TOR-mediated autophagy regulates cell death in *Drosophila* neurodegenerative disease

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Target of rapamycin (TOR) signaling is a regulator of cell growth. TOR activity can also enhance cell death, and the TOR inhibitor rapamycin protects cells against proapoptotic stimuli. Autophagy, which can protect against cell death, is negatively regulated by TOR, and disruption of autophagy by mutation of *Atg5* or *Atg7* can lead to neurodegeneration. However, the implied functional connection between TOR signaling, autophagy, and cell death or degeneration has not been rigorously tested. Using the *Drosophila melanogaster* visual system, we show in this study that hyperactivation of TOR leads to photoreceptor cell death in an age- and light-dependent manner and that this is because of TOR's ability to suppress autophagy. We also find that genetically inhibiting TOR or inducing autophagy suppresses cell death in *Drosophila* models of Huntington's disease and *phospholipase C* (*norpA*)–mediated retinal degeneration. Thus, our data indicate that TOR induces cell death by suppressing autophagy and provide direct genetic evidence that autophagy alleviates cell death in several common types of neurodegenerative disease.

Introduction

The evolutionarily conserved serine/threonine protein kinase target of rapamycin (TOR), a central effector of cell growth, controls diverse metabolic outputs, including protein translation, mRNA transcription, cytoskeletal organization, autophagy, and cell death (Wullschleger et al., 2006). Although most of the research on TOR has focused on its growth-promoting activity, hyperactive TOR signaling can also enhance the induction of cell death by various signals (Shah et al., 2004; Ghosh et al., 2006), and inhibition of TOR signaling can protect cells against proapoptotic insults (Ravikumar et al., 2006). The mechanisms underlying the relationship between TOR signaling and cell death are largely unknown, but the induction of autophagy that occurs when TOR activity is suppressed has been proposed to alleviate cellular toxicity in several neurodegenerative disease models (Rubinsztein et al., 2007; Mizushima et al., 2008).

Although unchecked autophagy can sometimes lead to cell death (Berry and Baehrecke, 2007; Scott et al., 2007), in vivo autophagy is mainly involved in eliminating damaged organelles or proteins with compromised function and can actually protect cells against death signals (Levine and Yuan, 2005; Klionsky, 2007). In fact, disruption of basal autophagy by loss of function of either *Atg5* or *Atg7*, genes which are necessary for autophagosome formation, can lead to neurodegeneration in otherwise normal neurons (Hara et al., 2006; Komatsu et al., 2006, 2007).

The prosurvival function of autophagy might have a protective role against the development of several diseases, most notably neurodegenerative diseases involving formation of intracellular aggregates (Rubinsztein et al., 2007). Many pathogenic aggregate-prone proteins, including polyglutamine (polyQ)-expanded huntingtin are strongly dependent on autophagy for their degradation (Ravikumar et al., 2002; Berger et al., 2006; Shibata et al., 2006), and rapamycin attenuates accumulation of mutated huntingtin proteins and cell death in Huntington's disease (HD) models (Ravikumar et al., 2004; Shibata et al., 2006). These findings have led to the proposition that autophagy induction might be used to treat neurodegenerative diseases such as HD (Ravikumar et al., 2002). However, it has not

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Abbreviations used in this paper: 4EBP, eukaryotic initiation factor 4E–binding protein; *GMR*, *glass multiple reporter*; HD, Huntington's disease; PI3K, phosphatidylinositol 3 kinase; polyQ, polyglutamine; PTEN, phosphatase and tensin homologue; Rheb, Ras homologue enriched in brain protein; S6K, P70 ribosomal S6 kinase; TOR, target of rapamycin; TSC, tuberous sclerosis complex; *UAS*, *upstream activator sequence*.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 186 No. 5 703–711
www.jcb.org/cgi/doi/10.1083/jcb.200904090

been genetically tested whether inhibition of TOR activity can suppress neuronal cell death in disease models or whether such suppression is autophagy dependent. Furthermore, it is not yet clear whether controlling TOR pathway activity and autophagy might suppress neurodegenerative diseases not associated with protein aggregation. Moreover, TOR activity, autophagy, and neurodegenerative cell death have not been functionally linked by genetic tests, although their association has been discussed (Ravikumar et al., 2004).

The *Drosophila melanogaster* visual system has proven to be a powerful genetic model for dissecting the molecular mechanisms underlying neurodegenerative diseases (Bilen and Bonini, 2005; Wang and Montell, 2007). Using the *Drosophila* visual system, we found that hyperactivation of TOR signaling led to neuronal cell death in an age- and light-dependent manner, and this was mediated via the suppression of autophagy. Either genetically inhibiting TOR or directly inducing autophagy also suppressed cell death in HD model. We further demonstrate that autophagy induction also greatly suppressed retinal degeneration associated with *norpA* mutations, possibly by mediating the turnover of toxic rhodopsin–arrestin complexes generated during phototransduction.

Results and discussion

TOR kinase is activated by the small GTPase Ras homologue enriched in brain protein (Rheb). The tuberous sclerosis complex (TSC) gene products Tsc1 and Tsc2 form a GTPase-activating protein that suppresses Rheb activity and thereby negatively regulates TOR kinase activity (Wullschleger et al., 2006). Therefore, to activate the TOR pathway, we overexpressed Rheb in *Drosophila* compound eyes using the *Gal4/upstream activator sequence* (*UAS*) system. Using the transgene combination *UAS-rheb* and *glass multiple reporter* (*GMR*)*–Gal4* (an eye-specific *Gal4* driver), we selectively overexpressed Rheb in photoreceptor neurons. The *Drosophila* compound eye consists of ∼800 repetitive units, known as ommatidia, each of which includes seven photoreceptor cells in any given plane of a tangential section. Each photoreceptor cell contains a microvillar structure, the rhabdomere, which is the photoreception organelle and is centrally located in the ommatidium (Fig. 1 A, R). Control retinas (*GMR-Gal4/+*) had all seven photoreceptor cells in each ommatidium regardless of their age or whether or not they were maintained in the light or dark (Fig. 1, A and G). Young *GMR-Gal4 UAS-rheb/+* (*GMR>rheb*) flies had normal eye morphology, although the ommatidium size was much larger than in controls (Fig. S1). However, *GMR>rheb* flies underwent an age-dependent loss of photoreceptor cells when cultured on a 12-h light/12-h dark cycle (Fig. 1, B, C, and G). Interestingly, *GMR>rheb* flies cultured in continuous darkness lost photoreceptor cells more slowly than those exposed to light (Fig. 1, D and G), indicating that photoreceptor degeneration in the Rheb-overexpressing eyes was dependent on phototransduction.

We also assayed photoreceptor cell death associated with mutation of *tsc1*, an essential negative regulator of Rheb. Using the *ey-Gal4 UAS-FLP/hid* system (Stowers and Schwarz, 1999), we generated eyes homozygous for a *tsc1* loss of function allele. Whereas young $tsc1^{29}$ mosaic flies (<2 d old) had all seven rhabdomeres (Fig. 1 E), almost no rhabdomeres were present in the eyes of $tsc1^{29}$ mosaic flies after 20 d of light/dark cycling (Fig. 1, F and G). This is consistent with our observation that overexpressed Rheb led to photoreceptor cell degeneration.

