LETTERS

# HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS

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**A prominent feature of late-onset neurodegenerative diseases is accumulation of misfolded protein in vulnerable neurons[1]. When levels of misfolded protein overwhelm degradative pathways, the result is cellular toxicity and neurodegeneration[2]. Cellular mechanisms for degrading misfolded protein include the ubiquitin-proteasome system (UPS), the main non-lysosomal degradative pathway for ubiquitinated proteins, and autophagy, a lysosome-mediated degradative pathway[3]. The UPS and autophagy have long been viewed as complementary degradation systems with no point of intersection[4,5]. This view has been challenged by two observations suggesting an apparent interaction: impairment of the UPS induces autophagy *in vitro*, and conditional knockout of autophagy in the mouse brain leads to neurodegeneration with ubiquitin-positive pathology[6–9]. It is not known whether autophagy is strictly a parallel degradation system, or whether it is a compensatory degradation system when the UPS is impaired; furthermore, if there is a compensatory interaction between these systems, the molecular link is not known. Here we show that autophagy acts as a compensatory degradation system when the UPS is impaired in *Drosophila melanogaster*, and that histone deacetylase 6 (HDAC6), a microtubule-associated deacetylase that interacts with polyubiquitinated proteins[10], is an essential mechanistic link in this compensatory interaction. We found that compensatory autophagy was induced in response to mutations affecting the proteasome and in response to UPS impairment in a fly model of the neurodegenerative disease spinobulbar muscular atrophy. Autophagy compensated for impaired UPS function in an HDAC6-dependent manner. Furthermore, expression of HDAC6 was sufficient to rescue degeneration associated with UPS dysfunction *in vivo* in an autophagy-dependent manner. This study suggests that impairment of autophagy (for example, associated with ageing or genetic variation) might predispose to neurodegeneration. Morover, these findings suggest that it may be possible to intervene in neurodegeneration by augmenting HDAC6 to enhance autophagy.**

DTS7 is a temperature sensitive, dominant negative mutant of the β2 subunit of the proteasome[11]. Using the UAS/GAL4 system[12], we targeted DTS7 expression to the *Drosophila* eye to cause tissue-restricted proteasome impairment. At 22 °C, proteasome function is intact and eye morphology was normal (Fig. 1a). However, at 28 °C substantial degeneration of the retina occurred owing to proteasome impairment (Fig. 1b). To investigate the role of HDAC6 in the setting of misfolded protein stress, we generated transgenic flies expressing wild-type *Drosophila* HDAC6 as well as wild-type and mutant versions of human HDAC6. Expression of either *Drosophila* HDAC6 or human HDAC6 strongly suppressed the degenerative phenotype associated with proteasome impairment (Fig. 1c, d). However, expression of a catalytically dead mutant of human HDAC6 (H216A;H611A) failed to modify the degenerative phenotype, indicating that the deacetylase function of HDAC6 is required for suppression (Fig. 1e). To assess the role of endogenous HDAC6, we used RNAi knockdown (Supplementary Fig. S3). Targeted knockdown of *Drosophila* HDAC6 did not noticeably alter eye morphology on its own (Supplementary Fig. S4), but strongly enhanced degeneration when the proteasome was impaired (Supplementary Fig. S5). HDAC6 did not modify the rough eye phenotype caused by ectopic expression of the positive regulator of cell death *reaper*, indicating that HDAC6 is not a general suppressor of cell death pathways (Supplementary Fig. S6). Ectopic expression of *Drosophila* HDAC3 and *Drosophila* HDAC11 did not suppress degeneration caused by proteasome impairment, indicating that this is not a general response of HDACs (not shown).

Impaired UPS function has been implicated in a broad array of neurodegenerative disorders, but *in vivo* evidence is lacking[13]. Spinobulbar muscular atrophy (SBMA) is an inherited neurodegenerative disease that is caused by polyglutamine (polyQ) repeat expansion in the androgen receptor (AR)[14]. Like most adult-onset neurodegenerative diseases, SBMA pathology features accumulation of ubiquitin-positive protein aggregates in vulnerable neurons[15]. To develop a *Drosophila* model of SBMA, we generated transgenic flies expressing full-length human AR with 12–121 glutamine repeats using the UAS/GAL4 system. Flies expressing polyQ-expanded AR recapitulate key features of human SBMA, including ligand-dependent, polyQ length-dependent degeneration (Fig. 1f, g and Supplementary Fig. S7), as previously reported[16].

