

Rapamycin alleviates toxicity of different aggregate-prone proteins

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Many neurodegenerative diseases are caused by intracellular, aggregate-prone proteins, including polyglutamine-expanded huntingtin in Huntington’s disease (HD) and mutant tau in fronto-temporal dementia/tauopathy. Previously, we showed that rapamycin, an autophagy inducer, enhances mutant huntingtin fragment clearance and attenuated toxicity. Here we show much wider applications for this approach. Rapamycin enhances the autophagic clearance of different proteins with long polyglutamines and a polyalanine-expanded protein, and reduces their toxicity. Rapamycin also reduces toxicity in *Drosophila* expressing wild-type or mutant forms of tau and these effects can be accounted for by reductions in insoluble tau. Thus, our studies suggest that the scope for rapamycin as a potential therapeutic in aggregate diseases may be much broader than HD or even polyglutamine diseases.

INTRODUCTION

Proteinopathies are a growing family of human disorders associated with the aggregation of misfolded proteins in specific tissues (1). These conditions include Alzheimer’s disease (AD), Parkinson’s disease, amyotrophic lateral sclerosis (1), diseases caused by abnormally expanded polyglutamine tracts in mutant proteins, exemplified by Huntington’s disease (HD) and spinocerebellar ataxia types 1, 2, 3, 6, 7 and 17 (2) and certain diseases caused by polyalanine expansion mutations, like oculopharyngeal muscular dystrophy (3). The role of aggregates in these diseases has been a subject of vigorous debate. However, the accumulation of the causative mutant protein either in aggregates, microaggregates or soluble oligomers has been correlated with toxicity (4–10). The mutations causing many of these diseases confer novel toxic functions on the specific protein, and disease severity frequently correlates with the expression levels of the mutant protein. Thus, it is important to understand the

factors that regulate the clearance of these aggregate-prone proteins.

The ubiquitin–proteasome and autophagy–lysosomal pathways are the two major routes for protein and organelle clearance in eukaryotic cells (11). While the narrow barrel of the proteasome precludes entry of oligomers, aggregates and organelles, such substrates can be degraded by macroautophagy (which we will call autophagy). Autophagy involves the formation of double-membrane structures called autophagosomes, which fuse with lysosomes to form autolysosomes where their contents are then degraded by acidic lysosomal hydrolases (12).

We have previously shown that mutant huntingtin fragments, expanded polyalanines tagged to enhanced green fluorescent protein and forms of α -synuclein are autophagy substrates in cell models (13,14). Their clearance is delayed by autophagy inhibitors like 3-methyl adenine (3MA) and bafilomycin A1 (Baf), whereas rapamycin enhances their clearance. Rapamycin induces autophagy by inhibiting the

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mammalian target of rapamycin (mTOR), which negatively regulates the pathway (15,16). We recently showed that rapamycin attenuated toxicity of mutant huntingtin fragments in cells (14), transgenic *Drosophila* and mouse models (17). We believe that autophagy is probably clearing monomeric and oligomeric precursors of aggregates, rather than the large inclusions themselves, as we and others have not observed inclusions that are membrane-bound and inclusions are much larger than mammalian autophagosomes (18).

In this study, we wanted to test if rapamycin treatment could be beneficial across a variety of diseases associated with aggregation. We therefore tested if rapamycin could enhance clearance of and decrease toxicity of a range of intracellular aggregate-prone proteins.

RESULTS

Rapamycin enhances clearance of diverse polyglutamine proteins

We first studied the effects of rapamycin in cell models expressing a variety of non-HD polyglutamine expansions, to test if rapamycin was effective in other polyglutamine diseases. We transiently transfected COS-7 cells with constructs encoding mutant ataxin1 with 92 glutamines (ataxin1-Q92), expanded polyglutamines tagged with enhanced green fluorescent protein (EGFP-Q81) and EGFP-Q97 containing a nuclear localization signal (NLS) (EGFP-NLS-Q97). Our rationale for using some constructs encoding proteins with NLSs is that they may still be accessible to autophagy as the NLS does not preclude some presence in the cytoplasm but simply shifts the equilibrium to favour nuclear localization. Furthermore, all of these proteins are initially translated in the cytoplasm and many of these make some cytosolic aggregates in cell models (e.g. ataxin1-Q92; EGFP-NLS-A37) (19).