TOR kinase controls cell growth in part via two well-characterized regulators of protein translation, the P70 ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E–binding protein (4EBP). Therefore, we considered the possibility that TOR might affect retinal degeneration by inducing cellular overgrowth, as promoted by S6K and 4EBP. Both *GMR>rheb* flies and $tsc1^{29}$ mosaic flies had hypertrophic eyes (Fig. S1). To reduce this hypertrophy, we used *UAS-rheb/+;ninaE-Gal4/+* (*ninaE>rheb*) flies, which only expressed Rheb in adult R1–R6 photoreceptors. The eyes of *ninaE>rheb* flies were similar in size to controls (Fig. S1) and had normal retinal morphology when the flies were young (<2 d old; Fig. 2 A). However, few R1–R6 rhabdomeres were detected in light-exposed 30-d-old *ninaE>rheb* flies, although R7 rhabdomeres were generally present (Fig. 2, B and F). To further test the idea that cell overgrowth might be responsible for neuronal degeneration in the presence of excess TOR activity, we examined the ommatidial morphology of both *GMR-Gal4/UAS-*$s6k^{TE}$ (*GMR>s6k*) flies, which overexpress constitutively active S6K, and $thor^2$ flies, which are mutant for *4EBP* (Teleman et al., 2005). After 30 d of light/dark cycling, photoreceptors were preserved normally in both the *GMR>s6k* flies and the $thor^2$ flies (Fig. 2, C, D, and F). This indicates that Rheb-induced photoreceptor cell death occurs independently of S6K and 4EBP and is most likely not a consequence of increased cell growth.

Because TOR drives negative feedback regulation of phosphatidylinositol 3 kinase (PI3K) signaling via S6K and IRS1 (Harrington et al., 2004; Shah et al., 2004), increases in TOR activity can decrease the activity of AKT, which is a universal survival signal (Datta et al., 1999). To test whether Rheb induced neuronal cell death through PI3K/AKT, we overexpressed PTEN (phosphatase and tensin homologue), a negative effector of PI3K signaling (Gao et al., 2000). No photoreceptor cell death was detected in 30-d-old *GMR-Gal4/UAS-pten* (*GMR>pten*) flies exposed to light (Fig. 2, E and F), suggesting that a reduction of PI3K/AKT activity is probably not responsible for Rheb-induced photoreceptor cell death.

In addition to growth signaling, TOR is important as a negative regulator of autophagy. To assess the possibility that TOR activation might cause neuronal degeneration by suppressing autophagy, we first tested whether a mutation in *atg7* (Juhász et al., 2007), a gene required for autophagy, could induce photoreceptor cell loss. Whereas all seven rhabdomeres were typically detected in the ommatidia of young (<2 d old) $atg7^{d77}$ flies, after 20 d of light/dark cycling, few rhabdomeres were detected (Fig. 3, A–D).

We reasoned that if retinal degeneration caused by hyperactive TOR is caused by the suppression of autophagy, the reactivation of autophagy might suppress cell death caused by excessive TOR activity. Because overexpression of Atg1 is sufficient to induce high levels of autophagy in *Drosophila*

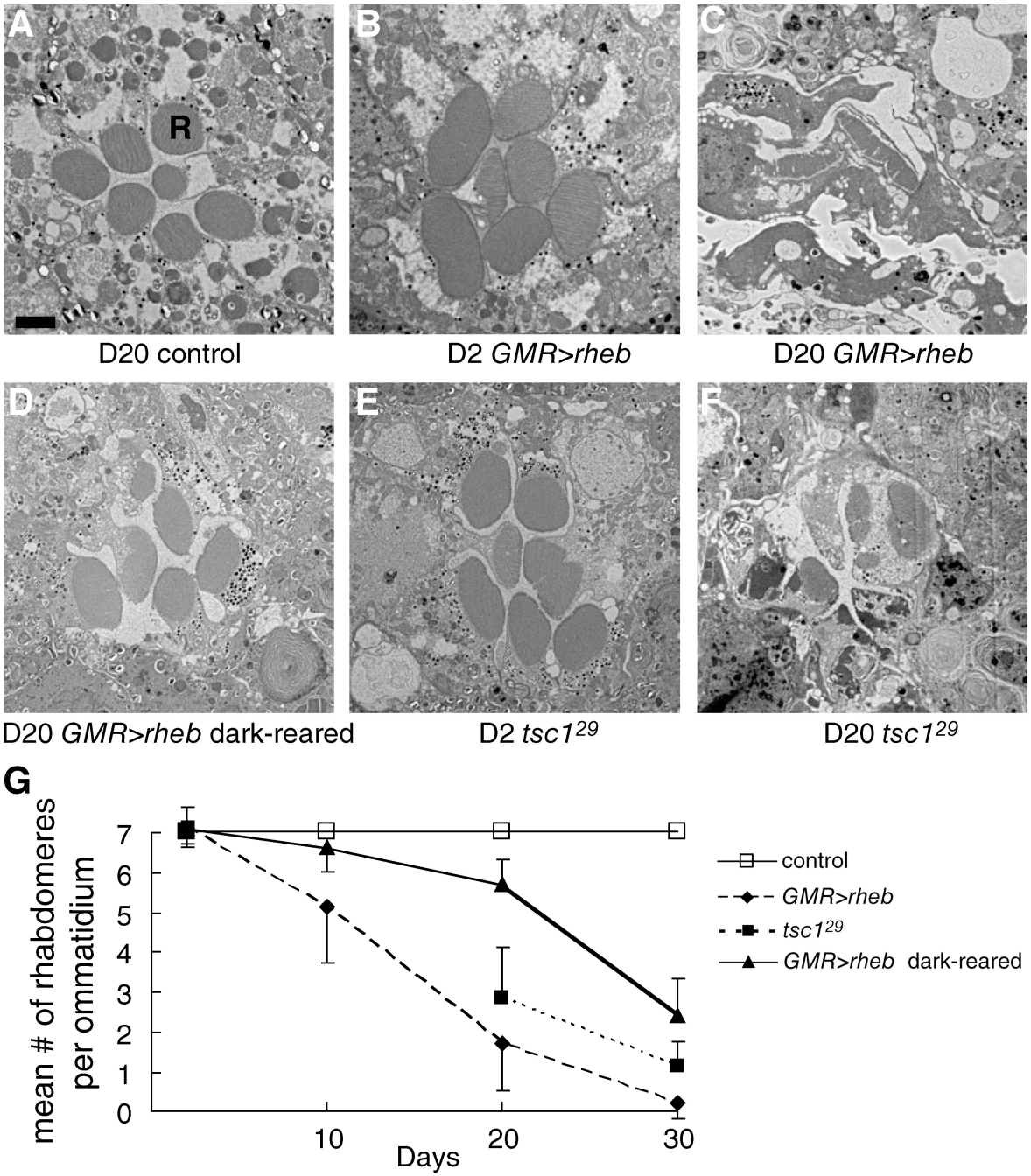

Figure 1. **Light-dependent photoreceptor degeneration in flies with overexpression of Rheb.** (A–F) Examination of the morphology by transmission EM. Cross sections were obtained from 2 (D2)- or 20-d-old (D20) flies maintained under 12-h light/12-h dark cycle, with the exception of the dark-reared *GMR>rheb*. 20-d-old control (*GMR-Gal4/+*; A), 2-d-old *GMR>rheb* (B), 20-d-old *GMR>rheb* (C), 20-d-old *GMR>rheb* reared in the dark (D), 2-d-old *tsc1*^{29} (E), and 20-d-old *tsc1*^{29} (F) are shown. (G) The time course of retinal degeneration, as determined by examining the number of rhabdomeres per ommatidium. Error bars indicate SD. R, rhabdomere. Bar, 2 μm.

