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Bax-like protein Drob-1 protects neurons from expanded polyglutamine-induced toxicity in *Drosophila*

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Bcl-2 family proteins regulate cell death through the mitochondrial apoptotic pathway. Here, we show that the *Drosophila* Bax-like Bcl-2 family protein Drob-1 maintains mitochondrial function to protect cells from neurodegeneration. A pan-neuronal knockdown of Drob-1 results in lower locomotor activity and a shorter lifespan in adult flies. Either the RNAi-mediated downregulation of Drob-1 or overexpression of Drob-1 antagonist Buffy strongly enhances the polyglutamine-induced accumulation of ubiquitinated proteins and subsequent neurodegeneration. Furthermore, ectopic expression of Drob-1 suppresses the neurodegeneration and premature death of flies caused by expanded polyglutamine. Drob-1 knockdown decreases cellular ATP levels, and enhances respiratory inhibitor-induced mitochondrial defects such as loss of membrane potential ($\Delta\psi_m$), morphological abnormalities, and reductions in activities of complex I + III and complex II + III, as well as cell death. Taken together, these results suggest that Drob-1 is essential for neuronal cell function, and that Drob-1 protects neurons from expanded polyglutamine-mediated neurodegeneration through the regulation of mitochondrial homeostasis.

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Introduction

A balance between cell proliferation and apoptosis is important for the normal development of multicellular organisms. Superfluous or damaged cells must be removed by apoptosis, while cells required for the subsequent stages of development must be protected by cell survival factors (reviewed in Baehrecke, 2002). The Bcl-2 family of proteins, which includes both anti- and proapoptotic members, plays key regulatory roles in apoptosis. Accumulating evidence in mammalian systems suggests that antiapoptotic Bcl-2 family proteins (e.g., Bcl-2 and Bcl-xL) prevent the mitochondrial release of cytochrome *c*, which is required for the formation of the Apaf-1 apoptosome and therefore caspase activation (Zou *et al*, 1997). Conversely, in response to cell-death stimuli, proapoptotic Bcl-2 proteins (e.g., Bax and Bak) facilitate the release of cytochrome *c* and other death-promoting factors from the mitochondria by forming pores or channels, or by altering the mitochondrial membrane permeability and the structural architecture of the mitochondria (Martinou and Green, 2001; Scorrano *et al*, 2002). Thus, the antideath Bcl-2 family members counteract the function of the prodeath family members.

Bcl-2 family proteins are conserved throughout evolution (Igaki and Miura, 2004). CED-9, a Bcl-2 family protein in the nematode *Caenorhabditis elegans*, plays an essential role in preventing programmed cell death; however, the mechanisms by which Bcl-2 family proteins prevent cell death may not be conserved between *C. elegans* and mammals. A major role of CED-9 is to sequester the caspase-activating protein CED-4 to the mitochondria and to inhibit CED-4 function; this is different from the major role of mammalian Bcl-2 family proteins that control mitochondrial cytochrome *c* release. The *Drosophila* genome encodes Apaf-1 and Bcl-2 family proteins that are structurally and functionally related to their mammalian orthologs (Igaki and Miura, 2004). However, cytochrome *c* may not be required to promote cell death in *Drosophila* S2 cells (Dorstyn *et al*, 2002, 2004; Zimmermann *et al*, 2002), suggesting that *Drosophila* Bcl-2 family proteins play another role that may not be involved in cytochrome *c* release. *Drosophila* has two Bcl-2 family proteins, Drob-1/Debcl/dBorg-1/dBok-1 (Brachmann *et al*, 2000; Colussi *et al*, 2000; Igaki *et al*, 2000; Zhang *et al*, 2000) and Buffy/dBorg-2 (Brachmann *et al*, 2000; Quinn *et al*, 2003). Drob-1 and Buffy share BH1, BH2, BH3, and weak BH4 homology regions and the C-terminal transmembrane region, and structurally belong to the Bax subfamily. Drob-1 has been shown to be a proapoptotic protein based on the observations that (i) ectopic expression of Drob-1 in fly eyes or *Drosophila* S2 cells results in cell death, and that (ii) the functional knockdown of Drob-1 by RNAi leads to the inhibition of cell death in embryos (Brachmann *et al*, 2000; Colussi *et al*, 2000; Igaki *et al*, 2000). In contrast to the proapoptotic function of Drob-1, Buffy can function as an antiapoptotic factor by inhibiting Drob-1 function (Quinn *et al*, 2003).

The aim of the present study was to elucidate the *in vivo* role of Drob-1 in *Drosophila*. The RNAi-mediated knockdown of Drob-1 in embryos prevented most cell deaths, supporting its proapoptotic role. Unexpectedly, a pan-neuronal knockdown of Drob-1 caused lower locomotor behavior activity

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and a shorter lifespan, and it also enhanced polyglutamine-mediated neurodegeneration. The inhibition of Drob-1 function by RNAi or by overexpressing Buffy caused a reduction in cellular ATP levels and an increase in the accumulation of ubiquitinated proteins. In the presence of respiratory inhibitors, Drob-1 knockdown enhanced abnormalities in mitochondrial morphology, loss of $\Delta\psi_m$, and cell death, and decreased complex I + III and complex II + III activities. Furthermore, the overexpression of Drob-1 protected cells from polyglutamine-mediated neurodegeneration. These findings indicate that Drob-1 functions as an antiapoptotic protein in neuronal cells by regulating mitochondrial homeostasis.

Results

Drob-1 positively regulates programmed cell death during embryogenesis

To elucidate the physiological role of Drob-1 in *Drosophila*, we established Drob-1 'knockdown' transgenic lines bearing an inverted-repeat (IR) construct of *drob-1* (*UAS-drob-1-IR*) (Supplementary Figure 1A). The functionality of the *UAS-drob-1-IR* transgene was confirmed by crossing *UAS-drob-1-IR* flies to *UAS-drob-1*^{debcl} (Colussi *et al*, 2000) flies using an eye-specific GAL4 driver *GMR-GAL4*. The expression of the *drob-1-IR* transgene completely blocked the small-eye phenotype caused by Drob-1 overexpression (Supplementary Figure 1B–E). On the other hand, knockdown of Drob-1 did not affect the ablated eye phenotype induced by overexpression of other proapoptotic proteins such as Rpr, Hid, Grim, and Dmp53 (data not shown).

To examine the phenotypes caused by the knockdown of Drob-1, the *UAS-drob-1-IR* transgene was expressed in selected tissues or at selected stages using different GAL4 drivers. As a control, we used a transgenic fly bearing an IR construct of *lac-Z* (*UAS-lacZ-IR*), which showed no detectable phenotypes with GAL4 drivers used. Ubiquitous expression of the *drob-1-IR* transgene at the entire developmental stages using *da-GAL4* driver resulted in a larval lethality (Supplementary Table 1). TUNEL analysis of stage 12–13 *da > drob-1-IR* embryos showed a remarkable reduction in the number of dying cells (Supplementary Figure 1I–K) compared with control *da > lacZ-IR* embryos (Supplementary Figure 1F–H), consistent with previous studies using *drob-1* dsRNA (Brachmann *et al*, 2000; Colussi *et al*, 2000). Knockdown of Drob-1 in the nervous system using *elav-GAL4* caused a semilethal phenotype at the larval–pupal polyphasic stages (Supplementary Table 1). On the other hand, *drob-1-IR* expression targeted to the developing retina or photoreceptor neurons using *GMR-GAL4* or *sev-GAL4* had no effect on eye morphology (data not shown). These observations indicate that the developmental programmed cell death is positively regulated by Drob-1, and that Drob-1 may play different roles at different developmental stages or in different cell types such as neurons.

Pan-neuronal Drob-1 knockdown results in lower locomotor activity and a shorter lifespan

Bcl-2 family proteins play an important role in the nervous system (reviewed in Lossi and Merighi, 2003; Becker and Bonni, 2004). To elucidate the physiological role of Drob-1 in neurons, we examined the effect of a pan-neuronal knockdown of Drob-1 on locomotor behavior and lifespan. The lifespans of two independent pan-neuronal Drob-1 knockdown fly lines (*elav > drob-1-IR#3* and *elav > drob-1-IR#10*) were markedly shorter than those of control *elav > lacZ-IR* flies (Figure 1A) (mean lifespans: *elav > lacZ-IR*, 71.1 ± 3.9 days; *elav > drob-1-IR#3*, 46.2 ± 2.1 days; *elav > drob-1-IR#10*, 44.6 ± 1.8 days; Supplementary Table 2). The frequency of locomotion in *elav > drob-1-IR* flies (at days 13–16) was significantly reduced compared with that in control flies (Figure 1B). These data reveal that Drob-1 is essential for neuronal cells to maintain flies' normal locomotion and lifespan.