Cells were treated with rapamycin or 3MA and the proportions of transfected cells with aggregates were assessed—this proportion correlates with the expression levels of the transgenes when aggregation itself is not perturbed (20). In ataxin1-Q92-transfected cells, rapamycin decreased the percentage of cells with aggregates and the proportion of cells with apoptotic nuclear morphology, whereas 3MA had the opposite effects (Fig. 1A and B). As we observed with mutant huntingtin constructs (14), rapamycin also decreased the levels of soluble ataxin1-Q92 relative to GFP (transfection control), whereas Baf A1 had the opposite effect (Fig. 1E and F). However, these agents had less pronounced and not always significant effects on cells expressing wild-type ataxin1 which aggregates much less than ataxin1-Q92 and is less toxic when overexpressed in COS-7 cells (Fig. 1C–F)—this is entirely consistent with our previous data suggesting that the more aggregate-prone species have a greater dependence on autophagy for their clearance (11,13,18). Both 3MA and Baf inhibit autophagy (14).

The effects of rapamycin and 3MA on mutant ataxin1 aggregation were also observed with EGFP-Q81 and EGFP-NLS-Q97 that express polyglutamine expansions outside the context of disease proteins (Fig. 2A and B). We used low amounts of EGFP-Q81 and EGFP-NLS-Q97 constructs in these experiments in order to obtain a system with an

optimal aggregation rate. These low amounts cause minimal toxicity. These constructs containing isolated long polyglutamines aggregate very rapidly—1 μ g of EGFP-NLS-Q97 will lead to the presence of the aggregates in 90% of transfected cells at 72 h (data not shown). Such a high aggregation rate is not desirable, as it does not resemble the disease pathology. In addition, we have previously shown that aggregates sequester mTOR leading (17) to rapamycin-insensitivity, once sufficient aggregation has occurred. Also, we believe that autophagy can degrade monomeric and oligomeric precursors of aggregates (at least preferably), rather than the large inclusions.

We tested the effects of these compounds on the clearance of a non-HD polyglutamine expansion by studying a stable Tet-ON inducible cell line expressing mutant ataxin3 with 70Q, which does not form obvious aggregates seen by light microscopy (data not shown). Because this line is inducible, we could assess transgene clearance by switching the expression on or off. We tested the effects of rapamycin and Baf A1 by adding the drugs to the medium and measuring the levels of ataxin3 after a brief switch-off period, similar to what we have done previously with huntingtin fragments (14). Mutant ataxin3 clearance was enhanced by rapamycin and was retarded by Baf (Fig. 2C and D). These effects were confirmed in another stable inducible ataxin3 cell line (data not shown)—Tet-OFF mesencephalic CSM14.1 cell lines expressing mutant ataxin3 with 70 glutamines (21).

To test whether rapamycin could also protect against non-HD polyglutamine-mediated toxicity *in vivo*, we studied flies expressing an isolated 108 residue polyglutamine stretch tagged with a myc/flag epitope (Q108) in neurons (22). Proteins with long polyglutamines (even shorter than Q108) have been shown to aggregate in flies in various tissues, whereas short stretches do not (23–28). Flies homozygous for the neuronal driver *elav-GAL4* were crossed to *UAS-Q108/TM3* flies, and the percentage of adult progeny carrying the *UAS-Q108* transgene was evaluated. Expression of Q108 in neurons caused dramatic pre-adult lethality, which was partially rescued by rapamycin treatment (Fig. 3A). Expression of the wild-type *Q22* transgene did not have any effect on survival to adulthood—the percentage of progeny carrying this transgene is slightly higher than 50% due to the mild toxic effects of the *TM3* balancer (Fig. 3B). Rapamycin treatment does not increase the survival of *Q22* flies (Fig. 3B), confirming the specificity of our read-out. In summary, these data suggest that rapamycin can have beneficial effects for different non-HD proteins with long polyglutamines.