(Scott et al., 2007), we generated transgenic flies expressing tagged Atg1 under the control of the *ninaE* promoter (*ninaE-atg1*). In *ninaE-atg1* flies, high levels of Atg1-MYC protein were detected in the head, and autophagy, as assayed by LysoTracker staining, was strongly increased in photoreceptor cell bodies (Fig. S2). Although it has been hypothesized that autophagy can down-regulate TOR (Lee et al., 2007), we observed that S6K phosphorylation was not affected by the *ninaE-atg1* transgene (Fig. S2), implying that induction of autophagy in the eye by overexpression of Atg1 did not suppress TOR activity. Compared with *ninaE>rheb* flies, which lost most major photoreceptor neurons after 30 d of light/dark culture, *ninaE>rheb/ninaE-atg1* flies retained most of their photoreceptor cells (Fig. 3, E–H). Thus, these results show that enforced autophagy can suppress TOR-dependent neural degeneration, strongly supporting the idea that TOR-induced neuronal cell death is a consequence of the inhibition of autophagy.

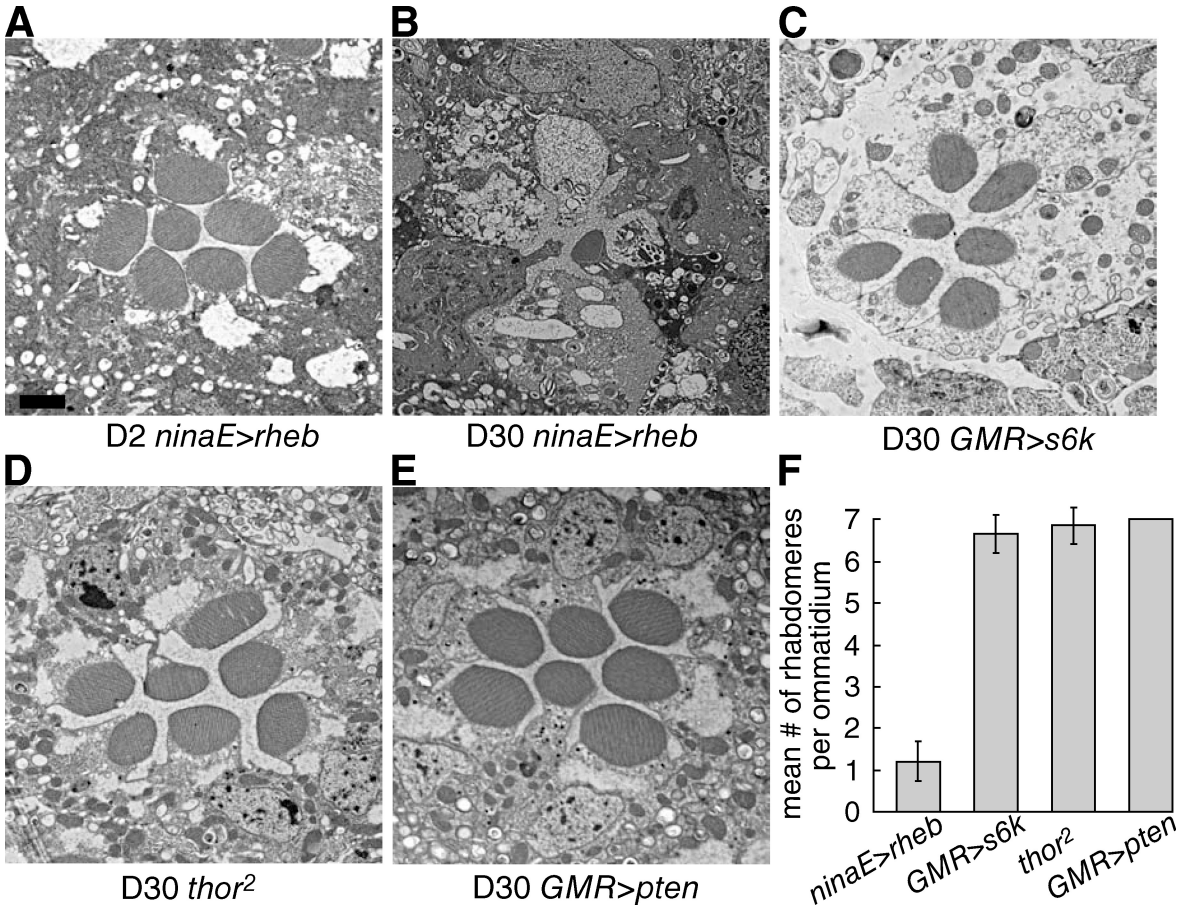

Figure 2. **Rheb induced cell death independent of cell growth.** (A–E) Examination of the morphology of 2-d-old (D2) *ninaE>rheb* (A), 30-d-old (D30) *ninaE>rheb* (B), 30-d-old *GMR>s6k* (C), 30-d-old *thor*^{2} (D), and 30-d-old *GMR>pten* ommatidia (E) by transmission EM. Cross sections of retinas were obtained from 30-d-old flies kept under a 12-h light/12-h dark cycle except D2 *ninaE>rheb* flies (A), which were <2 d old. (F) Mean number of rhabdomeres per ommatidium from 30-d-old flies. Error bars indicate SD. Bar, 2 µm.

It has been proposed that suppression of TOR activity and the induction of autophagy have protective roles toward many neurodegenerative diseases (Ravikumar et al., 2004). One such condition is HD, a devastating autosomal dominant neurodegenerative disease caused by polyQ-expanded huntingtin protein. In animal models of HD, the TOR inhibitor rapamycin can attenuate cell death (Ravikumar et al., 2004), and mutations in autophagy genes can enhance polyQ aggregation and neurodegeneration (Jia et al., 2007). However, whether the effects of TOR on polyQ-mediated neurodegeneration are caused by its regulation of autophagy has not been directly or genetically tested. On the contrary, one recent study using the *Drosophila* eye implicated S6K and PDK1 rather than autophagy as enhancers of polyQ-mediated neurodegeneration (Nelson et al., 2005). To explore the role of TOR-dependent autophagy in HD, we expressed N-terminal fragments of human huntingtin that contain 120 glutamine residues in photoreceptor neurons (Jackson et al., 1998). The *GMR-htt.Q120* flies (*HQ120*) manifested strong age-dependent loss of rhabdomeres and photoreceptor cells in an optical neutralization assay and by EM (Fig. 4, A and B). Inhibition of TOR by overexpression of TSC1 and TSC2 (*GMR>Tsc1/2*) largely suppressed neural degeneration caused by *HQ120* (Fig. 4, A–C). Furthermore, direct induction of autophagy by overexpressed ATG1 (*ninaE-atg1*) also suppressed photoreceptor cell death in the *HQ120* flies (Fig. 4, A and D). These results support the idea that inducing autophagy by inhibiting TOR can suppress neurodegenerative diseases involving protein aggregates such as HD.