Downregulation of Drob-1 leads to cellular ATP depletion

Progressive mitochondrial dysfunction is thought to be an important pathogenic mechanism that leads to irreversible damage in neuronal cells (reviewed in Orth and Schapira, 2001). Mitochondrial dysfunction is also involved in the aging process in both invertebrates and vertebrates, including humans (reviewed in Lenaz *et al*, 2000, 2002; Golden *et al*, 2002; Pollack *et al*, 2002; Tsang and Lemire, 2003). We therefore analyzed the ATP levels in Drob-1 knockdown flies as a marker of mitochondrial metabolism. The ATP levels in Drob-1 knockdown flies were significantly less than those in control fly lines (Figure 1C). On the other hand, overexpression of Drob-1 did not affect the ATP levels (Figure 1C). Another *Drosophila* Bcl-2 family protein, Buffy, can bind to and inactivate Drob-1 (Quinn *et al*, 2003). We found that overexpression of Buffy reduced the ATP levels in adult flies (Figure 1C). We confirmed that the levels of *drob-1* mRNA were markedly reduced, but the levels of *buffy* were unaffected in flies expressing *drob-1-IR* (data not shown). The *buffy* mRNA levels were also unaffected in flies overexpressing Drob-1 (data not shown). These results suggest that the inactivation of Drob-1 induces changes in mitochondrial energy metabolism and leads to ATP depletion.

Downregulation of Drob-1 enhances polyglutamine-induced toxicity

Since we found that Drob-1 plays a crucial role in neurons, we next asked whether the reduction of Drob-1 would affect neurodegeneration. We investigated the role of Drob-1 in the pathogenesis of a fly model of polyglutamine disease, since the expression of expanded polyglutamine decreases the cellular concentrations of ATP (Sanchez *et al*, 2003). A fly model of Machado–Joseph disease (MJD), generated by overexpression of a truncated form of the human MJD protein with an expanded polyglutamine stretch (MJDtr-Q78), shows progressive neural degeneration (Warrick *et al*, 1998). Consistent with previous findings (Warrick *et al*, 1998, 1999), we found that the targeted expression of expanded polyglutamine in neurons using the *elav-GAL4* driver resulted in early adult death (Figure 2A and B). Flies expressing both *drob-1-IR* and MJDtr-Q78 in their neurons showed a significantly shorter lifespan than did the flies expressing MJDtr-Q78 alone, suggesting that the downregulation of Drob-1 increases the neural toxicity caused by expanded polyglutamine (Figure 2A and B).

The expression of pathogenic human expanded polyglutamine proteins (e.g., MJD protein and huntingtin) in *Drosophila* compound eyes elicits late-onset degeneration and the loss of photoreceptor neurons. The ectopic expression

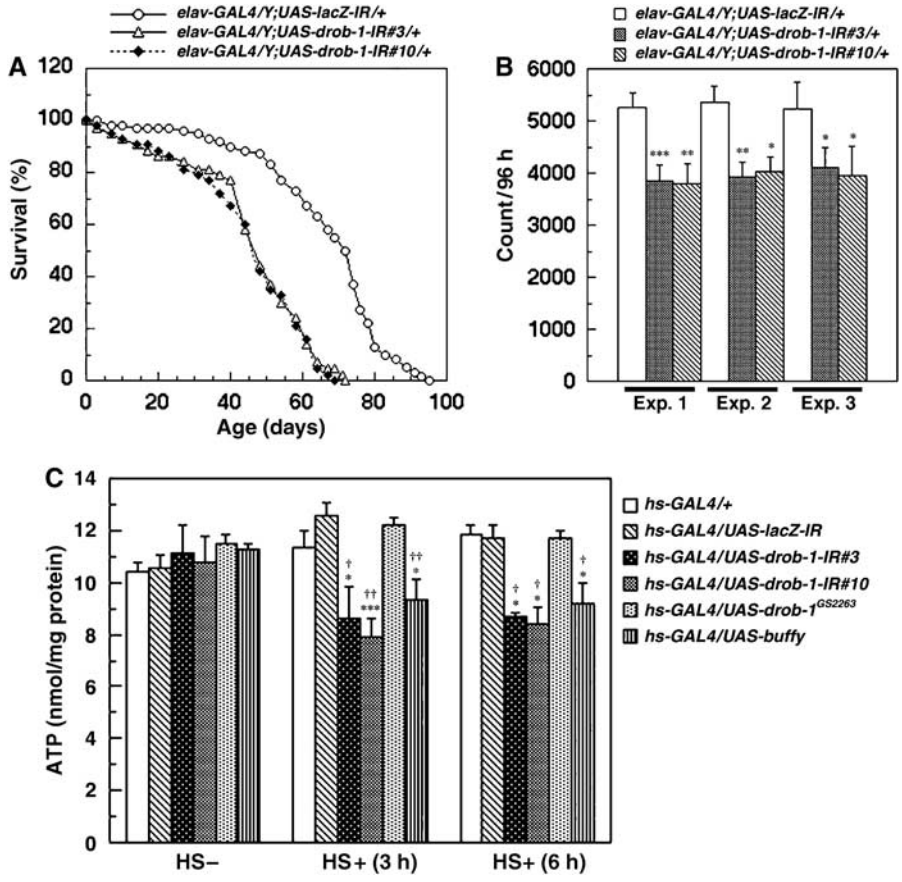

Figure 1 RNAi-mediated knockdown of Drob-1 results in a shorter lifespan, lower locomotor activity, and ATP depletion. (**A**) Expression of the *UAS-drob-1-IR* or *UAS-lacZ-IR* transgene was targeted to cells in the peripheral and central nervous systems using an *elav-GAL4* driver. Two independent *elav>drob-1-IR* fly lines (#3 and #10) show significantly shorter lifespans than do control flies (*elav>lacZ-IR*). (**B**) Locomotor activity was analyzed using the DAM system as described in Materials and methods. Two independent *elav>drob-1-IR* fly lines (#3 and #10) show lower locomotion activity for 96 h (13–16 days after eclosion) than do control flies (*elav>lacZ-IR*). Each bar in the graph shows the mean ± s.e.m. of 32 flies ($n=32$) of each genotype. Three independent experiments (Exp. 1–3) were performed. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.005$ relative to control by Student's *t*-test. (**C**) ATP levels in *hs-GAL4/+*, *hs-GAL4/UAS-lacZ-IR*, *hs-GAL4/UAS-drob-1-IR#3*, *hs-GAL4/UAS-drob-1-IR#10*, *hs-GAL4/UAS-drob-1*^{GS2263}, and *hs-GAL4/UAS-buffy* adult flies (4 days after eclosion) 3 or 6 h after treatment with or without heat shock (twice at 37°C for 30 min with a 30 min interval) were measured as described in Materials and methods. Each value shows the mean ± s.e.m. of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.005$ for each value as compared with *hs-GAL4/+*, $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, and $^{\dagger\dagger\dagger}P<0.005$ for each value as compared with *hs-GAL4/UAS-lacZ-IR* by Student's *t*-test. GS2263 is a fly line that can overexpress untagged Drob-1 in a GAL4-dependent manner.

of *MJDtr-Q78* led to late-onset degeneration in adult eyes (Figure 2C–F) (Jackson *et al*, 1998; Warrick *et al*, 1998). We found that the coexpression of *drob-1-IR* with *MJDtr-Q78* remarkably accelerated the onset of the neurodegeneration (Figure 2G–J and Supplementary Figure 2). As shown in Figure 2K–N (external eyes) and Figure 2O–R (tangential sections of the eyes), the knockdown of Drob-1 strongly enhanced the polyglutamine-induced lack of pigment and severe loss of retinal structure in day 1 flies (Figure 2L, M, P, and Q). The *GMR>drob-1-IR* eye was normal as compared with the control eye bearing only the promoter transgene, *GMR-GAL4* (Figure 2K, N, O, and R). The overexpression of Buffy also enhanced the polyglutamine-induced neurodegeneration (Figure 3D and E) and lethality (Figure 3A and B and Supplementary Table 2), suggesting that Buffy acts as a proneurodegenerative factor by inactivating Drob-1. Indeed, the eyes coexpressing Drob-1, Buffy, and MJDtr-Q78 showed significantly weaker degenerative eye phenotype than the eyes expressing Buffy and MJDtr-Q78 (Supplementary Figure 3), supporting the notion that Buffy enhances neurodegeneration by suppressing Drob-1.

Overexpression of Drob-1 suppresses expanded polyglutamine-induced neurodegeneration

Overexpression of HA-tagged Drob-1 in the *Drosophila* compound eye causes a small-eye phenotype (Colussi *et al*, 2000; Igaki *et al*, 2000); however, nontagged Drob-1 does not (Brachmann *et al*, 2000). We used nontagged Drob-1 (*UAS-drob-1*^{GS2263}) to examine whether Drob-1 could function as a protective factor against neurodegeneration induced by expanded polyglutamine. The pan-neuronal moderate expression of Drob-1 slightly expanded the lifespan (mean lifespans: *elav>lacZ*, 72.3 ± 1.3; *elav>drob-1*^{GS2263}, 78.3 ± 2.3; $P<0.05$) (Figure 4A and Supplementary Table 2). On the other hand, the pan-neuronal overexpression of Buffy caused a shorter lifespan (mean lifespan: *elav>buffy*, 63.6 ± 4.7; $P<0.05$) (Figure 4A and Supplementary Table 2). The ectopic expression of Drob-1 at moderate levels, which showed no detectable phenotype in the eye on its own (Figure 4F and I), suppressed the MJDtr-Q78-induced neurodegeneration (Figure 4D, E, G, and H). Similar results were also obtained using flies bearing the *GMR-drob-1* transgene, which did not cause the rough-eye phenotype by itself (Supplementary