Rapamycin enhances clearance of polyalanine expansion *in vivo*

We have previously shown that a mutant protein with an expanded polyalanine tract can be also degraded by autophagy in cell models (14). We tested if rapamycin would be beneficial against the toxic effects of such a model aggregate-prone protein *in vivo* using a *Drosophila* model expressing long polyalanines fused to an NLS (EGFP-NLS-A37) that we have generated (29) (and Berger Z. *et al.*, submitted). In this *Drosophila* model, independent insertions of EGFP-NLS-A37 cause toxicity in a variety of tissues when expressed

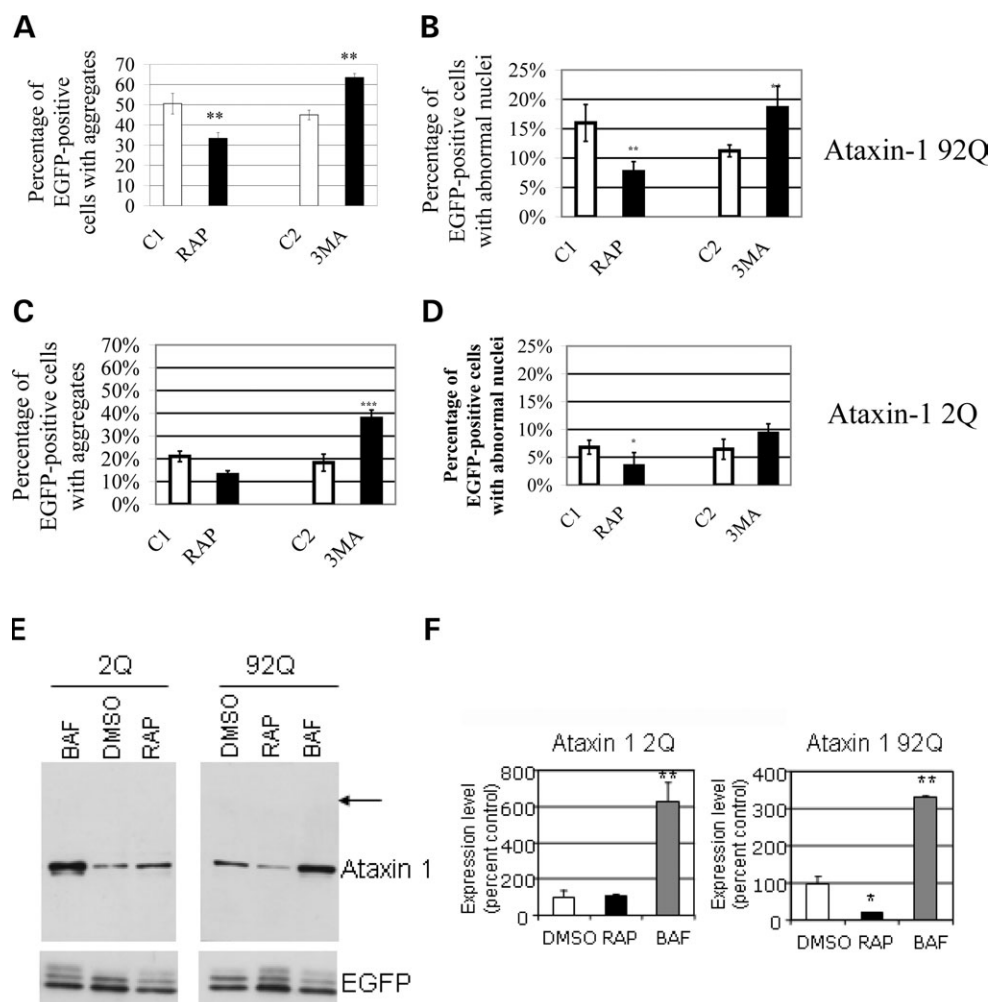


Figure 1. Rapamycin decreases aggregation and levels of mutant ataxin1. (A and B) COS-7 cells were transfected with 1.5 μ g of ataxin1 with ataxin1-97Q and 0.5 μ g of EGFP and drugs were added in the medium for the following 24 h, after which the cells were fixed. We evaluated the percentage of transfected cells with inclusions (A) or abnormal nuclei (B). Rapamycin decreased inclusion formation and toxicity and 3MA had opposite effects. C1 shows cells treated with DMSO (vehicle of rapamycin), C2 without any treatment (3MA is soluble in plain medium), RAP, rapamycin. The same applies to other panels. Means with standard deviations are shown. Aggregates, rapamycin: $P = 0.001$, OR = 0.661, 95% CI 0.517–0.845; 3MA: $P = 0.003$; OR = 1.412, 95% CI 1.123–1.776. Cell death, rapamycin: $P = 0.001$; OR = 0.486; 95% CI 0.322–0.734; 3MA: $P = 0.008$; OR = 1.630; 95% CI 1.136–2.340. (C and D) COS-7 cells were transfected and treated as described in A and B, but ataxin1 with 2Q was used. Aggregates, rapamycin: $P = 0.053$, OR = 0.709, 95% CI 0.501–1.005; 3MA: $P < 0.0001$, OR = 2.702, 95% CI 1.994–3.660. Cell death, rapamycin: $P = 0.012$, OR = 0.460, 95% CI 0.251–0.844; 3MA: $P = 0.095$, OR = 2.478, 95% CI 0.930–2.478. (E) Rapamycin decreased the soluble levels of mutant ataxin1 although this effect was not seen with