To evaluate UPS function in this fly model of SBMA, we generated transgenic flies expressing a fluorescent reporter of UPS function. CL1–GFP is a fusion protein created by introducing a degradation signal to otherwise stable green fluorescent protein (GFP)[17]. This protein is rapidly degraded by the UPS, and its steady state levels reflect the functional status of this pathway[18]. When stable GFP was expressed in eye imaginal discs from third-instar larvae, a robust fluorescent signal was detected by confocal microscopy (Fig. 1k). In contrast, eye imaginal discs from control flies expressing the CL1–GFP reporter emitted a low fluorescent signal, reflecting an active UPS (Fig. 1l). To test the ability of the UPS reporter flies to

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detect proteasome impairment *in vivo*, we co-expressed CL1–GFP in the eye with DTS7. At 22 °C, CL1–GFP reporter levels remained low in eye imaginal discs co-expressing DTS7, consistent with normal proteasome function (Fig. 1m). In contrast, at 28 °C, there was a significant increase in the CL1–GFP signal, demonstrating the ability of the reporter to detect proteasome impairment associated with a degenerative phenotype *in vivo* (Fig. 1n and Supplementary Fig. S8). UPS reporter RNA levels were not altered by the conditions used in our experiments (Supplementary Fig. S9).

We next expressed the CL1–GFP reporter in SBMA flies. In AR121 flies not exposed to ligand, fluorescent signal from the UPS reporter remained low, indicating that proteasome function was normal despite high expression of polyQ-expanded AR (Fig. 1o). However, flies reared on dihydrotestosterone (DHT), the natural ligand of AR, exhibited a significant increase in reporter signal, indicating proteasome impairment in association with induction of toxicity (Fig. 1p and Supplementary Fig. S8). The ligand-dependent nature of this finding indicates that UPS impairment is not merely a consequence of overexpressed AR121. Proteasome impairment by AR expression is a polyQ length-dependent phenomenon, because no impairment was observed in flies expressing AR12 (Supplementary Fig. S8). The finding of proteasome impairment in SBMA flies is consistent with a prior report that polyQ toxicity *in vivo* is enhanced by proteasome mutations[19].

The determination that there is impairment of the UPS in SBMA flies led us to examine the ability of HDAC6 to modify the degenerative phenotype in this model of human neurodegenerative disease. Consistent with the results using proteasome mutant flies, ectopic expression of either *Drosophila* or human HDAC6 suppressed the ligand-dependent degenerative phenotype in flies expressing polyQ-expanded AR (Fig. 1h, i). Expression of the catalytically dead mutant of human HDAC6 (H216A;H611A) failed to modify the degenerative phenotype, indicating that the deacetylase function of HDAC6 is also required for suppression of polyQ toxicity (Fig. 1j). Knockdown of endogenous HDAC6 with RNAi enhanced ligand-dependent degeneration in AR52 flies (Supplementary Fig. S5). Thus, endogenous HDAC6 also plays a role in protecting cells from polyQ toxicity.

We previously reported induction of autophagy and sequestration of polyQ-expanded AR in autophagic vacuoles *in vitro*[20]. Induction of autophagy *in vitro* in response to proteasome impairment has also been described[6,7]. To determine whether autophagy is induced *in vivo* when the UPS is impaired, we performed ultrastructural evaluation by transmission electron microscopy (TEM) in the DTS7 and SBMA flies. In both cases, we found a significant increase in morphological features of autophagy (Fig. 2f). These included autophagic vacuoles such as early autophagosomes in which membranes surrounded cytoplasmic components (Fig. 2a, b), more mature autophagic vacuoles (Fig. 2c), multilamellar bodies (MLBs, Fig. 2d) and multivesicular bodies (MVBs, Fig. 2e).

