterior to the MF further confirmed that the results were statistically significant (Fig. 3e). Our findings, taken together, suggest that the overexpression of dUCH induces apoptosis, which is followed by compensatory proliferation.

Overexpression of dUCH interferes with R7 photoreceptor cell differentiation by down-regulating the MAPK pathway

We next examined photoreceptor cell differentiation in eye imaginal discs of dUCH-overexpressing flies. Photoreceptor

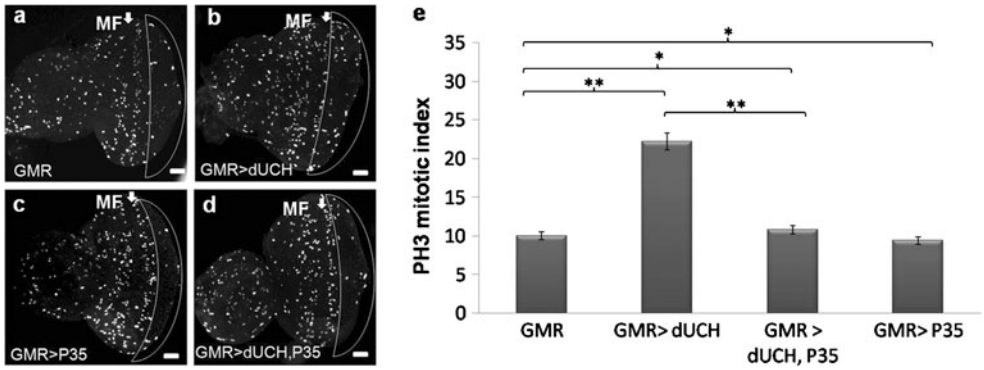

Fig. 3 Overexpression of dUCH induces ectopic mitosis in the eye imaginal disc depending on apoptotic function. Immunostaining of eye imaginal discs with anti-PH3 antibodies. **a** GMR-GAL4;+;+, **b** GMR-GAL4;UAS-dUCH/+;+, **c** GMR-GAL4;+;UAS-P35/+, **d** GMR-GAL4;UAS-dUCH/+;UAS-P35/+. Overexpression of dUCH increased ectopic mitotic signals in the posterior region (**b**), whereas co-expression of dUCH and P35 reduced aberrant mitotic cells (**d**). Regions with ectopic PH3-positive cells are marked with *crescents* (*arrows*/*MF* morphogenetic furrow). *Bars* 200 μm. **e** Quantification of the delayed PH3 mitotic index in the eye discs from GMR, GMR>dUCH, GMR>dUCH,P35 and GMR>P35 flies; n=5 discs per genotype. *P>0.5, **P<0.001. PH3 signals in the *crescent* region (in **a**–**d**) behind the synchronized M-phase stripe were quantified

cells have been found to be generated sequentially: R8 is generated first, with movement posterior from the MF, and then cells are added pairwise (R2 and R5, R3 and R4, R1 and R6), with R7 being the last photoreceptor to be added to the ommatidial cluster (Wolff and Ready 1993). Several enhancer trap lines have been developed to mark photoreceptor cells. In this study, we utilized enhancer trap lines P82 (featuring an insertion in *deadpan*; Kramer et al. 1995) and B38 (with an insertion in *klingon*; Butler et al. 1997), which specifically express the β-galactosidase marker in photoreceptor cells of early R3/R4/R7 and R7, respectively. After the mating of each enhancer trap line with transgenic flies overexpressing dUCH, eye imaginal discs of F1 larvae were immunostained with anti-β-galantibodies. With P82, the ommatidia of GMR-GAL4>UAS-dUCH flies were found to contain R3 and R4 signals, but no R7 signals (Fig. 4b). Loss of R7 signals in dUCH-overexpressing flies was also confirmed with R7-specific enhancer trap line B38 (Fig. 4d), although R7 signals of control lines were clearly detectable (Fig. 4a, c). Despite some background signals being detectable in other photoreceptor cells, the strongest signal was observed with the R7 cell in each ommatidium. Thus, we concluded that the overexpression of dUCH specifically impaired R7 cell differentiation.