To further explore the mechanisms underlying TOR's neural-protective function, we used two well-known genetic models of retinal degeneration. *NinaE*^{RH27} encodes a dominant mutant in the major rhodopsin Rh1, which promotes retinal cell death through apoptosis (Davidson and Steller, 1998). *NinaE*^{RH27} flies started to lose photoreceptor cells after 25 d of 12-h light/12-h dark cycling (Fig. S3, A and D). Overexpression of TSC1 and TSC2 (*GMR>Tsc1/2*) did not attenuate cell death in the *NinaE*^{RH27} flies and even had converse effects (Fig. S3, B and E). Moreover, direct induction of autophagy by overexpression of Atg1 did not suppress cell death caused by the dominant *NinaE*^{RH27} mutation (Fig. S3, C and F). Thus, the suppression of TOR activity or the induction of autophagy cannot protect cells from all kinds of cell death. In this case, caspase-dependent apoptosis appeared to be TOR independent.

In a second neurodegeneration model, mutations in the *Drosophila phospholipase C* gene *norpA* result in light-dependent retinal degeneration triggered by the accumulation of cytosolic

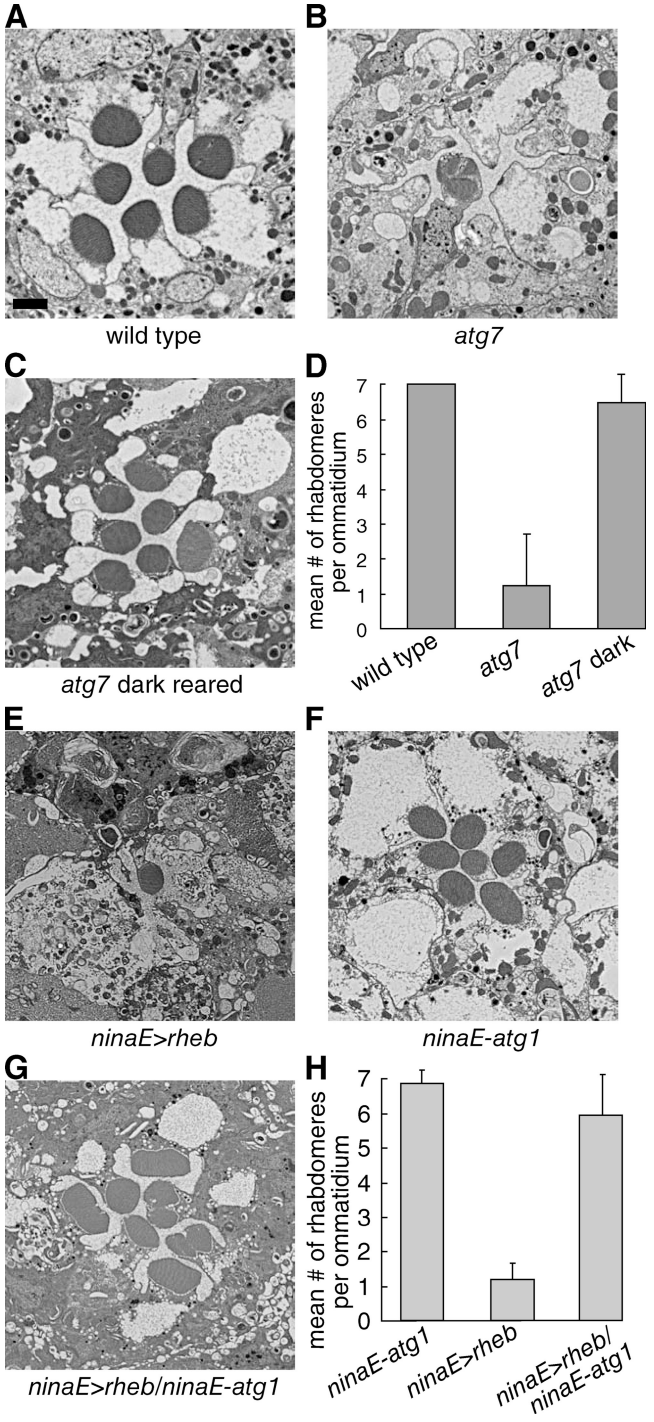

Figure 3. **Rheb/TOR signaling drives photoreceptor cell death by suppressing autophagy.** (A–C) Transmission EM images of cross sections from wild type (w^{1118}; A), $atg7^{d77}$ (B), and $atg7^{d77}$ dark reared (C). The sections were obtained from 30-d-old flies maintained under 12-h light/12-h dark cycle, with the exception of dark-reared $atg7^{d77}$ (C). (D) Histogram of the mean number of rhabdomeres per ommatidium from 30-d-old flies. (E–G) Retinal morphology of *ninaE>rheb* (E), *ninaE-atg1* (F), and *ninaE>rheb/ninaE-atg1* (G). The sections were obtained from 30-d-old flies maintained under 12-h light/12-h dark cycle. (H) Histogram of the mean number of rhabdomeres per ommatidium from 30-d-old flies. (D and H) Error bars indicate SD. Bar, 2 µm.

rhodopsin–arrestin complexes (Alloway et al., 2000). Neuronal degeneration in the *norpA* model cannot be suppressed by the inhibitors of apoptosis and thus seems not to be mediated by apoptosis (Hsu et al., 2004). We found that $norpA^{P24}$ mutants underwent rapid retinal degeneration, such that the animals had detectable loss of photoreceptors cells by 4 d and were almost devoid of photoreceptor cells by day 10 (Fig. 5, A and B). The *norpA*-mediated degeneration was significantly suppressed by overexpression of either TSC1 and TSC2 or Atg1 in photoreceptor cells (Fig. 5, A, C, and D). Thus, the suppression of autophagy by TOR activity is a critical component in *norpA*-mediated neurodegeneration.

Stable rhodopsin–arrestin complexes are known to trigger the cell death that occurs in the *norpA* mutants (Alloway et al., 2000), and therefore, we considered whether autophagy might suppress *norpA*-mediated cell death by clearing these toxic complexes. To test this, we assayed the percentage of major arrestin (Arr2) bound to rhodopsin using a coprecipitation assay. We found that exposure to light for 1 h caused ∼45% of the Arr2 to bind to rhodopsin in wild-type eyes, whereas >60% was bound in *norpA* mutants (Fig. 5, E and F). However, in both $norpA^{P24}$;*ninaE-atg1* and $norpA^{P24}$;*GMR>Tsc1/2* flies, the fraction of rhodopsin-bound Arr2 was decreased back nearly to the wild-type level without changing the overall rhodopsin/Arr2 ratio (Fig. 5, E and F). These data illustrate that autophagy can help eliminate rhodopsin–arrestin complexes and thus reduce their toxicity in the *norpA* model of retinal degeneration.