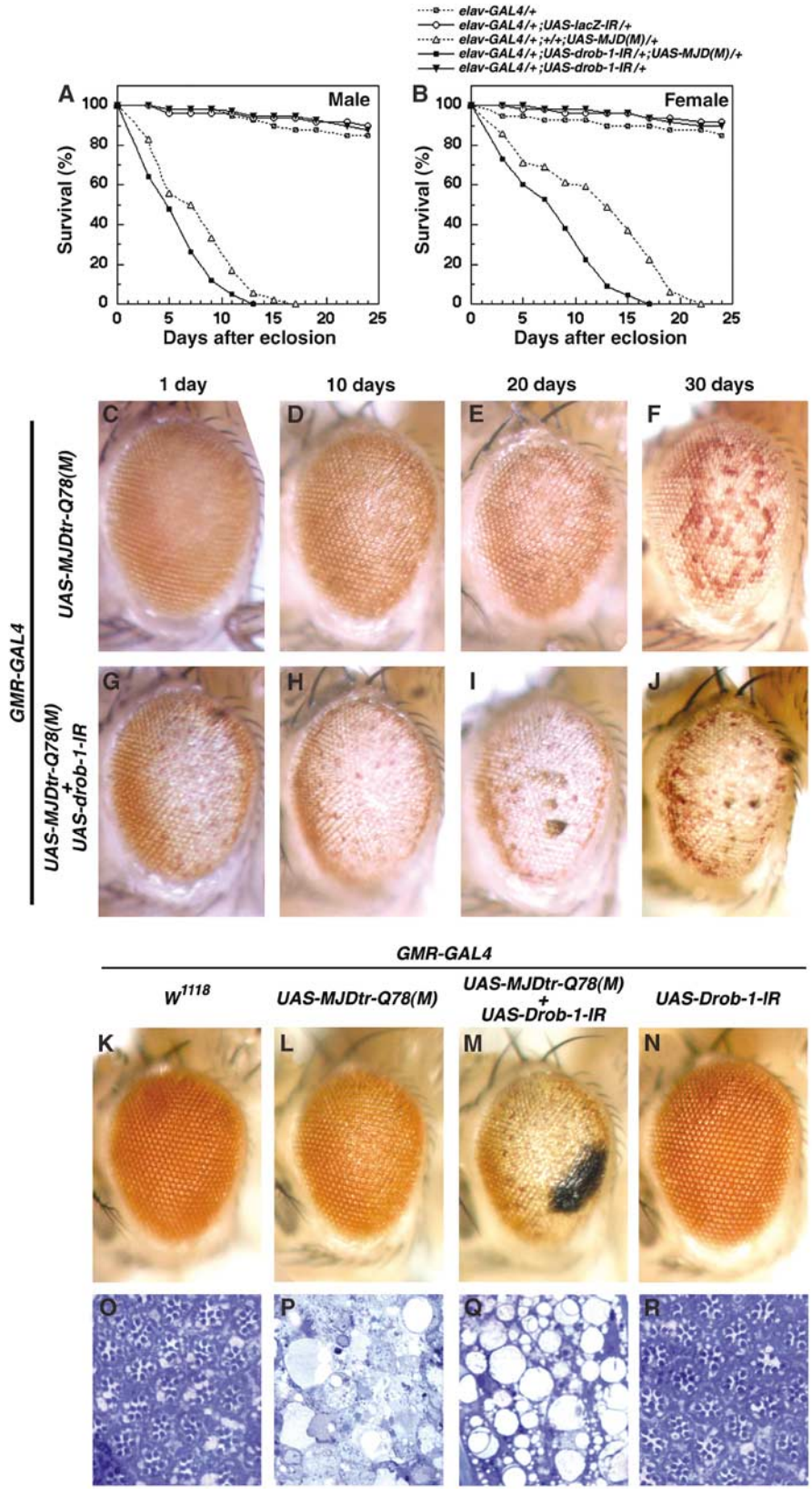

Figure 2 RNAi-mediated knockdown of Drob-1 enhances polyglutamine-induced neuronal toxicity. (**A, B**) Expression of the *UAS-drob-1-IR* or *UAS-MJDtr-Q78* transgene was targeted to the nervous system using the *elav-GAL4* driver. We used two control fly lines: one bears only the *elav-GAL4* transgene and the other bears both the *UAS-lacZ-IR* and *elav-GAL4* transgenes. The *elav>drob-IR, MJDtr-Q78(M)* flies died earlier than the *elav>MJDtr-Q78(M)* flies. (**C–J**) Light micrographs of the eyes of flies 1 day (C, G), 10 days (D, H), 20 days (E, I), and 30 days (F, J) after eclosion are shown. (**K–R**) Eyes (K–N) and tangential sections of the eyes (O–R) of flies 1 day after eclosion are shown. (K, O) Control flies bearing only the driver *GMR-GAL4* show normal eyes. Genotypes are as follows: *GMR-GAL4/CySM1; UAS-MJDtr-Q78(M)/+* (C–F), *GMR-GAL4/UAS-drob-1-IR; UAS-MJDtr-Q78(M)/+* (G–J), *GMR-GAL4/CySM1* (K, O), *GMR-GAL4/CySM1*; *UAS-MJDtr-Q78(M)/+* (L, P), *GMR-GAL4/UAS-drob-1-IR*; *UAS-MJDtr-Q78(M)/+* (M, Q), and *GMR-GAL4/UAS-drob-1-IR*; *TM3, Sb/+* (N, R).

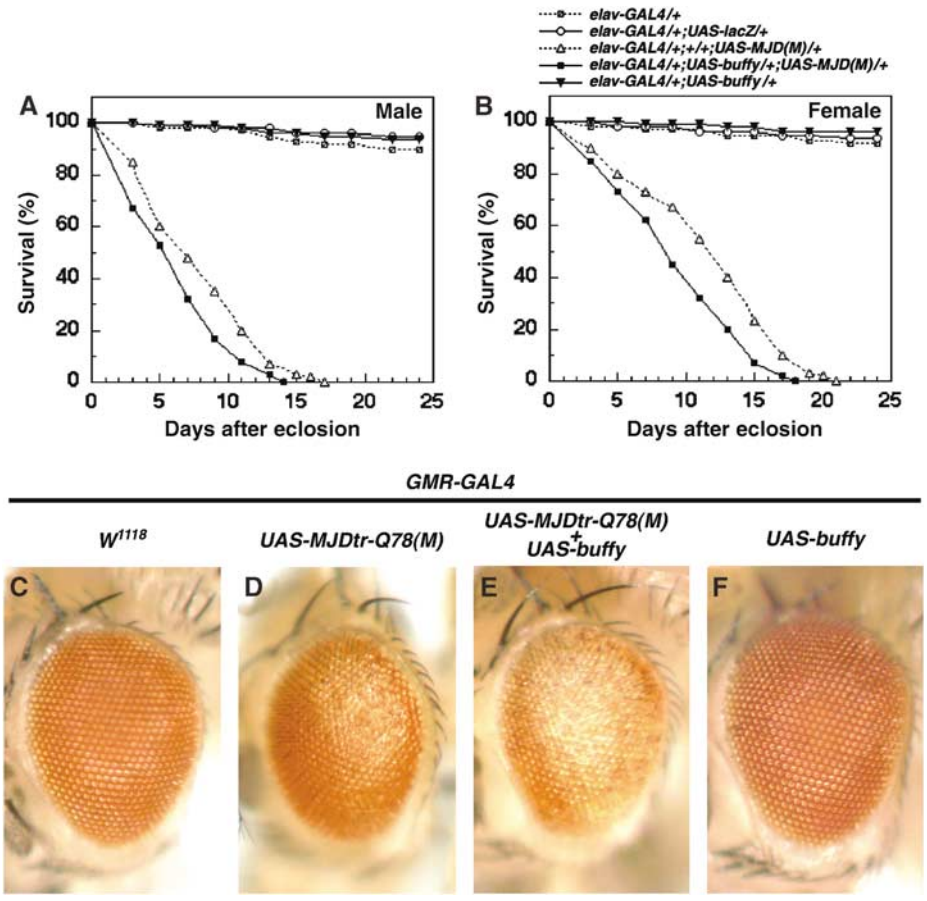

Figure 3 Buffy, a Drob-1 antagonist, enhances polyglutamine-induced neurodegeneration. (**A, B**) Longevity curve of each genotype indicated is shown. (**C–F**) Adult eyes of 1-day-old flies with the following genotypes are shown: (C) *GMR-GAL4/CySM1*, (D) *GMR-GAL4/CySM1; UAS-MJDtr-Q78/+*, (E) *GMR-GAL4/UAS-buffy; UAS-MJDtr-Q78/+*, and (F) *GMR-GAL4/UAS-buffy.*

Figure 4). In addition, Drob-1 markedly rescued the early adult death of flies expressing MJDtr-Q78 in their neurons (Figure 4B and C and Supplementary Table 2). Together, these results suggest that Drob-1 can protect neurons from polyglutamine-mediated neurodegeneration.