The MAPK or ERK pathway is at the heart of signalling networks that govern proliferation, differentiation and cell survival (Kolch 2000). In *Drosophila* eye imaginal discs, the proper differentiation of the R7 cell depends on local activation of the Sevenless receptor tyrosine kinase followed by sequential activation of the MAPK signalling cascade. Genetic screens have identified many essential components of the MAPK signalling cascade involved in R7 cell-fate specification such as Drk, Ras1 and Draf (Simon et al. 1991; Dickson et al. 1996; Karim et al. 1996; Wolff and Ready 1993). Therefore, overexpression of dUCH probably down-regulates the MAPK

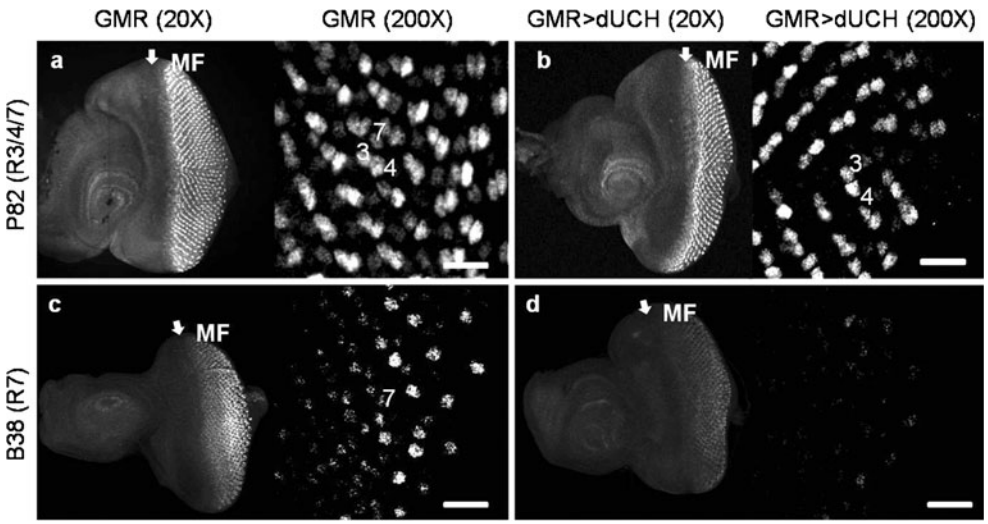

Fig. 4 Overexpression dUCH impairs R7 cell differentiation. Confocal images of eye imaginal discs stained with anti-β-gal antibodies. **a** GMR-GAL4;P82/+;+, **b** GMR-GAL4;P82/UAS-dUCH;+, **c** GMR-GAL4;+;B38/+, **d** GMR-GAL4;UAS-dUCH/+;B38/+ (*numbers* R3, R4 and R7 photoreceptor cells, *arrows*/*MF* morphogenetic furrow). Magnification ×20 (*left*), ×200 (*right*). *Bars* 50 μm