Overall, our results indicate that constraining TOR activity is crucial for neuronal survival, especially in highly active neurons, and that this is caused by, in large part, TOR's function as a suppressor of autophagy. Importantly, we found that the direct induction of autophagy by overexpression of Atg1 largely suppressed Rheb-induced photoreceptor cell death as well as photoreceptor neural degeneration in *Drosophila* models of HD and *phospholipase C* deletion. Our results support the idea that the continuous clearance of cytosolic components through autophagy is important for preventing the accumulation of toxic proteins that would otherwise disrupt neural function and ultimately lead to neurodegeneration.

Autophagy has been shown to be important in the clearance of cellular aggregates in several neurodegenerative disease models (Ravikumar et al., 2002; Webb et al., 2003; Iwata et al., 2005). Moreover, recent studies have suggested that the TOR inhibitor rapamycin can attenuate cell death in several intracytosolic proteinopathies and that this might occur through the up-regulation of autophagy (Ravikumar et al., 2004; Berger et al., 2006). Thus, our genetic demonstration that suppression of TOR or activation of autophagy can cell-autonomously suppress neurodegeneration in a polyQ aggregation model provides direct genetic evidence to support this prevailing view of neurodegenerative diseases. Interestingly, heat shock proteins such as Hsp70 and Hsp40, which function as chaperones to prevent protein aggregation, as well as several components of the ubiquitin proteasome can suppress polyQ-induced degeneration (Warrick et al., 1999; Fernandez-Funez et al., 2000; Kazemi-Esfarjani and Benzer, 2000). Like autophagy, both pathways have key roles in protein quality control, emphasizing the significant

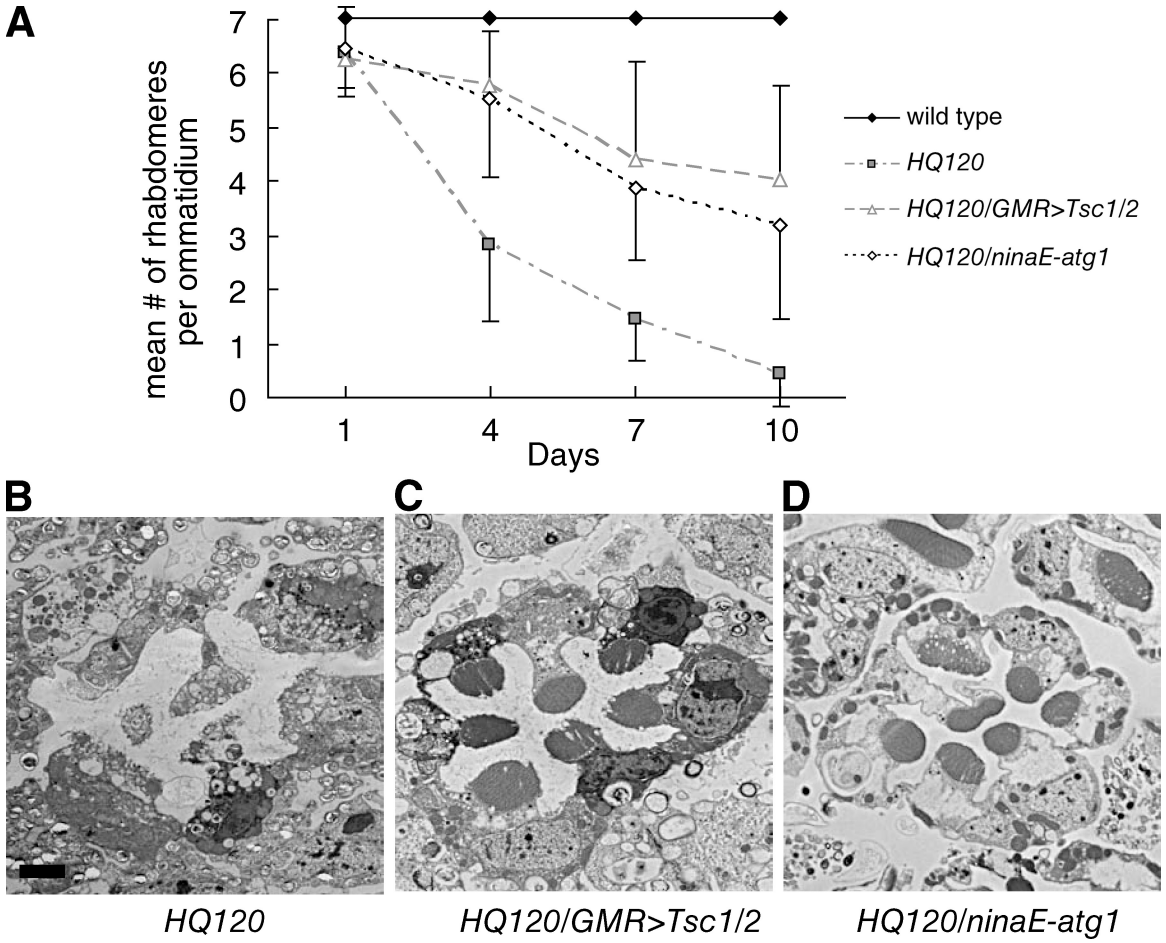

Figure 4. **Neuronal degeneration in *HQ120* flies was suppressed by overexpression of TSC1/2 or Atg1.** (A) Time course of photoreceptor degeneration was determined by the optical neutralization technique. The flies were reared under a 12-h light/12-h dark cycle. Each data point was based on the examination of ≥60 ommatidia from at least four flies. Error bars indicate SD. (B–D) Retinal morphology of 10-d-old flies examined by transmission EM. *HQ120* (B), *HQ120;GMR>Tsc1/2* (C), and *HQ120;ninaE-atg1* (D) are shown. Bar, 2 µm.

role of protein quality control in protecting cells from toxic aggregates such as those formed from polyQ aggregation.

However, many neural cell death models and most retinal degeneration diseases are not known to involve the formation of protein aggregates. Thus, one outstanding question was whether TOR signaling and autophagy could modulate the cellular toxicity of unaggregated proteins. The toxic rhodopsin–arrestin complexes that form as a result of phototransduction, promoting retinal degeneration in *norpA* mutants, provided a good test case because rhodopsin–arrestin complexes are not detected in aggregates. We found that either TOR-dependent or -independent autophagy could suppress the accumulation of rhodopsin–arrestin in *norpA* mutants and that either mode of autophagy activation could alleviate retinal degeneration. We believe that these data support a model in which autophagy can suppress neural degeneration by removing toxic soluble proteins as well as toxic protein aggregates. Therefore, therapy involving the inhibition of TOR or other means for inducing autophagy might be applied to neurological diseases rather generally.

TSC disease is a heritable disease caused by the hyperactivation of TOR signaling that occurs after somatic loss of heterozygosity in either *Tsc2* or *Tsc1*. Although benign neural tumors that arise via unchecked cell growth are commonly believed to be the principal adverse effect in TSC disease, cell death has been detected inside these tumors (Maldonado et al., 2003; Zeng et al., 2007). Thus, an alternative explanation for some of the symptoms of TSC such as seizures is that they arise as a consequence of neural cell death and neurodegeneration.