wild-type ataxin1. Baf increased the soluble levels of both mutant and wild-type ataxin1. The arrow represents the boundary between the stacking gel and resolving gel, we did not observe ataxin1 staining at high-molecular weights suggesting that the decrease in soluble levels is not due to a reduction in solubility of the protein. (F) Quantification of ataxin1 levels from western blots run in triplicate. 2Q, DMSO versus RAP, $P = 0.852$, DMSO versus Baf, $P = 0.009$; 92Q, DMSO versus RAP, $P = 0.012$, DMSO versus Baf, $P = 0.003$ (unpaired t -test). In all panels, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

at similar levels to EGFP-NLS-A7 insertions, which are not toxic (Berger Z. *et al.*, submitted). Expression of this construct leads to the formation of aggregates both inside and outside of the nucleus (19) (data not shown). The survival of flies expressing the A37 construct was analysed in a manner similar to that described for the Q108 flies.

Survival of flies carrying UAS-EGFP-NLS-A37 was increased by rapamycin treatment (Fig. 4A), whereas survival of flies with the equivalent non-toxic A7 transgene was not affected (Fig. 4B). Rapamycin also decreased the toxicity of long polyalanines, assessed by another read-out—the presence of abnormal eyes (Fig. 4C). Rapamycin

decreased the soluble EGFP-NLS-A37 levels in these flies (Fig. 4D and E).

We then tested if the rapamycin effect was dependent on autophagy, by analysing toxicity of long polyalanines (assessed by abnormal eyes) in the presence of a mutation of the *Drosophila* homologue of the *Atg1* gene, which is essential for autophagy (30). As homozygous loss of *Atg1* is pupal lethal (30), we analysed flies that had one functional copy of *Atg1* absent. Absence of one functional copy of *Atg1* did not enhance polyalanine toxicity in the absence of drug treatment, but almost entirely abolished the rescue effect of rapamycin (Fig. 4C). This suggests that absence of