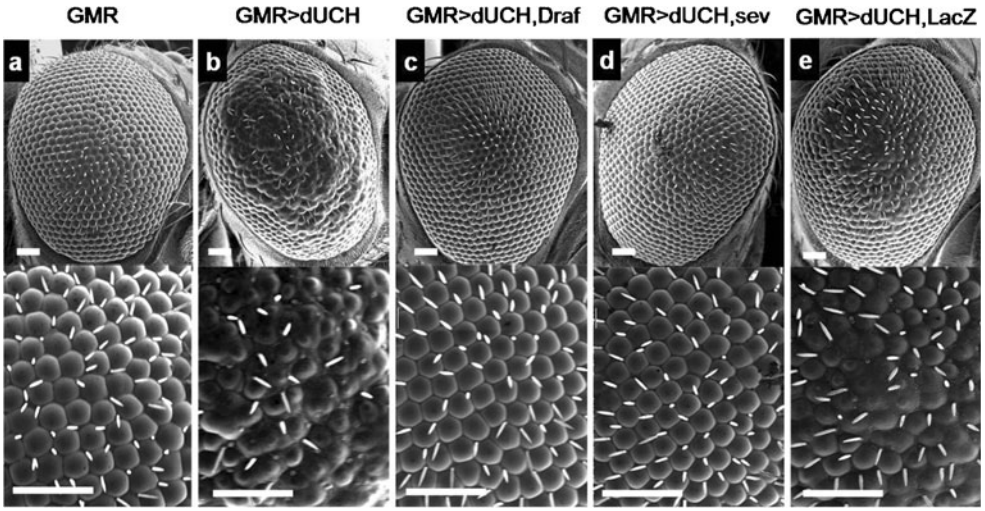

Fig. 5 Suppression of the dUCH-induced rough eye phenotype by co-expression of *sev* or *Draf*. **a** GMR-GAL4;+, **b** GMR-GAL4;UAS-dUCH/+, **c** GMR-GAL4;UAS-dUCH/+; *hsp-Draf*/+, **d** GMR-GAL4/*hsp-sev*;UAS-dUCH/+, **e** GMR-GAL4;+;UAS-*LacZ*/+. Flies were reared at 28°C. Magnifications ×200 (*top*), ×700e (*bottom*). *Bars* 50 μm

pathway, resulting in impairment of R7 cell differentiation. Consistent with this, co-expression with either Sevenless or Draf was able to suppress the rough eye phenotype induced by overexpressing dUCH (Fig. 5b–d), whereas co-expression with LacZ exerted no effect (Fig. 5e). Both *hsp-Draf* and *hsp-sevenless* lines have been successfully used to rescue the defect in the ERK pathway (Yoshioka et al. 2011). This was further confirmed by immunostaining of eye imaginal discs with anti-dpERK antibodies to monitor ERK activation (Fig. 6). Overexpression of dUCH extensively decreased dpERK signals (6.5-fold) in the eye discs (Fig. 6b, f), whereas co-expression of Sevenless or Draf brought about recovery to the level in control lines (Fig. 6a, c, d, f). Taking the results together, we concluded that overexpression of dUCH interfered with R7 photoreceptor cell differentiation by down-regulating the MAPK pathway.

dUCH causes cell differentiation defects independently of its apoptotic function

Cell proliferation, differentiation and death are fundamental processes in multicellular organisms. In the experiments described above, we showed that the overexpression of dUCH interfered with these processes during development of the *Drosophila* compound eye. Whereas proliferation was a response to apoptotic function, the relationship between apoptosis and differentiation caused by overexpressing dUCH was unclear. We therefore designed other crosses to explore this question. First, we co-expressed P35 to inhibit apoptosis in dUCH-overexpressing flies and then the eye imaginal discs were stained with anti-dpERK antibodies. Although flies co-expressing dUCH and P35 showed an apparently normal eye morphology (Fig. 2c), signals of dpERK were still down-regulated in the eye discs (Fig. 7b) and ectopic expression of P35 alone did not affect the dpERK signals (Fig. 6c). Quantitative analyses further confirmed these results (Fig. S2).