Drob-1 is required to reduce the accumulation of undegraded proteins caused by expanded polyglutamine

The overexpression of expanded polyglutamine reduces proteasome activity and increases the accumulation of undegraded proteins in S2 cells and flies (Kanuka *et al*, 2003). We found that the downregulation of Drob-1 or overexpression of Buffy resulted in an increase of ubiquitinated proteins in *Drosophila* heads (Figure 5A). In addition, coexpression of *MJDtr-Q78* with *drob-1-IR* or *buffy* enhanced the accumulation of ubiquitinated proteins (Figure 5A). We therefore pursued the role of Drob-1 in the accumulation of ubiquitinated proteins and subsequent cell death in *Drosophila* S2 cells. The accumulation of ubiquitinated proteins caused by proteasome inhibitors such as lactacystin or MG-132 was accelerated by knocking down of Drob-1 (Figure 5B and Supplementary Figure 5A). The proteasome inhibitors caused a severe reduction in cell viability that was greatly enhanced by Drob-1 knockdown (Figure 5C and Supplementary Figure 4B). On the other hand, the knockdown of Buffy, which may activate Drob-1 function, significantly suppressed the proteasome inhibition-induced cell death (Figure 5C and Supplementary Figure 5B). In contrast, the knockdown of either Drob-1 or Buffy did not affect the cell death induced by tunicamycin (Figure 5D). These observations suggest that Drob-1 protects cells from cytotoxicity induced by the disruption of proteasome function. Supporting this idea, the downregulation of Drob-1, but not of Buffy, induced cellular ATP depletion (Figure 5E) that may cause a suppression of proteasome function (Beal *et al*, 1993; Sanchez *et al*, 2003).

Downregulation of Drob-1 enhances mitochondrial dysfunction

Expanded polyglutamine protein causes ATP loss, a mitochondrial dysfunction (Sanchez *et al*, 2003). The mitochondrial complex II enzyme activity is selectively decreased in the striatum in Huntington's disease (HD) patients (reviewed in Browne and Beal, 2004). In addition, mitochondrial membrane depolarization may be involved in neuronal cell death in HD (Panov *et al*, 2002; Ruan *et al*, 2004). These studies suggest that an impairment of mitochondrial respiratory function may play a role in the pathogenesis of polyglutamine diseases. We therefore examined in S2 cells whether the knockdown of Drob-1 affects mitochondrial membrane depolarization and cell death induced by mitochondrial respiratory chain inhibitors such as rotenone, a complex I inhibitor, or 3-nitropropionic acid (3-NP), a mitochondrial complex II

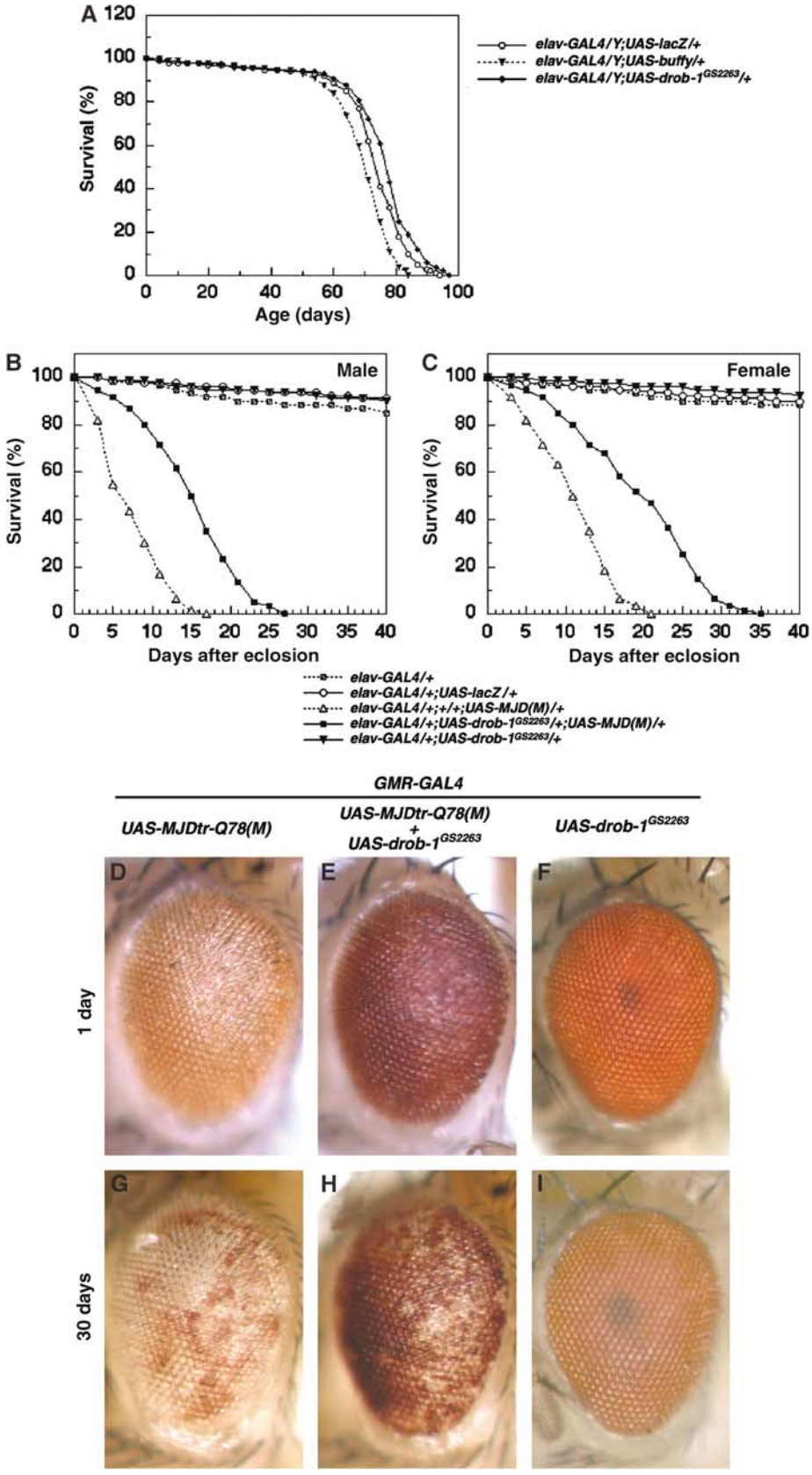

Figure 4 Drob-1 protects neurons from polyglutamine-induced toxicity. (**A–C**) Longevity curve of each genotype indicated is shown. (**D–I**) External eye phenotypes of (D, G) *GMR-GAL4/+; UAS-MJDtr-Q78/+*, (E, H) *GMR-GAL4/UAS-drob-1^{GS2263}; UAS-MJDtr-Q78/+*, and (F, I) *GMR-GAL4/UAS-drob-1^{GS2263}*, 1 day (D–F) or 30 days (G–I) after eclosion are shown. GS2263 is a fly line that can overexpress untagged Drob-1 in a GAL4-dependent manner.