To assess the role of autophagy when the UPS is impaired, we inhibited autophagy by RNAi knockdown of the autophagy genes *atg6* and *atg12*. Knockdown of either *atg6* or *atg12* did not affect eye morphology (Supplementary Fig. S10), indicating that the *Drosophila* eye can tolerate reduced autophagy when UPS function is intact, at least in 1-day-old flies. In contrast, knocking down either *atg6* or *atg12* strongly enhanced the rough eye phenotype associated with UPS impairment in DTS7 flies reared at 28 °C (Fig. 2g–i) and in AR52 flies reared on DHT (Fig. 2j–l). From these data, we can infer that the autophagy induced by UPS impairment is compensatory.

We hypothesized that ectopic expression of HDAC6 suppressed degeneration by promoting autophagic degradation of aberrant

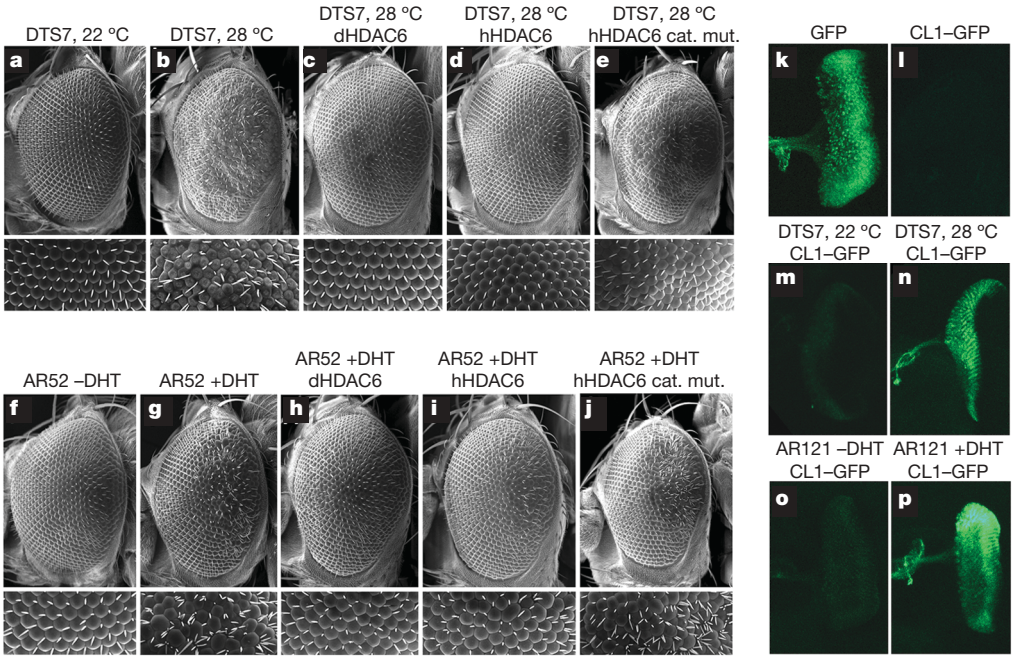


**Figure 1 | HDAC6 rescues degeneration in flies with proteasome impairment and in a fly model of SBMA that exhibits impaired UPS function.** **a**–**e**, Scanning electron microscopy (SEM) images of fly eyes expressing DTS7 with or without the indicated HDAC6 transgenes. Lower panels, magnification of ommatidia. **a**, Normal eyes in DTS7 flies reared at 22 °C. **b**, Rough eyes in DTS7 flies reared at 28 °C. **c**, **d**, Degeneration was suppressed by expression of *Drosophila* HDAC6 (dHDAC6; **c**) or human HDAC6 (hHDAC6; **d**), but not a catalytically dead mutant of hHDAC6 (cat. mut.; **e**). **f**–**j**, SEM images of fly eyes expressing AR52 with or without the indicated HDAC6 transgenes. Lower panels, magnification of ommatidia. **f**, Normal eyes in AR52 flies reared without (−) DHT. **g**, Rough eyes in AR52 flies reared with (+) DHT. Degeneration was suppressed by expression of *Drosophila* HDAC6 (**h**) or human HDAC6 (**i**), but not a catalytically dead mutant of human HDAC6 (**j**). **k**–**p**, Detection of UPS reporter in imaginal eye discs from third-instar larvae by confocal microscopy. High level fluorescence was found in flies expressing GFP (**k**, positive control), but fluorescence was barely detectable in control flies expressing CL1–GFP (**l**, negative control). CL1-GFP accumulates in DTS7 flies with temperature-dependent proteasome impairment (compare **m** to **n**) and in AR52 flies with ligand-dependent degeneration (compare **o** to **p**). The retinal phenotypes of 200 to >1,000 flies of each genotype were examined. Quantitative analyses of eye phenotypes and proteasome impairment are presented in Supplementary Figs S2 and S3, respectively. (DHT, dihydrotestosterone.)