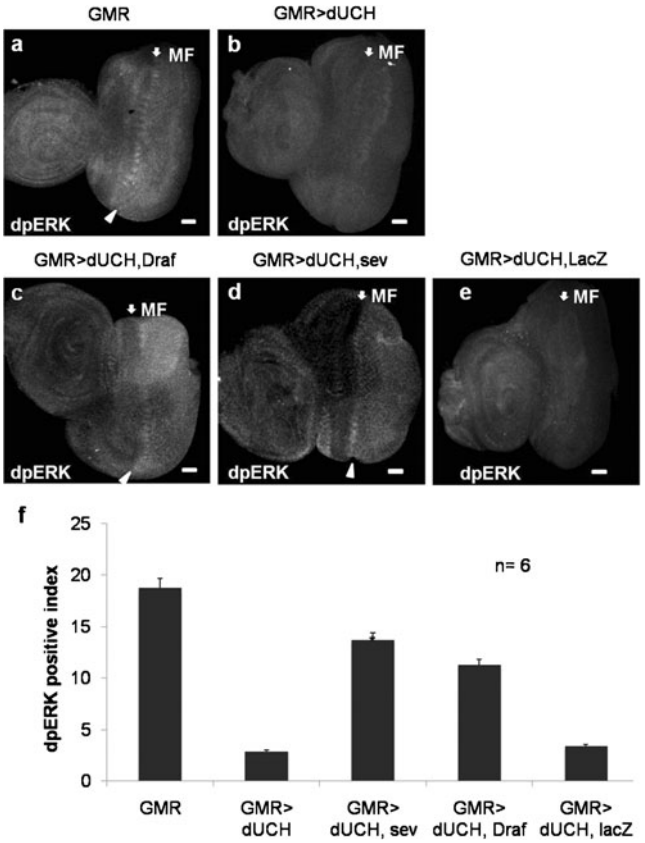

Fig. 6 Overexpression of dUCH reduces dpERK signals. **a** GMR-GAL4, **b** GMR-GAL4;UAS-dUCH/+, **c** GMR-GAL4;UAS-dUCH/+; *hsp-Draf*/+, **d** GMR-GAL4/*hsp-sev*;UAS-dUCH/+;+, **e** GMR-GAL4; UAS-dUCH/+;UAS-LacZ/+ (*arrowhead* dpERK-positive cells, *arrows*/*MF* morphogenetic furrow). The dpERK signals (*arrowheads*) detected in a control eye disc (**a**) were diminished in an overexpressing disc (**b**). However, signals were rescued by co-expressing dUCH with Draf (**c**) or Sevenless (**d**). *Bars* 200 μm. **f** dpERK-positive index in the eye discs from GMR, GMR>dUCH, GMR>dUCH,sev, GMR>dUCH, Draft, and GMR>dUCH,lacZ flies; n=6 discs per genotype, P<0.03

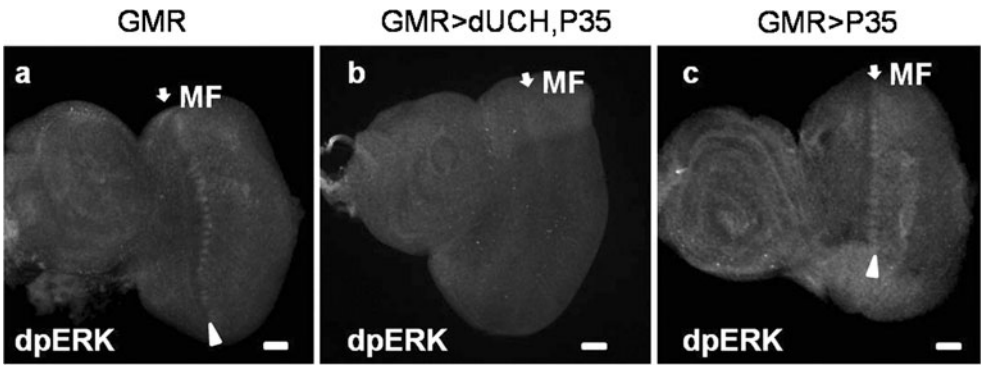

Fig. 7 Reduction of dpERK signals by dUCH overexpression was not rescued by co-expression of P35. Immunostaining of eye discs with anti-dpERK. **a** GMR-GAL4, **b** GMR-GAL4;UAS-dUCH/+;UAS-P35/+, **c** GMR-GAL4;+;UAS-P35/+ (*arrowhead* dpERK-positive cells, *arrow*/*MF* morphogenetic furrow). *Bars* 200 μm

In a parallel set of experiments, we examined apoptotic cells in the eye discs of flies co-expresssing dUCH with Sevenless or Draf. On immunostaining with anti-active caspase3 antibodies (Fig. 8), increased signals were still detected in the posterior region to MF in eye discs of the co-expressing flies (Fig. 8e, f, i), as observed in flies overexpressing dUCH alone (Fig. 8d) or co-overexpressing dUCH with LacZ (Fig. 8h), whereas no such signals were observed in control lines carrying GMR-GAL4 alone (Fig. 8a) or co-overexpressing dUCH with P35 (Fig. 8g) or in fly lines overexpressing Sevenless alone or Draf alone (Fig. 8b, c). In summary, these data revealed that overexpression of dUCH was able specifically to interfere with the differentiation of R7 photoreceptor cells in developing *Drosophila* eyes, independently of the pro-apoptotic function of the enzyme.