Materials and methods

Drosophila stocks

ninaE-Gal4, *GMR-Gal4*, and *GMR-htt.Q120* flies were obtained from the Bloomington Stock Center. *UAS-rheb*, *tsc1*^{29}, *UAS-Tsc1*, *UAS-Tsc2*, *UAS-pten*, *UAS-s6k*, and *thor*^{2} flies were previously described (Gao et al., 2000; Gao and Pan, 2001; Saucedo et al., 2003; Teleman et al., 2005; Scott et al., 2007). H. Steller (The Rockefeller University, New York, NY) provided *NinaE*^{RH27} flies. C. Montell (The Johns Hopkins University, Baltimore, MD) provided *norpA*^{P24} flies. T. Neufeld (University of Minnesota, Minneapolis, MN) provided *atg7*^{d77} flies.

Genotypes

Fig. 1 shows *GMR-Gal4/+*, *GMR>rheb*, and *tsc1*^{29} (*ey-Gal4 UAS-FLP;*P[*neoFRT*]*82B GMR-hid/*P[*neoFRT*]*82B tsc1*^{29}). Fig. 2 shows *ninaE>rheb*, *GMR>pten*, *thor*^{2}, and *GMR>s6k*. Fig. 3 shows *atg7*^{d77}, *ninaE-atg1* (*+;ninaE-atg1/+;ninaE-atg1/+*), and *ninaE>rheb/ninaE-atg1* (*UAS-rheb/ninaE-atg1;ninaE-Gal4/ninaE-atg1*). Fig. 4 shows *HQ120* (*GMR-HQ120*), *HQ120;GMR>Tsc1/2* (*GMR-Gal4/GMR-HQ120;UAS-Tsc1 UAS-Tsc2/+*),

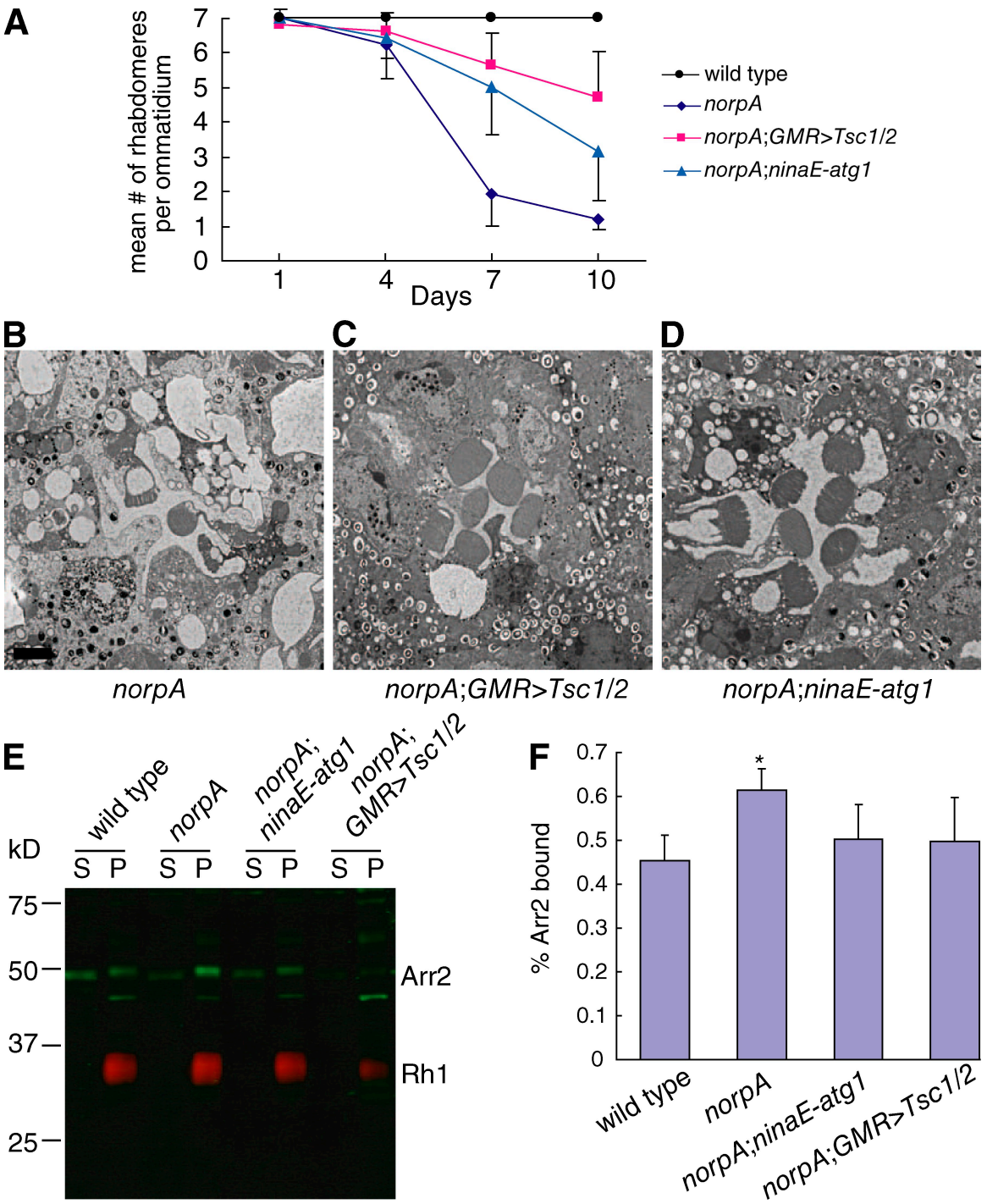

Figure 5. **Suppression of retinal degeneration in *norpA* flies by overexpression of either TSC1/2 or Atg1.** (A) The time course of retinal degeneration, as determined by optical neutralization. (B–D) Examination of retinal morphology by transmission EM. *norpA*^{P24} (B), *norpA*^{P24}*;GMR>Tsc1/2* (C), and *norpA*^{P24}*;ninaE-atg1* (D) are shown. 10-d-old flies under light/dark cycle were used. (E and F) Stable rhodopsin–arrestin complexes in *norpA*^{P24} flies were reduced by overexpression of either TSC1/2 or Atg1. (E) Western blot of fractions from fly heads exposed to light for 1 h. Pellet (P) and supernatant (S) fractions are indicated. Dark-reared flies <2 d old were used. (F) Histogram quantifying the percent of Arr2 bound to rhodopsin after treatment with 1 h of light. The asterisk indicates a statistically significant difference relative to wild type (Student's unpaired *t* test; *, $P < 0.05$). (A and F) Error bars indicate SD. Bar, 2 µm.

and *HQ120;ninaE-atg1* (*GMR-HQ120/ninaE-atg1;ninaE-atg1*). Fig. 5 shows *norpA*^{P24}, *norpA*^{P24}*;GMR>Tsc1/2* (*norpA*^{P24}*;GMR-Gal4/+;UAS-Tsc1 UAS-Tsc2/+*), and *norpA*^{P24}*;ninaE-atg1* (*norpA*^{P24}*;ninaE-atg1/+;ninaE-atg1/+*).