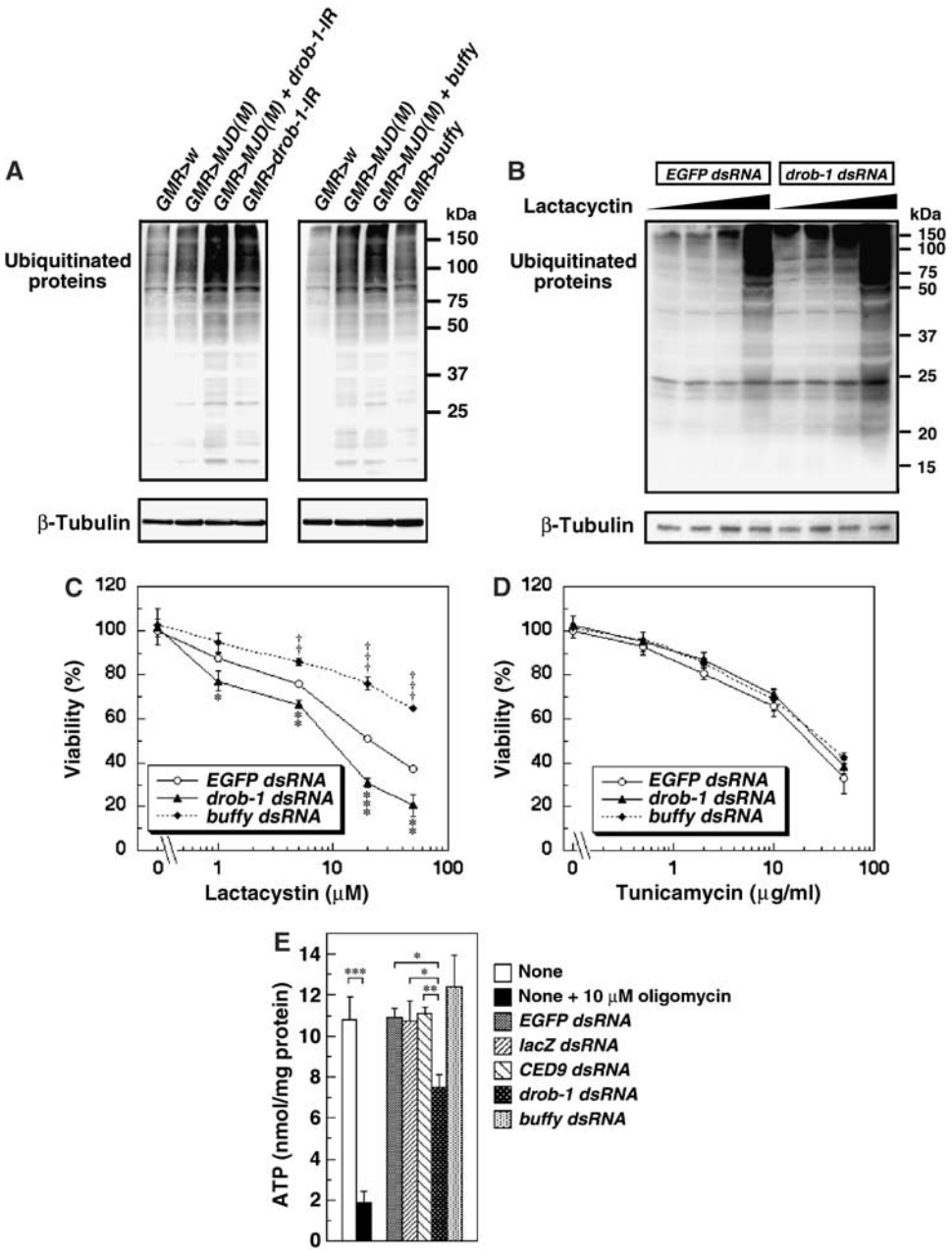

Figure 5 Knockdown of Drob-1 enhances the accumulation of ubiquitinated proteins. (**A**) Fly heads from each line of the indicated genotype (1 day after eclosion) were subjected to immunoblotting with an anti-ubiquitin antibody and anti-β-tubulin antibody. (**B**) S2 cells were cultured with either *EGFP* dsRNA or *drob-1* dsRNA for 48 h, and incubated with or without lactacystin (1, 5, and 20 μM) for an additional 24 h as described in Materials and methods. The cell lysate was subjected to Western analysis using the anti-ubiquitin and anti-β-tubulin antibodies. (**C, D**) S2 cells were transfected with *EGFP*, *drob-1*, or *buffy* dsRNA for 48 h, and left untreated or treated with lactacystin, a proteasome inhibitor (1, 5, 20, and 50 μM) (B), or tunicamycin, an inhibitor of N-glycosylation that induces the rapid unfolded protein response (UPR) (0.5, 2, 10, and 50 μg/ml) (C), for 24 h. Cell viability was determined by cell death assay as described in Materials and methods. Mean ± s.e.m., $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.0005$ for cells with *drob-1* dsRNA, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.005$, and $^{\dagger\dagger\dagger}P < 0.0005$ for cells with *buffy* dsRNA as compared with control cells (with *EGFP* dsRNA) by paired Student's *t*-test. (**E**) Cells were cultured with or without 10 μM oligomycin, an inhibitor of mitochondrial ATP synthase, for 1 h, or with *EGFP* dsRNA, *lacZ* dsRNA, *ced-9* dsRNA, *drob-1* dsRNA, or *buffy* dsRNA for 72 h. Cellular ATP content was measured as described in Materials and methods. Each value shows the mean ± s.e.m. of four ($n = 4$) independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.005$ by paired Student's *t*-test.

inhibitor that is used for generating animal models of HD (Beal *et al*, 1993; Browne and Beal, 2004). JC-1 is widely used to measure the mitochondrial depolarization in live cell. JC-1 monomer exhibits green fluorescence and JC-1 aggregate (J-aggregate) at high concentration exhibits red fluorescence (Smiley *et al*, 1991). It allows us to label the mitochondria as well as to observe membrane potential ($\Delta\psi_m$). Although TMRM or TMRE would be more appropriate for quantitative assay of membrane potential, J-aggregate formation increases linearly with applied membrane potential in a limited range and was therefore used for a qualitative analysis of $\Delta\psi_m$. Treatment with either rotenone or 3-NP reduced the presence of red J-aggregates, indicating a relative decrease of $\Delta\psi_m$, in a dose-dependent manner (Figure 6). The knockdown of Drob-1

strongly enhanced the rotenone- or 3-NP-induced reduction of $\Delta\psi_m$ (Figure 6). Moreover, the downregulation of Drob-1 enhanced cell death induced by either rotenone or 3-NP (Figure 7A and B). The knockdown of Drob-1, without complex I or complex II inhibition, did not affect $\Delta\psi_m$ and cell viability (Figures 6 and 7). On the other hand, the knockdown of Buffy significantly suppressed rotenone- or 3-NP-induced cell death (Figure 7A and B). In contrast, no difference was seen in the cell death induced by other mitochondrial inhibitors such as antimycin A (complex III), KCN (complex IV), or oligomycin (complex V) in S2 cells treated with control, *drob-1*, or *buffy* dsRNA (Figure 7C–E). We further analyzed morphological and biochemical alterations in mitochondria in Drob-1 knockdown S2 cells following the treatment with rotenone or 3-NP. In analyzing with transmission electron microscopy, we observed three types of previously described mitochondrial morphologies: normal mitochondria, swollen and higher electron-dense mitochondria, and swollen and lower electron-dense mitochondria with disrupted outer membrane (Ghadially, 1982; Angermuller *et al*, 1998; Sesso *et al*, 2004). It has been reported that the morphology of mitochondria with ruptured membrane is a sign of the very early stage of apoptosis, and it might be involved in mitochondrial permeability transition and loss of $\Delta\psi_m$ (Angermuller *et al*, 1998; Sesso *et al*, 2004). We found that Drob-1 knockdown significantly increased the percentage of mitochondria showing abnormal morphologies in S2 cells with intact nuclei (Figure 8A–G). In addition, the downregulation of Drob-1 significantly enhanced the reduction of activities of mitochondrial respiratory chain complex I + III (NADH-cytochrome *c* oxidoreductase) and complex II + III (succinate-cytochrome *c* oxidoreductase) in S2 cells treated with rotenone or 3-NP (Figure 8H and I). Together, these data suggest that Drob-1 plays an important role in the maintenance of mitochondrial homeostasis, and may protect cells from neurodegeneration caused by mitochondrial dysfunction through a defect in complex I or complex II.

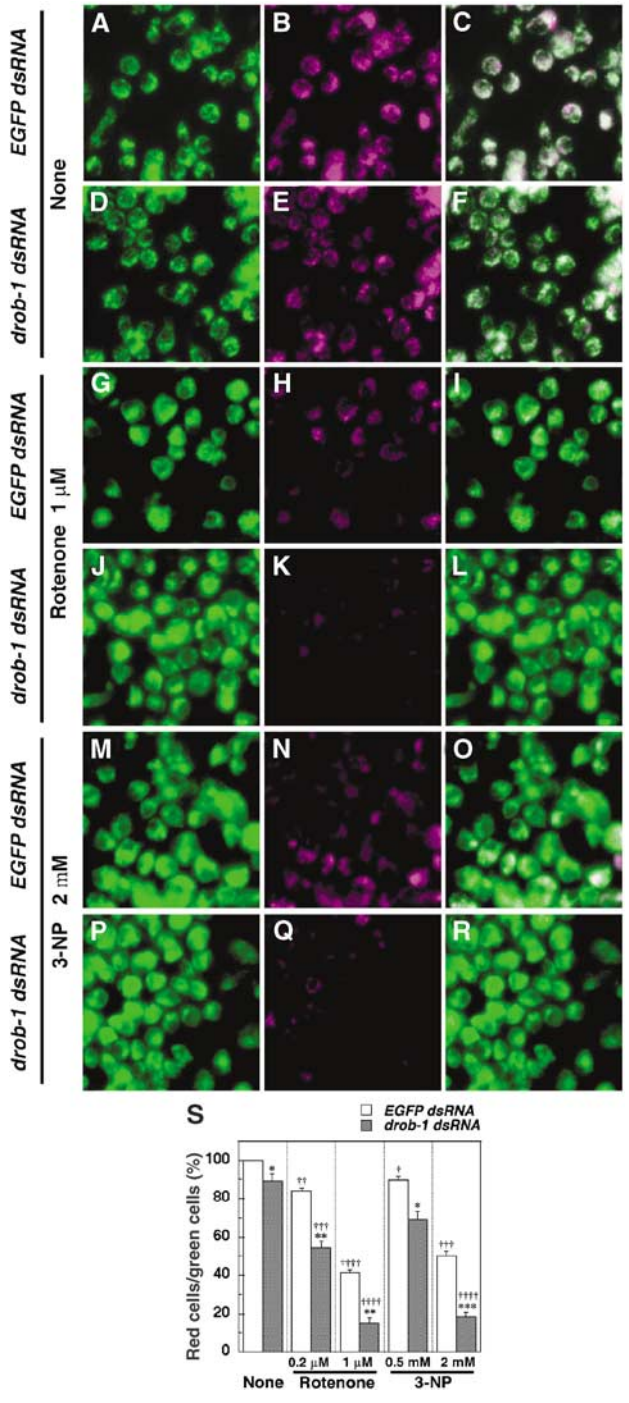

Figure 6 Knockdown of Drob-1 enhances rotenone- or 3-NP-induced loss of mitochondrial membrane potential ($\Delta\psi_m$) in S2 cells. (**A–R**) Cells were cultured with either *EGFP* dsRNA or *drob-1* dsRNA for 48 h, and then incubated with or without rotenone (0.2 and 1 μM) or 3-NP (0.5 and 2 mM) for an additional 24 h. After the treatment, cells were labeled with $\Delta\psi_m$-sensitive dye JC-1 and imaged with confocal microscope as described in Materials and methods. Total (inactive + active) and active mitochondria are labeled green JC-1 monomers (A, D, G, J, M, and P) and red J-aggregates (B, E, H, K, N, and Q) fluorescence, respectively (see Materials and methods). Overlay image of green and red JC-1 fluorescence is shown in panels C, F, I, L, O, and R. (**S**) Quantitative analysis of $\Delta\psi_m$ was performed by calculating the percentage of the number of red J-aggregates fluorescence-labeled cells out of that of total cells (green JC-1 monomers fluorescence-labeled cells) as described in Materials and methods. Each value shows the mean ± s.e.m. of three independent experiments. Approximately 300 cells were analyzed per each condition in each experiment. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.005$ for each value as compared with *EGFP* dsRNA-treated cells under each experimental condition, $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.005$, and $^{\dagger\dagger\dagger\dagger}P<0.001$ for each value as compared with control (cells treated with *EGFP* dsRNA only) by Student's *t*-test.