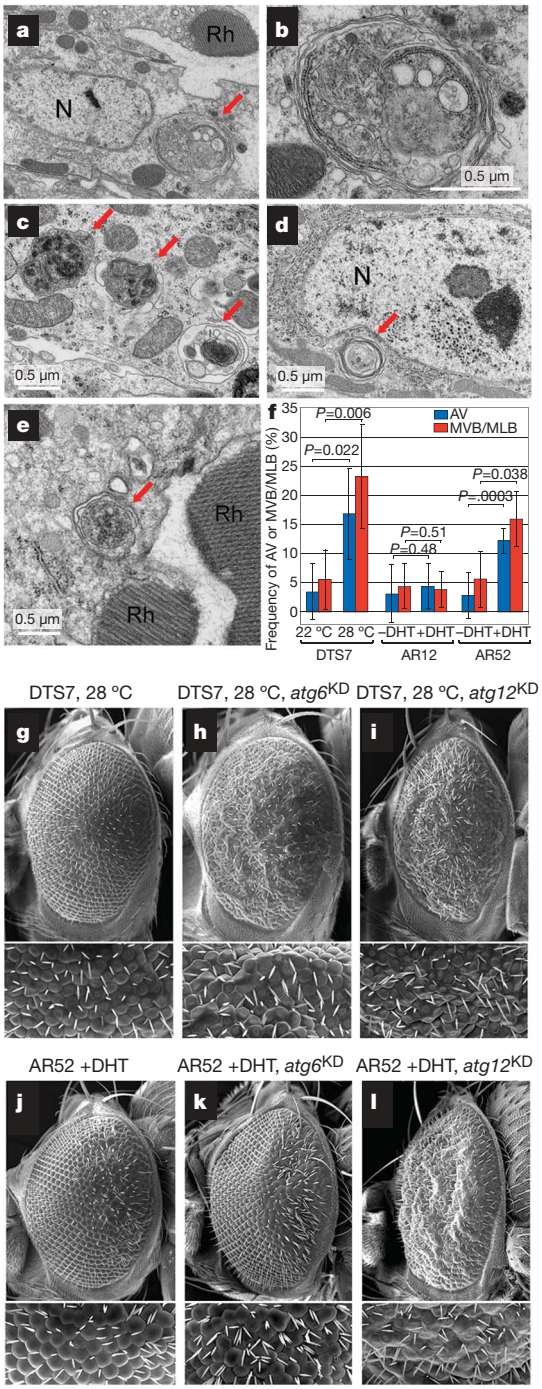


**Figure 2 | Induction of compensatory autophagy in flies with proteasome mutations and in SBMA flies.** **a**–**e**, Representative examples of autophagic vacuoles detected by TEM in retinal sections used to generate the quantitative data shown in (**f**). **a**, An autophagosome (red arrow) containing cytoplasmic contents in a photoreceptor neuron from an AR52 fly reared on DHT. **b**, Higher magnification of the autophagosome in **a**. **c**, Multiple autophagolysosomes (red arrows) containing dense, amorphous material from an AR52 fly reared on DHT. **d**, A juxtanuclear multilamellar body (red arrow) from a DTS7 fly reared at 28 °C. **e**, A multivesicular body (red arrow) from a DTS7 fly reared at 28 °C. **f**, A significant increase in the frequency of neurons with autophagic figures in DTS7 flies reared at 28 °C compared to those reared at 22 °C, and in AR52 flies reared on DHT compared to those reared off DHT. Data show mean ± s.d., $n = 59$–$82$ neurons in 5 sections per condition. No accumulation of autophagic figures was found in AR12 flies. **g**–**l**, SEM images of fly eyes expressing the indicated transgenes. RNAi knockdown (KD) of *atg6* and *atg12* enhances degeneration in DTS7 flies reared at 28 °C (compare **h**, **i** to **g**) and AR52 flies reared on DHT (compare **k**, **l** to **j**). 200 to >1,000 fly eyes of each genotype were examined. Quantitative analyses of eye phenotypes are presented in Supplementary Fig. S2. (N, nucleus; Rh, rhabdomere.)