Generation of transgenic *Drosophila*

To express MYC-tagged ATG1 under control of the *ninaE* (Rh1) promoter, the full-length *atg1* cDNA with a C-terminal MYC tag was subcloned into the pcNX vector (Wang and Montell, 2006). To express GFP-labeled Rh1 in the photoreceptor cells, a GFP tag was added to the C terminus of Rh1 and subsequently subcloned into the pcNX vector. The constructs were injected into w^{1118} embryos, and transformants were identified on the basis of eye pigmentation.

EM

Adult fly heads were dissected and fixed in a solution with 2% paraformaldehyde, 4% glutaraldehyde, and 0.1 M phosphate, pH 7.4, for 12 h at

4°C and in a solution with 1% osmium tetroxide and 0.1 M phosphate, pH 7.4, for 2 h at 4°C. The tissues were then dehydrated in a series of ethanol solutions (10-min washes in 10, 25, 50, 75, and 100% ethanol) and embedded in LR white resin. Thin sections (~85 nm) were prepared at a depth of 30–40 µm and were examined by transmission EM using a transmission electron microscope (model 1230; JEOL) at room temperature. The images were acquired using a bottom-mount charge-coupled device camera (Ultrascan 1000; Gatan, Inc.), and Digital Micrograph software (Gatan, Inc.) was used to convert images into tiff files.

Optical neutralization

The optical neutralization assay was described previously (Franceschini and Kirschfeld, 1971). In brief, heads were dissected and immersed in immersion oil. Pictures were acquired using a microscope (DMRB; Leica).

Arr2 binding assays

Fly heads were exposed to light for 1.5 h and homogenized in TBS buffer (150 mM NaCl, 20 mM Tris, pH 7.5, and 1× protease inhibitor cocktail) with a Pellet Pestle (Kimble-Kontes). Pellet and supernatant fractions were separated by centrifuge at 12,000 *g* for 20 min (centrifuge 5415c; Eppendorf). Proteins from each fraction were fractionated by SDS-PAGE and were transferred at 30 V to Immobilon-P transfer membranes (Millipore) in Tris-glycine buffer. The blots were probed with rabbit anti-Arr2 and mouse anti-Rh1 antibodies (Developmental Studies Hybridoma Bank) and subsequently with IRDye 800–labeled anti–rabbit IgG and IRDye 680–labeled mouse IgG (LI-COR Biosciences). Signals were then detected using an Odyssey infrared imaging system (LI-COR Biosciences).

LysoTracker staining of dissected ommatidia

Ommatidia from adult flies were dissected in glass culture dishes (MatTek) filled with Schneider's *Drosophila* medium (Wang et al., 2008). The dissociated ommatidia were attached to poly-D-lysine–coated glass surfaces by placing in an incubator for 12 h at room temperature. The ommatidia were washed once in PBS, and the cells were subsequently loaded with 100 nM LysoTracker red DND-99 for 20 min under light condition. Live cell images were acquired using a microscope (Eclipse; Nikon) with a Plan-Apochromat 40× air immersion objective, and images were acquired with a charge-coupled device camera (CoolSNAP HQ2; Roper Scientific) at room temperature and NIS-Elements software (Nikon). The images were transferred into Photoshop 7.0 (Adobe) to assemble the figures.

Online supplemental material

Fig. S1 shows normal retinal cell size in flies with overexpression of Rheb by the *ninaE* promoter. Fig. S2 shows expression of MYC-tagged Atg1 and increase of autophagy without affecting S6K phosphorylation in *ninaE-atg1* retinas. Fig. S3 shows that overexpression of TSC1/2 or Atg1 does not suppress the retinal degeneration in $NinaE^{RH27}$ flies. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200904090/DC1.

We thank the Bloomington Stock Center and Drs. T. Neufeld, H. Steller, and C. Montell for fly stocks. We also thank B. Schneider and J. Bousman for preparing sections of compound eyes, Dr. H. Brown for helpful comments on the manuscript, and members of the Edgar laboratory for helpful suggestions.

This work was supported by National Institutes of Health grant R01 NS058230 to B.A. Edgar and a Damon Runyon Cancer Research Foundation Fellowship to T. Wang.

Submitted: 17 April 2009
Accepted: 30 July 2009

References

Alloway, P.G., L. Howard, and P.J. Dolph. 2000. The formation of stable rhodopsin-arrestin complexes induces apoptosis and photoreceptor cell degeneration. *Neuron.* 28:129–138.

Berger, Z., B. Ravikumar, F.M. Menzies, L.G. Oroz, B.R. Underwood, M.N. Pangalos, I. Schmitt, U. Wullner, B.O. Evert, C.J. O'Kane, and D.C. Rubinsztein. 2006. Rapamycin alleviates toxicity of different aggregate-prone proteins. *Hum. Mol. Genet.* 15:433–442.

Berry, D.L., and E.H. Baehrecke. 2007. Growth arrest and autophagy are required for salivary gland cell degradation in *Drosophila*. *Cell.* 131:1137–1148.

Bilen, J., and N.M. Bonini. 2005. *Drosophila* as a model for human neurodegenerative disease. *Annu. Rev. Genet.* 39:153–171.

Datta, S.R., A. Brunet, and M.E. Greenberg. 1999. Cellular survival: a play in three Akts. *Genes Dev.* 13:2905–2927.

Davidson, F.F., and H. Steller. 1998. Blocking apoptosis prevents blindness in *Drosophila* retinal degeneration mutants. *Nature.* 391:587–591.

Fernandez-Funez, P., M.L. Nino-Rosales, B. de Gouyon, W.C. She, J.M. Luchak, P. Martinez, E. Turiegano, J. Benito, M. Capovilla, P.J. Skinner, et al. 2000. Identification of genes that modify ataxin-1-induced neurodegeneration. *Nature.* 408:101–106.

Franceschini, N., and K. Kirschfeld. 1971. [Pseudopupil phenomena in the compound eye of *Drosophila*]. *Kybernetik.* 9:159–182.

Gao, X., and D. Pan. 2001. TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. *Genes Dev.* 15:1383–1392.

Gao, X., T.P. Neufeld, and D. Pan. 2000. *Drosophila* PTEN regulates cell growth and proliferation through PI3K-dependent and -independent pathways. *Dev. Biol.* 221:404–418.

Ghosh, S., V. Tergaonkar, C.V. Rothlin, R.G. Correa, V. Bottero, P. Bist, I.M. Verma, and T. Hunter. 2006. Essential role of tuberous sclerosis genes TSC1 and TSC2 in NF-kappaB activation and cell survival. *Cancer Cell.* 10:215–226.

Hara, T., K. Nakamura, M. Matsui, A. Yamamoto, Y. Nakahara, R. Suzuki-Migishima, M. Yokoyama, K. Mishima, I. Saito, H. Okano, and N. Mizushima. 2006. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature.* 441:885–889.

Harrington, L.S., G.M. Findlay, A. Gray, T. Tolkacheva, S. Wigfield, H. Rebholz, J. Barnett, N.R. Leslie, S. Cheng, P.R. Shepherd, et al. 2004. The TSC1-2 tumor suppressor controls insulin–PI3K signaling via regulation of IRS proteins. *J. Cell Biol.* 166:213–223.