Discussion

In this report, we have shown that Drob-1 can either promote (cell death during embryogenesis) or inhibit (polyglutamine-induced neurodegeneration) cell death, depending on a variety of conditions. In addition, we have shown that Buffy also has dual functions, namely a survival function (for cell death during embryogenesis) (Quinn *et al*, 2003) and a proapoptotic function (for polyglutamine-induced neurodegeneration).

Certain members of the Bcl-2 family proteins can function as both anti- and prodeath factors. Antiapoptotic Bcl-2 and Bcl-xL can be converted into proapoptotic proteins when they are cleaved by caspases or by other proteases (Cheng *et al*, 1997; Clem *et al*, 1998). The resulting C-terminal fragments have a 'Bax-like' prodeath activity that induces cytochrome *c* release from mitochondria and forms pores in synthetic

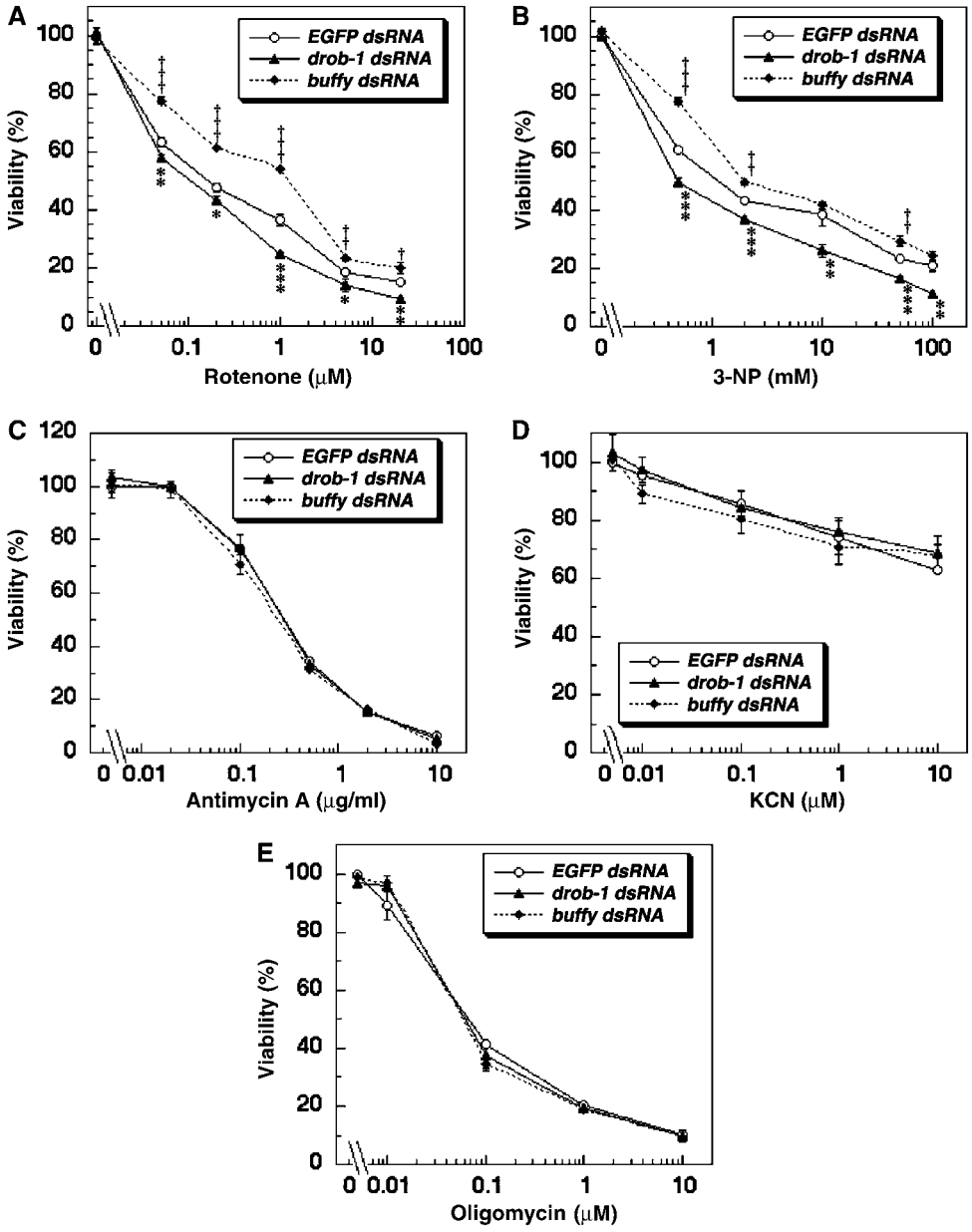

Figure 7 Knockdown of Drob-1 specifically enhances rotenone- or 3-NP-induced cell death in S2 cells. (**A–E**) Cells were transfected with *EGFP*, *drob-1*, or *buffy* dsRNA for 48 h, and left untreated or treated with rotenone (0.05, 0.2, 1, 5, and 20 μM) (A), 3-NP (0.5, 2, 10, 50, and 100 mM) (B), antimycin A (0.02, 0.1, 0.5, 2, and 10 μg/ml) (C), KCN (0.01, 0.1, 1, and 10 μM) (D), or oligomycin (0.01, 0.1, 1, and 10 μM) (E). Mean±s.e.m., $n=3$, $^{*}P<0.05$, $^{**}P<0.005$, and $^{***}P<0.0005$ for cells with *drob-1* dsRNA, $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.005$, and $^{\dagger\dagger\dagger}P<0.0005$ for cells with *buffy* dsRNA as compared with control cells (with *EGFP* dsRNA) by paired Student's *t*-test.

membranes (Kirsch *et al*, 1999; Basanez *et al*, 2001). *C. elegans* CED-9 also exhibits prodeath as well as antideath activity (Hengartner and Horvitz, 1994). Proapoptotic Bax and Bak may promote or inhibit neuronal death depending on the specific death stimulus, neuron subtype, and stage of postnatal development (Lewis *et al*, 1999; Fannjiang *et al*, 2003). Bax promotes the survival of trigeminal ganglia neurons during development in mice that are deficient in NGF or TrkA, while it promotes the death of superior cervical ganglia neurons in the same models (Middleton and Davies, 2001). Bax potently protects mice and cultured hippocampal neurons from Sindbis virus-induced apoptosis, whereas it promotes the death of Sindbis virus-infected dorsal root ganglia neurons (Lewis *et al*, 1999). Bak protects hippocampal neurons from the cell death caused by excitotoxicity or viral infection; however, as mice mature, Bak function is converted from anti- to prodeath in virus-infected spinal cord neurons (Fannjiang *et al*, 2003). Bak also protects mice from kainate-induced seizures, suggesting a possible role in regulating synaptic activity (Fannjiang *et al*, 2003). Drob-1 has also been shown to have a protective activity against serum-deprivation- or CED-3-induced S2 cell death (Brachmann *et al*, 2000). Thus, individual Bcl-2 family proteins can have a pro- or antiapoptotic function, depending on the cellular context or specific stimulus. These findings, combined with our observations in this study, suggest that the dual-function nature of Bcl-2 family proteins may be evolutionarily conserved from nematodes to mammals.