protein. Thus, we examined AR levels *in vivo* and determined that expression of HDAC6 led to lower steady state levels of polyQ-expanded AR *in vivo*, whereas inhibition of autophagy by knockdown of *atg6* or *atg12* resulted in higher steady state levels (Fig. 3a). These altered steady state levels occurred despite no significant change in RNA levels (Supplementary Fig. S11), suggesting that HDAC6 accelerates the rate of AR degradation. To investigate this further, we adapted the inducible Geneswitch expression system[21] to monitor protein turnover. In *elav*-GS;UAS-AR52 flies, no expression was detected before exposure to the inducing agent RU486 (data not shown). To induce expression, starved flies were fed sucrose media containing RU486 for one hour, which resulted in a pulse of expression that became detectable within 2 h, peaked after approximately 10 h, and then gradually decayed with a half-life of ~100 min (Fig. 3b, c and Supplementary Fig. S12). In *elav*-GS;UAS-AR52;UAS-dHDAC6 flies (dHDAC6 indicates *Drosophila* HDAC6), there was a parallel induction of AR52 expression, but an accelerated rate of decay, with a half-life of ~50 min (Fig. 3c, d and Supplementary Fig. S12). Importantly, co-expression of *Drosophila* HDAC6 accelerated the turnover of not only AR52 monomers, but also high molecular weight aggregates that were trapped in the stacking gel (Fig. 3d).

We determined that treatment with rapamycin suppressed degeneration caused by either proteasome impairment or polyQ toxicity (Fig. 4a–d). This finding is consistent with a prior report, in which rapamycin suppressed degeneration in fly and mouse models of Huntington's disease[22]. Rescue by rapamycin has been attributed to inhibition of TOR and induction of autophagy, although a role for other TOR-regulated pathways could not be excluded[22,23]. We found that knockdown of *atg12* blocked the ability of rapamycin to suppress degeneration when the proteasome was impaired, verifying that rapamycin rescue is autophagy-dependent (Fig. 4e, f). Importantly,