Hsu, C.D., M.A. Whaley, K. Frazer, D.A. Miller, K.A. Mitchell, S.M. Adams, and J.E. O'Tousa. 2004. Limited role of developmental programmed cell death pathways in *Drosophila norpA* retinal degeneration. *J. Neurosci.* 24:500–507.

Iwata, A., J.C. Christianson, M. Bucci, L.M. Ellerby, N. Nukina, L.S. Forno, and R.R. Kopito. 2005. Increased susceptibility of cytoplasmic over nuclear polyglutamine aggregates to autophagic degradation. *Proc. Natl. Acad. Sci. USA.* 102:13135–13140.

Jackson, G.R., I. Salecker, X. Dong, X. Yao, N. Arnheim, P.W. Faber, M.E. MacDonald, and S.L. Zipursky. 1998. Polyglutamine-expanded human huntingtin transgenes induce degeneration of *Drosophila* photoreceptor neurons. *Neuron.* 21:633–642.

Jia, K., A.C. Hart, and B. Levine. 2007. Autophagy genes protect against disease caused by polyglutamine expansion proteins in *Caenorhabditis elegans*. *Autophagy.* 3:21–25.

Juhász, G., B. Erdi, M. Sass, and T.P. Neufeld. 2007. Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in *Drosophila*. *Genes Dev.* 21:3061–3066.

Kazemi-Esfarjani, P., and S. Benzer. 2000. Genetic suppression of polyglutamine toxicity in *Drosophila*. *Science.* 287:1837–1840.

Klionsky, D.J. 2007. Autophagy: from phenomenology to molecular understanding in less than a decade. *Nat. Rev. Mol. Cell Biol.* 8:931–937.

Komatsu, M., S. Waguri, T. Chiba, S. Murata, J. Iwata, I. Tanida, T. Ueno, M. Koike, Y. Uchiyama, E. Kominami, and K. Tanaka. 2006. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature.* 441:880–884.

Komatsu, M., Q.J. Wang, G.R. Holstein, V.L. Friedrich Jr., J. Iwata, E. Kominami, B.T. Chait, K. Tanaka, and Z. Yue. 2007. Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. *Proc. Natl. Acad. Sci. USA.* 104:14489–14494.

Lee, S.B., S. Kim, J. Lee, J. Park, G. Lee, Y. Kim, J.M. Kim, and J. Chung. 2007. ATG1, an autophagy regulator, inhibits cell growth by negatively regulating S6 kinase. *EMBO Rep.* 8:360–365.

Levine, B., and J. Yuan. 2005. Autophagy in cell death: an innocent convict? *J. Clin. Invest.* 115:2679–2688.

Maldonado, M., M. Baybis, D. Newman, D.L. Kolson, W. Chen, G. McKhann II, D.H. Gutmann, and P.B. Crino. 2003. Expression of ICAM-1, TNF-α, NF κ B, and MAP kinase in tubers of the tuberous sclerosis complex. *Neurobiol. Dis.* 14:279–290.

Mizushima, N., B. Levine, A.M. Cuervo, and D.J. Klionsky. 2008. Autophagy fights disease through cellular self-digestion. *Nature.* 451:1069–1075.

Nelson, B., S. Nishimura, H. Kanuka, E. Kuranaga, M. Inoue, G. Hori, H. Nakahara, and M. Miura. 2005. Isolation of gene sets affected specifically by polyglutamine expression: implication of the TOR signaling pathway in neurodegeneration. *Cell Death Differ.* 12:1115–1123.

Ravikumar, B., R. Duden, and D.C. Rubinsztein. 2002. Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum. Mol. Genet.* 11:1107–1117.

Ravikumar, B., C. Vacher, Z. Berger, J.E. Davies, S. Luo, L.G. Oroz, F. Scaravilli, D.F. Easton, R. Duden, C.J. O'Kane, and D.C. Rubinsztein. 2004. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine

expansions in fly and mouse models of Huntington disease. *Nat. Genet.* 36:585–595.

Ravikumar, B., Z. Berger, C. Vacher, C.J. O'Kane, and D.C. Rubinsztein. 2006. Rapamycin pre-treatment protects against apoptosis. *Hum. Mol. Genet.* 15:1209–1216.

Rubinsztein, D.C., J.E. Gestwicki, L.O. Murphy, and D.J. Klionsky. 2007. Potential therapeutic applications of autophagy. *Nat. Rev. Drug Discov.* 6:304–312.

Saucedo, L.J., X. Gao, D.A. Chiarelli, L. Li, D. Pan, and B.A. Edgar. 2003. Rheb promotes cell growth as a component of the insulin/TOR signalling network. *Nat. Cell Biol.* 5:566–571.

Scott, R.C., G. Juhász, and T.P. Neufeld. 2007. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. *Curr. Biol.* 17:1–11.

Shah, O.J., Z. Wang, and T. Hunter. 2004. Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. *Curr. Biol.* 14:1650–1656.

Shibata, M., T. Lu, T. Furuya, A. Degterev, N. Mizushima, T. Yoshimori, M. MacDonald, B. Yankner, and J. Yuan. 2006. Regulation of intracellular accumulation of mutant Huntingtin by Beclin 1. *J. Biol. Chem.* 281:14474–14485.

Stowers, R.S., and T.L. Schwarz. 1999. A genetic method for generating *Drosophila* eyes composed exclusively of mitotic clones of a single genotype. *Genetics.* 152:1631–1639.

Teleman, A.A., Y.W. Chen, and S.M. Cohen. 2005. 4E-BP functions as a metabolic brake used under stress conditions but not during normal growth. *Genes Dev.* 19:1844–1848.

Wang, T., and C. Montell. 2006. A phosphoinositide synthase required for a sustained light response. *J. Neurosci.* 26:12816–12825.

Wang, T., and C. Montell. 2007. Phototransduction and retinal degeneration in *Drosophila*. *Pflugers Arch.* 454:821–847.

Wang, T., X. Wang, Q. Xie, and C. Montell. 2008. The SOCS box protein STOPS is required for phototransduction through its effects on phospholipase C. *Neuron.* 57:56–68.

Warrick, J.M., H.Y. Chan, G.L. Gray-Board, Y. Chai, H.L. Paulson, and N.M. Bonini. 1999. Suppression of polyglutamine-mediated neurodegeneration in *Drosophila* by the molecular chaperone HSP70. *Nat. Genet.* 23:425–428.

Webb, J.L., B. Ravikumar, J. Atkins, J.N. Skepper, and D.C. Rubinsztein. 2003. Alpha-Synuclein is degraded by both autophagy and the proteasome. *J. Biol. Chem.* 278:25009–25013.

Wullschleger, S., R. Loewith, and M.N. Hall. 2006. TOR signaling in growth and metabolism. *Cell.* 124:471–484.

Zeng, L.H., Y. Ouyang, V. Gazit, J.R. Cirrito, L.A. Jansen, K.C. Ess, K.A. Yamada, D.F. Wozniak, D.M. Holtzman, D.H. Gutmann, and M. Wong. 2007. Abnormal glutamate homeostasis and impaired synaptic plasticity and learning in a mouse model of tuberous sclerosis complex. *Neurobiol. Dis.* 28:184–196.