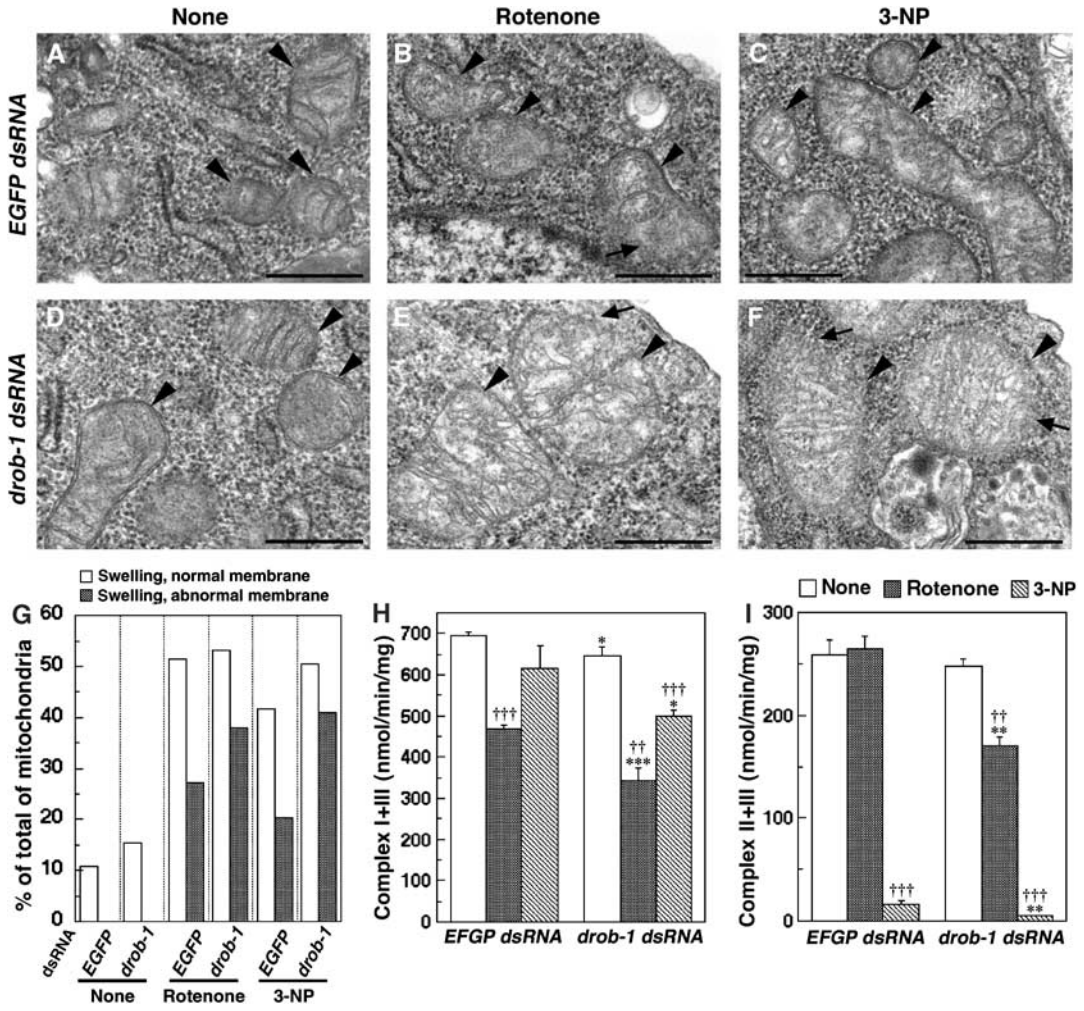

Figure 8 Knockdown of Drob-1 enhances rotenone- or 3-NP-induced mitochondrial morphological abnormalities and decreases activities of respiratory chain complexes in S2 cells. (**A–F**) Electron microscopy images of mitochondria in *EGFP* dsRNA- or *drob-1* dsRNA-treated S2 cells with or without the treatment with rotenone or 3-NP (arrowheads: mitochondria). Rotenone or 3-NP induced swollen and lower electron-dense mitochondria with disrupted outer membranes, signs of degeneration and derangement of the membranes (arrows). Scale bars correspond to 500 nm. (**G**) Quantification of mitochondrial morphological abnormalities. Mitochondria were classified into three types based on their morphologies: normal mitochondria, swollen and higher electron-dense mitochondria, and swollen and lower electron-dense mitochondria with abnormal membrane structures. Approximately 150 mitochondria in at least 15 cells were analyzed in an experimental group. (**H, I**) Mitochondrial complex I + III and complex II + III activities. Mean ± s.e.m., $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.0005$ for cells with *drob-1* dsRNA compared with cells with *EGFP* dsRNA, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.005$, and $^{\dagger\dagger\dagger}P < 0.0005$ for cells with rotenone or 3-NP as compared with cells without drugs (none) by paired Student's *t*-test.

Mitochondrial function (i.e., the production of ATP, regulation of apoptosis, and production of reactive oxygen species (ROS)) is crucial for the maintenance of postmitotic tissues (e.g., muscles and brain) in normal aging, and plays a role in degenerative diseases in humans and in animal models (reviewed in Lenaz *et al*, 2000, 2002; Orth and Schapira, 2001; Golden *et al*, 2002; Pollack *et al*, 2002; Tsang and Lemire, 2003). During normal aging and the progression of human degenerative diseases, a decrease in the total number of cells in some postmitotic tissues (e.g., heart, skeletal muscle, and brain) is associated with a reduction in mitochondrial metabolic activity (reviewed in Lenaz *et al*, 2000, 2002; Orth and Schapira, 2001; Pollack *et al*, 2002). We have shown that Drob-1 plays an important role in the survival of postmitotic neurons under both physiological and pathological conditions. Importantly, our finding that downregulation of Drob-1 results in a decrease in cellular ATP levels suggests that Drob-1 may be involved in the maintenance of mitochondrial metabolism (Figures 1 and 5). In addition, Drob-1 protects cells from stresses that cause mitochondrial dysfunction (Figures 6 and 7). Thus, our results suggest that Drob-1 may regulate the homeostasis of neurons and the aging process by maintaining mitochondrial metabolism. The ubiquitin–proteasome system plays a crucial role in preventing the polyglutamine-induced accumulation of unfolded proteins (reviewed in Ciechanover and Brundin, 2003). This system acts in an ATP-dependent manner. Inhibition of the mitochondrial respiratory chain by the complex I inhibitor rotenone reduces the ubiquitin-proteasomal activity in both rat primary dopaminergic neurons and human SH-SY5Y neuroblastoma cells (Hoglinger *et al*, 2003; Shamoto-Nagai *et al*, 2003). Expanded polyglutamine protein has been reported to cause mitochondrial dysfunction, ATP loss, a defect in complex II enzyme activity, and subsequent

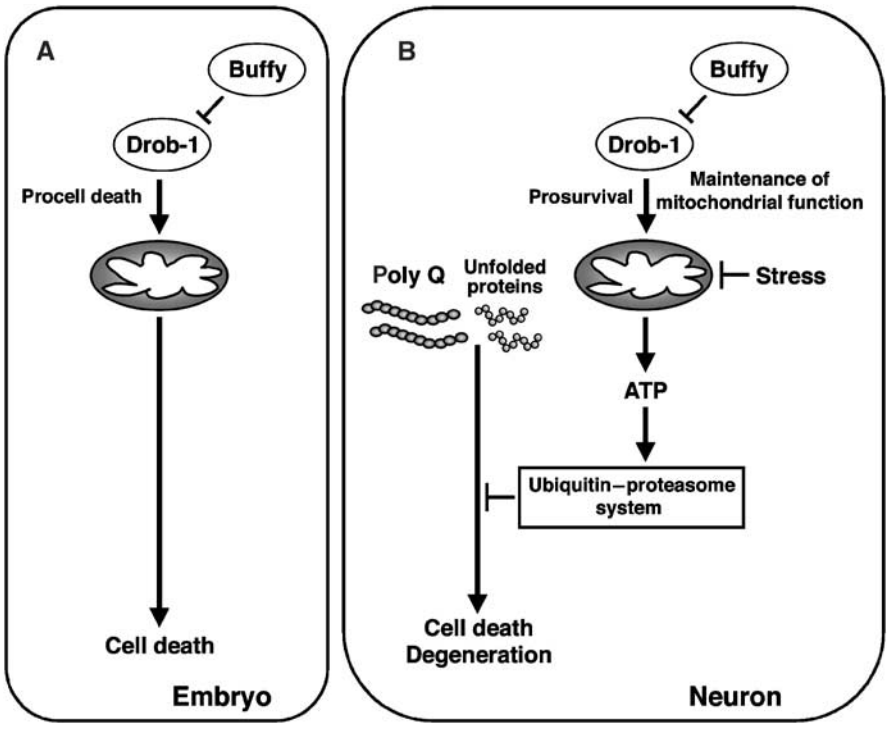

Figure 9 A model for the context-dependent dual function of Drob-1. Drob-1 plays an important role in facilitating programmed cell death during embryogenesis (**A**). Drob-1 can also protect neurons from polyglutamine-, unfolded protein-, or mitochondrial inhibition-induced pathological cell death and degeneration (**B**). Drob-1 may regulate mitochondrial ATP homeostasis thereby attenuating the toxicity caused by disruption of the ubiquitin–proteasome system. Buffy can antagonize both the pro- and anti-cell-death function of Drob-1.

inhibition of the ATP-dependent ubiquitin–proteasome system (Beal *et al*, 1993; Kanuka *et al*, 2003; Sanchez *et al*, 2003). Our results, combined with these previous findings, led us to propose a model in which Drob-1 suppresses polyglutamine-induced ATP depletion, thereby facilitating the subsequent activation of the ubiquitin–proteasome system, which protects neurons from cell death and degeneration. Buffy can antagonize this survival function of Drob-1 in neurons (Figure 9).

We have shown that downregulation of Drob-1 induces ATP depletion and a shorter lifespan in flies. In *C. elegans*, mitochondrial complex II deficiency causes a shorter lifespan, hypersensitivity to oxidative stress, energy depletion, ROS overproduction, and CED-3- and CED-4-dependent supernumerary cell death (Ishii *et al*, 1998; Senoo-Matsuda *et al*, 2001, 2003). In the complex II-deficient *C. elegans* mutant *mev-1*, the shorter lifespan is partially rescued by a loss-of-function mutation of CED-3, suggesting that the supernumerary apoptosis may contribute to shortening the lifespan in *C. elegans* (Senoo-Matsuda *et al*, 2003). Interestingly, the shorter lifespan in the *mev-1* mutant may be associated with a decrease in the mitochondrial localization of CED-9 and its downregulation (Senoo-Matsuda *et al*, 2003). In mammals, Bcl-xL can prevent the perturbation of mitochondrial ATP/ADP exchange caused by growth factor deprivation, and can maintain oxidative phosphorylation under the growth-factor-withdrawal condition (Vander Heiden *et al*, 1999). The proapoptotic Bcl-2 family protein Bad is required to assemble the mitochondria-based glucokinase complex, which regulates glycolysis (Danial *et al*, 2003). Thus, the regulation of mitochondrial homeostasis may be an evolutionarily conserved role of Bcl-2 family proteins.