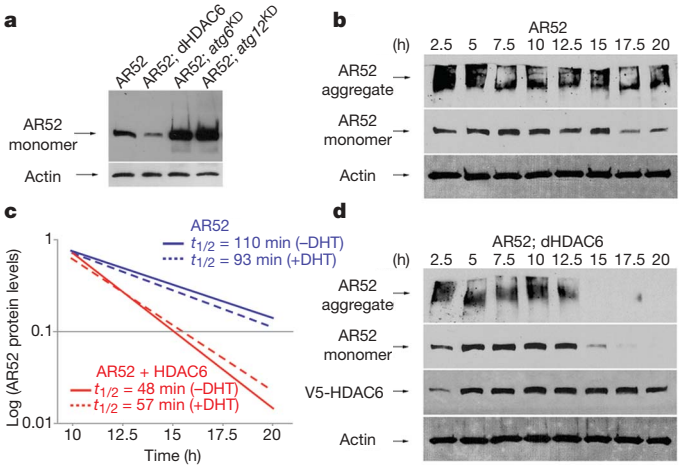


**Figure 3 | HDAC6 accelerates the turnover of polyQ-expanded AR.** **a**, **b**, **d**, Western blots from flies expressing the indicated transgenes. **a**, Steady state levels of AR52 protein are reduced in flies overexpressing *Drosophila* HDAC6, but are elevated in flies in which *atg6* or *atg12* has been knocked down. **b**, Western blots showing the temporal profile of AR52 protein monomer and high molecular weight aggregate levels after a brief pulse of expression. AR52 protein became detectable by 2.5 h after treatment with RU486, reached a peak at 10 h, and then slowly decayed. **c**, A logarithmic plot of AR52/actin ratios was used to determine the line of best fit by regression analysis ($y = Ae^{-Kx}$). $R^2 = 0.9117$ (AR52 − DHT), $R^2 = 0.7808$ (AR52 + DHT), $R^2 = 0.9719$ (HDAC6 + (AR52 − DHT)), $R^2 = 0.9644$ (HDAC6 + (AR52 + DHT)). Half-life was determined by the slope of the best fit line with the equation $t_{1/2} = 0.693/K$. Half-life of AR52 *in vivo* was reduced ~2-fold in flies co-expressing *Drosophila* HDAC6 and did not differ significantly in the presence (broken lines) or absence (solid lines) of DHT. Plots of the mean AR52/actin ratios are shown in Supplementary Fig. S12. **d**, Flies co-expressing *Drosophila* HDAC6 showed a nearly identical profile of induced expression as in **b**, but AR protein decayed at an accelerated rate. Exogenous *Drosophila* HDAC6 was detected by immunoblot against the V5 epitope.

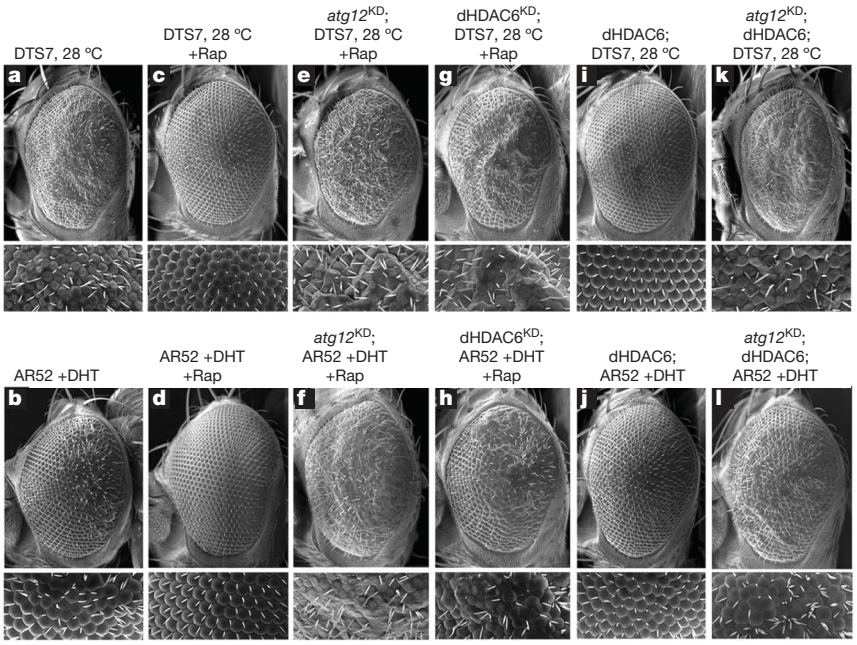


**Figure 4 | Rescue of degeneration by HDAC6 is autophagy-dependent.** **a–l**, SEM images of fly eyes expressing the indicated transgenes. The rough eye phenotypes caused by proteasome mutation (**a**) or by expression of polyQ-expanded AR (**b**) were both suppressed by rearing flies on the TOR inhibitor rapamycin (**c**, **d**). **e**, **f**, Rapamycin failed to suppress degeneration in an autophagy-deficient background created by knockdown of *atg12*, confirming that rescue by rapamycin is autophagy-dependent. **g**, **h**, Rapamycin also failed to suppress degeneration when HDAC6 levels were knocked down, demonstrating that suppression via the TOR pathway is HDAC6-dependent. **k**, **j**, HDAC6 failed to suppress degeneration in an autophagy-deficient background, confirming that rescue by HDAC6 is dependent on autophagy (compare **k**, **l** to **i**, **j**). 200 to >1,000 fly eyes of each genotype were examined. Quantitative analyses of eye phenotypes are presented in Supplementary Fig. S2.

we also determined that knockdown of *Drosophila* HDAC6 blocked the ability of rapamycin to suppress degeneration, indicating that *Drosophila* HDAC6 is essential in order for induction of autophagy to compensate for proteasome impairment (Fig. 4g, h). Furthermore, we determined that the ability of *Drosophila* HDAC6 to suppress degeneration was autophagy-dependent, as rescue was blocked by knockdown of *atg12* (Fig. 4i–l). Thus, HDAC6 is integral to rescue of degeneration by autophagy and essential for autophagy to compensate for impaired UPS function.