Discussion

Idiopathic PD is the second most common neurodegenerative disease and the most frequent that affects the subcortex. Overexpression of the de-ubiquitinating enzyme UCH-L1 in cultured mammalian cells leads to inclusion formation in response to proteasome impairment (Liu et al. 2002). In the present study, to explore further the biological impact of

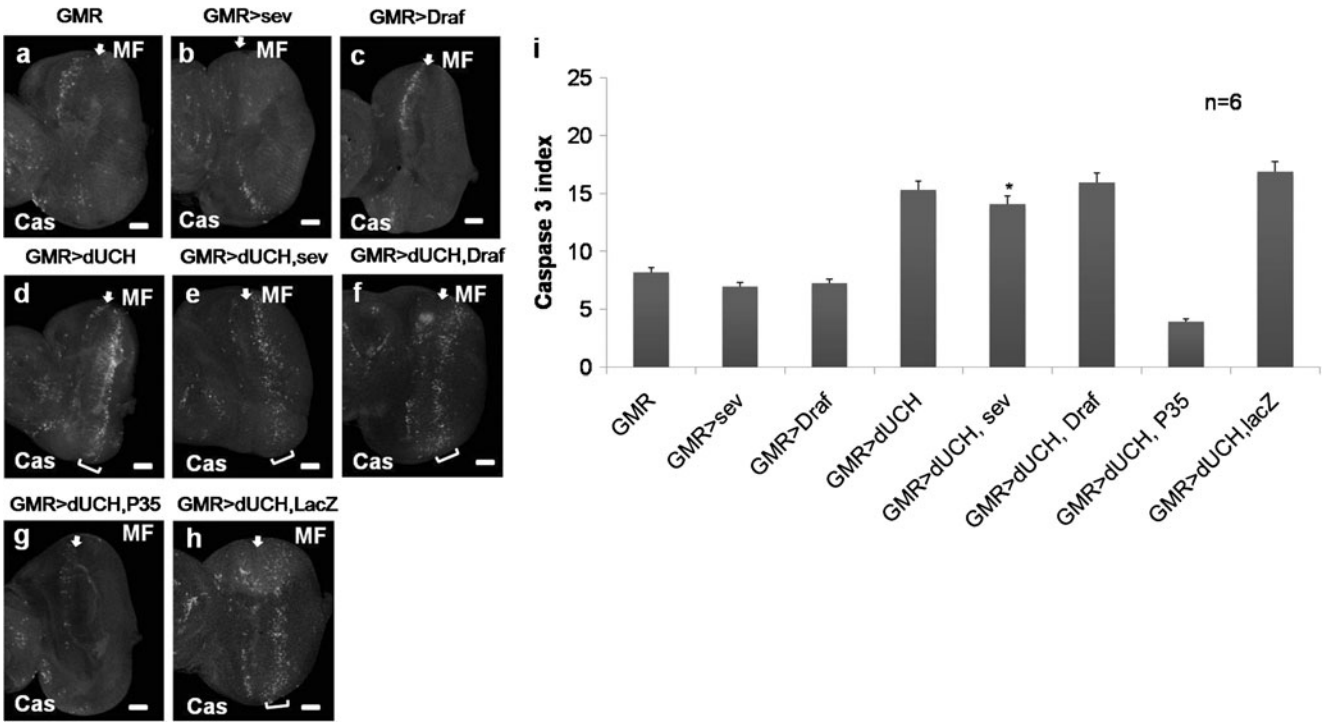

Fig. 8 Ectopicapoptosis induced by dUCH-overexpression was not rescued by co-expression of *sev* or *Draf*. **a** GMR-GAL4, **b** GMR-GAL4/*hsp-sev*, **c** GMR-GAL4;*hsp-Draf*, **d** GMR-GAL4; UAS-dUCH/+, **e** GMR-GAL4/*hsp-sev*;UAS-dUCH/+, **f** GMR-GAL4;UAS-dUCH/+;*hsp-Draf*/+, **g** GMR-GAL4;UAS-dUCH/+;UAS-P35/+, **h** GMR-GAL4;UAS-dUCH/+;UAS-LacZ/+. Flies co-overexpressing dUCH with Senvenless (**e**) or Draf (**f**) still showed apoptotic signals despite the rescue of the dpERK signal (*brackets* caspase3-positive cells, *arrow*/*MF* morphogenetic furrow). *Bars* 200 μm. **i** Caspase3 index in the eye discs from GMR, GMR>sev, GMR>Draf, GMR>dUCH, GMR>dUCH,sev, GMR>dUCH,Draft, GMR>dUCH, P35 and GMR>dUCH,lacZ flies; n=6 discs per genotype, P<0.02