Our findings also suggest that Bcl-2 family proteins may play a crucial role in the pathogenesis of polyglutamine diseases. It would be greatly informative to determine whether Bcl-2 family proteins also play a crucial role in mammalian systems that can be a therapeutic target for neurodegenerative disorders. Further study of Drob-1 should increase our understanding of the universal roles of Bcl-2 family proteins and may contribute to the development of new therapeutic applications, not only for polyglutamine diseases, but also for other abnormal-protein-accumulating neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis.

Materials and methods

Fly stocks

Fly culture and crosses were carried out at 25°C. *Canton-S* or *white*^{1118} was used as a wild-type strain. *da-GAL4*, *GMR-GAL4*, and *elav-GAL4* fly lines were used as driver strains. All general fly stocks and GAL4 lines including *UAS-reaper*, *GMR-reaper*, and *GMR-hid* were obtained from *Drosophila* stock centers. We also used *UAS-MJDtr-Q78* (Warrick *et al*, 1998), *UAS-lacZ-IR* (Kennerdell and Carthew, 2000), *UAS-buffy* (Quinn *et al*, 2003), *UAS-drob-1*^{debcl} (Colussi *et al*, 2000), *GMR-Dmp53* (Ollmann *et al*, 2000), and *GMR-drob-1* (Igaki *et al*, 2000) flies. *UAS-drob-1*^{GS2263} was a kind gift from Toshiro Aigaki. The *UAS-lacZ* fly line was a gift from Yasushi Hiromi.

Generation of RNAi transgenic fly lines

IR transgenic fly lines of *drob-1* were generated using a modified transformation vector, pUAST-D13 (a kind gift from Ryu Ueda). A 500-bp-long cDNA fragment was amplified by PCR and inserted as an IR into pUAST-D13. In all cases, IRs were constructed in a head-to-head orientation. Transgenic flies were generated by general P-element-mediated transformation.

Plasmids and dsRNAs

pUAST-HA-drob-1 and *pBSSK-HA-drob-1* were described previously (Igaki *et al*, 2000). A driver plasmid that expresses GAL4 under control of the *actin5C* promoter (*pWAGAL4*) was a kind gift from Yasushi Hiromi. *pCaspeR-hs-lacZ* was described previously (Hisahara *et al*, 1998). dsRNAs for *drob-1*, *buffy*, or *EGFP* were synthesized and the cells were treated with dsRNA essentially as described previously (Igaki *et al*, 2002).

ATP assay, immunoblotting, and imaging of mitochondrial membrane potential ($\Delta\psi_m$) using RNAi in Drosophila S2 cells

S2 cells were cultured in six-well plates (5×10^5 cells/well). The cells were washed with serum-free medium and treated with 20 μg/ml dsRNA in the serum-free medium for 30 min. Two volumes of Schneider's medium containing 10% FCS were then added to the medium, and the cells were cultured for 48 or 72 h. For immunoblotting or imaging of $\Delta\psi_m$, cells were treated with various amounts of the indicated inhibitors for an additional 24 h. And then, cells were subjected to the assays.

Cell-death assay using RNAi in Drosophila S2 cells

For the cell death assay, S2 cells were cultured in 24-well plates (1×10^5 cells/well) and were cotransfected using CellFectin (Invitrogen) with a driver plasmid pWAGAL4 and pCaspeR-hs-lacZ, a reporter plasmid that encodes β-galactosidase under control of the *hsp70* promoter, together with 25 ng of *EGFP* or *drob-1* dsRNA. At 48 h after the transfection, the cells were left untreated or treated with various amounts of the indicated inhibitors at 26°C for 24 h. Cells were then heat-shocked at 37°C for 2 h as described (Hisahara *et al*, 1998), and cultured at 26°C for another 24 h. The cells were lysed in 300 μl of 1 × Reporter lysis buffer (Promega) and each lysate was assayed for β-galactosidase activity in a reaction mixture containing 1 mg/ml *o*-nitrophenyl-β-D-galactopyranoside, as described (Igaki *et al*, 2000).

Longevity assay

For the longevity assay, more than 100 flies of each sex were collected for each genotype within 24 h after eclosion and maintained at 25°C. Flies were transferred to fresh food every 2 or 3 days and the numbers of dead flies were counted.

Locomotor activity assay

Male flies of each genotype were collected within 24 h after eclosion and entrained to a 12 h light:12 h dark cycle (LD12:12) at 25°C for at least 3 days. Flies were transferred to glass tubes for *Drosophila* activity monitoring (DAM) system (Trikinetics, Waltham, MA) interfaced with an Apple computer and locomortor activity was recorded under LD12:12 at 25°C for 14 days.

Assay for ATP levels

Heat-shocked *hs-GAL4/UAS-lacZ-IR*, *hs-GAL4/UAS-drob-1-IR*, *hs-GAL4/UAS-drob-1^{GS2263}*, or *hs-GAL4/UAS-buffy* adult flies were homogenized in 1 × Reporter lysis buffer (Promega), and were quickly frozen. The frozen fly samples were boiled for 15 min to destroy ATPase activity, then spun at 17 800 *g* for 5 min and the supernatant was diluted 100-fold with the same buffer. S2 cells were lysed in 0.5 ml of 1 × Reporter lysis buffer (Promega), and the lysate was quickly frozen in a dry ice/methanol bath. After thawing on ice, the cells were diluted 100-fold with the same buffer. The cellular ATP content in fly tissues or S2 cells was quantified by a luciferin- and luciferase-based assay using an ATP Determination Kit (Molecular Probes). Luminofluorescence was measured using the Wallac ARVO SX 1420 Multilabel Counter (Perkin Elmer Life Sciences), and the data were normalized to the protein content.

Histology

Flies were prepared for semithin sections and the sections were subjected to toluidine blue staining as described (Kanuka *et al*, 1999). For the light microscopic images of adult eyes, flies were anesthetized and examined with a Nikon SMZ1000 microscope (Nikon) equipped with an AxioCam digital camera (Carl Zeiss).

Immunoblotting

For adult heads, 12 fly heads were carefully dissected from anesthetized flies (1 day after eclosion) and lysed in 48 μl of SDS sample buffer. The S2 cells or adult head lysates were then separated by 10% SDS–PAGE and subjected to immunoblotting using an anti-ubiquitin mouse monoclonal antibody (1:250; Stressgen), an anti-β-tubulin mouse monoclonal antibody (1:500; CHEMICON), and an anti-mouse IgG-HRP antibody (1:1000; Promega). Signals were visualized using ECL plus (Amersham).

Imaging of $\Delta\psi_m$

$\Delta\psi_m$ was analyzed using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1; Molecular Probes), a lipophilic cationic fluorescence dye. JC-1 is driven into mitochondria in a membrane potential-dependent manner. At high mitochondrial membrane potentials, JC-1 accumulates sufficiently in the mitochondria to form aggregates that fluoresce red. At lower mitochondrial potentials, less dye enters mitochondria resulting in monomers that fluoresce green (Smiley *et al*, 1991). This assay allowed one to quantify the percentage of highly energized mitochondria (with both red and green fluorescence) and depolarized mitochondria (with green fluorescence only). S2 cells were incubated with 5 μg/ml JC-1 (made up as a 1 mg/ml stock in dimethyl sulfoxide) for 10 min at room temperature in the dark. Then, cells were washed three times with PBS and live cell imaging was performed with a Zeizz LSM 510 META laser scanning confocal microscopy system. The ratio of number of cells with highly energized red mitochondria to total number of cells with green mitochondria was calculated. Approximately 500 cells were analyzed per experimental condition.

Mitochondrial isolation

S2 cells were cultured with 25 μg/ml *EGFP* dsRNA or *drob-1* dsRNA for 72 h, and incubated with or without 0.05 μM rotenone or 0.5 mM 3-NP for an additional 16 h. Cells were then harvested and homogenized in isolation buffer (210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, and 5 mM Tris–HCl, pH 7.4). Mitochondria were isolated by differential centrifugation and suspended in Tris–EDTA buffer (0.1 mM EDTA and 50 mM Tris–HCl, pH 7.4) as described (Senoo-Matsuda *et al*, 2001).

Mitochondrial complex I and complex II assay

The activities of complex I + III and complex II + III in mitochondria isolated from S2 cells were measured as described (Senoo-Matsuda *et al*, 2001).

Transmission electron microscopy

S2 cells were cultured with 25 μg/ml *EGFP* dsRNA or *drob-1* dsRNA for 72 h, and incubated with or without 0.05 μM rotenone or 0.5 mM 3-NP for an additional 16 h. Cells were fixed with 2.5% glutaraldehyde in 0.1 M Sorenson's buffer (pH 7.2) for 12 h. The samples were then postfixed with 1% OsO_4 in 0.1 M Sorenson's buffer (pH 7.2) for 1 h. Enblock staining was performed using 1% tannic acid. After dehydration, samples were embedded in Lx-112 (Ladd Research Industries Inc.). Semithin 1 μm and thin 60 nm sections were cut on the MT-7000 urtramicrotome. Thin sections were stained with uranyl acetate and lead citrate and examined under a JEOL JEM-1200 EXII transmission electron microscope operating at 80 kV.

Statistical analysis

Data are given as means ± s.e.m. Student's *t*-tests were performed on all quantitative analyses.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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