Our findings extend previous studies in three important ways. First, we determined that induction of autophagy is sufficient to rescue degeneration associated with UPS impairment, dramatically illustrating the compensatory relationship between autophagy and the UPS. Second, we determined that HDAC6 activity is essential for autophagy to compensate for impaired UPS function. Finally, we determined that ectopic expression of HDAC6 alone is sufficient to rescue degeneration caused by proteasome mutations and polyQ toxicity, and does so in an autophagy-dependent manner. These observations are consistent with a mechanism in which HDAC6 facilitates turnover of aberrant proteins by autophagy, lowering their steady state levels and mitigating toxicity. We recently determined that overexpression of HDAC6 also suppressed degenerative phenotypes in additional models of neurodegenerative disease, including flies expressing pathologic Aβ fragments and other polyQ-expanded proteins (U.B.P. and J.P.T., unpublished results). Thus, the HDAC6-mediated pathway of protein clearance may have broad relevance to degenerative proteopathies.

Although the current study indicates that the mechanism of HDAC6 rescue involves accelerated turnover of misfolded protein by autophagy, further study is required to determine the precise details of how this occurs. The mechanism could involve modulation of HSP90 activity, as this chaperone is a substrate of HDAC6 deacetylase activity[24]. Alternatively, HDAC6 may be involved in shuttling polyubiquitinated substrates to a location conducive to engulfment by autophagosomes, consistent with a known role for HDAC6 in the formation of aggresomes *in vitro*[10]. A third possibility is that HDAC6 may contribute to the transport of lysosomes to the site of autophagy, as suggested by the observation that HDAC6 knockdown results in dispersal of lysosomes away from the microtubule organizing centre[6]. Elucidating the precise role of HDAC6 in linking autophagy and the UPS promises substantial insights into cellular management of misfolded protein.

## METHODS

**Fly stocks and phenotypes.** *Drosophila* stocks were maintained on standard cornmeal agar media at 25 °C unless otherwise noted. Food was supplemented with DHT (Steraloids) and rapamycin (Sigma) once it had cooled to <50 °C to final concentrations of 1 mM and 1 μM, respectively. Eye phenotypes of 200 to >1,000 flies of each genotype and condition were examined and scored as described in Supplementary Methods. Quantification of eye phenotypes is shown in Supplementary Fig. S2.

**Immunoblots and protein turnover.** Immunoblots were performed using antibodies against GFP (ab6556, Novus Biologicals), AR (N20, Santa Cruz Biotech), 119 β-actin (119, Santa Cruz Biotech), tubulin (Sigma), V5 epitope (Invitrogen), and affinity-purified antibody against *Drosophila* HDAC6 as described[25]. To monitor protein turnover *in vivo*, 1-day-old adult flies of the appropriate genotype were starved for 12 h in a vial that contained only a Kimwipe soaked with 3 ml of water. After starvation, flies were placed in a vial that contained a Kimwipe soaked with 3 ml of 500 μM RU486 (Steraloids) dissolved in a 2% sucrose solution (minus DHT condition) or 500 μM RU486 and 1 mM DHT in a 2% sucrose solution (plus DHT condition) for 1 h, and then transferred to a vial containing standard food or food containing 1 mM DHT until collected for extract preparation.

**TEM.** To quantify morphologic features by TEM, we used longitudinal sections through the retina and identified photoreceptor neurons by the presence of rhabdomeres. 59–82 neurons from 5 flies per condition were scored for the presence of autophagic vacuoles, multivesicular bodies and multilamellar bodies, and comparisons between conditions were made with a paired *t*-test. A detailed description of all methods used in this study may be found in Supplementary Methods.

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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