overexpression of UCH-L1 in living organisms, we have utilized the *Drosophila* system. Overexpression of dUCH, a *Drosophila* homologue of human UCH-L1 in living flies, has revealed that the enzyme interferes with various biological processes, such as cell cycling, apoptosis and photoreceptor cell differentiation during the development of the *Drosophila* compound eye.

In multicellular organisms, proteins are sensitive to damage and, when they become misfolded under stressed conditions, they may undergo refolding through chaperon pathways or be ubiquitinated for degradation via the proteosome pathway (Neefjes et al. 2004). As observed with UCH-L1 in mammalian cells (Liu et al. 2002), overexpression might result in the loss of normal functions, leading to an impairment of proteasome actions and consequently might affect multiple pathways during eye development (Neefjes et al. 2004).

In the present study, we have detected increased apoptotic signals in the eye discs of dUCH-overexpressing flies, suggesting a close link with apoptosis in vivo, in agreement with findings from a nasopharyngeal carcinoma cell model in which UCH-L1 forms a complex with p53/p14^{ARF}/MDM2 and activates p53 signalling (Li et al. 2010). Although we have examined the p53 level in eye discs from flies overexpressing dUCH, no significant increase has been observed (data not shown). Therefore, the activation of the p53 pathway by dUCH might not occur in *Drosophila*. Genetic screening to identify mutations that can modify the dUCH-induced rough eye phenotype might give us clues to clarify the link between dUCH and apoptosis. The dUCH-overexpressing flies established in the present study should be useful for such approaches in future.

Our analyses have shown that the dUCH-induced apoptosis is followed by compensatory proliferation to keep the compound eye a constant size. Such a process is commonly observed at various *Drosophila* sites, including the wing (Huh et al. 2004). Although the induction of apoptosis appears to be a primary effect of dUCH overexpression in the eye disc, dUCH might also participate in the activation of compensatory proliferation.

We have additionally demonstrated that the overexpression of dUCH specifically impairs R7 photoreceptor cell differentiation, which is regulated by Sevenless and the ERK signalling pathway in *Drosophila* (Nagaraj and Banerjee 2004). Overexpression of dUCH completely diminishes the activated ERK signals during the eye development and co-expression of Sevenless or Draf restores the ERK signals and consequently the eye morphology. Notably, human UCH-L1 has been reported to regulate tumour-necrosis-factor-α mediated vascular smooth muscle proliferation negatively via the suppression of ERK activity (Ichikawa et al. 2010). As is well documented, the MAPK/ERK pathway plays an important role in signalling networks that govern proliferation, differentiation and cell survival depending on the cell and tissue context (Kolch 2000).

In conclusion, dUCH can participate in multiple biological pathways such as apoptosis, proliferation and R7 cell differentiation during eye development. *Drosophila* has the practical advantages of being small and highly fertile and of having a short generation time. These characteristics allow large-scale genetic screening and high-throughput screening of candidate drugs for therapy; such screening can be performed in a relatively short period of time and at low cost (Leyssen and Hassan 2007; Bier 2005). In addition to PD, human UCH-L1 is also related to other neurodegenerative diseases, including Alzheimer's disease (Choi et al. 2004) and Huntington's disease (Xu et al. 2009). Recent findings also indicate complex roles of UCH-L1 in tumourigenesis from tumour-suppressive to oncogenic, depending on the tumour type (Tezel et al. 2000; Takase et al. 2003; Yu et al. 2008; Seliger et al. 2009; Lee et al. 2006). Thus, the dUCH-overexpressing flies established in this study might also find application for the identification of chemicals and natural substances that are able to modify dUCH activity. This might be the first step to finding candidate drugs for the therapy of various human diseases in which UCH-L1 is involved.

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