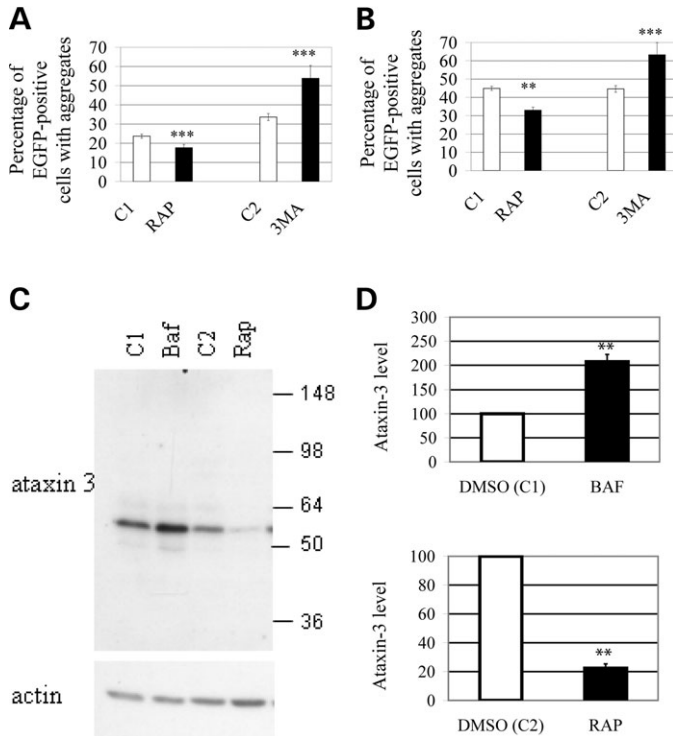


Figure 2. Rapamycin decreases aggregation and levels of other polyglutamine constructs. (A and B) COS-7 cells were transfected with 0.05 μ g of EGFP-Q81 (A) or with 0.05 μ g of EGFP-NLS-Q97 (B) and aggregation was evaluated as in Fig. 1A and B. Rapamycin decreased inclusion formation, whereas 3MA had the opposite effect. Means with standard deviations are shown. EGFP-Q81, rapamycin: $P < 0.001$, OR = 0.339, 95% CI 0.270–0.424; 3MA, $P < 0.001$; OR = 1.597, 95% CI 1.329–1.919. EGFP-NLS-Q97, rapamycin: $P = 0.002$; OR = 0.733, 95% CI 0.604–0.890; 3MA, $P < 0.001$; OR = 1.416, 95% CI 1.189–1.686. (C) Expression of mutant ataxin3 with 70Q was induced by adding doxycycline for 24 h (C1, Baf) or 72 h (C2, RAP). Doxycycline was then removed and clearance was measured after 14 h. RAP and DMSO control (C2) were added both in the switch-on and switch-off period, Baf and DMSO control (C1) were added only in switch-off period. Rapamycin decreases ataxin3 levels and Baf has opposite effects. (D) Quantification of ataxin3 70Q levels from the experiment in (C). DMSO and RAP $P = 0.006$ ($n = 6$), DMSO and BAF $P = 0.004$ ($n = 6$). Unpaired t -test was used. *** $P < 0.001$, ** $P < 0.01$.

one functional copy of *Atg1* is only limiting when autophagy is up-regulated but not under basal (non-rapamycin conditions) conditions. In addition, this suggests that the protective effect of rapamycin *in vivo* in the context of an aggregate-prone protein is mostly due to induction of autophagy.

Rapamycin enhances clearance of tau and decreases tau toxicity *in vivo*

In order to test whether rapamycin was protective against an aggregate-prone protein that did not result from a polyglutamine or polyalanine codon reiteration mutation, we used *Drosophila* models expressing wild-type and mutant tau R406W in the eyes. Expression of wild-type tau in *Drosophila* eyes using the *ey-GAL4* driver leads to three different classes of phenotype: normal-looking eyes, abnormal eyes

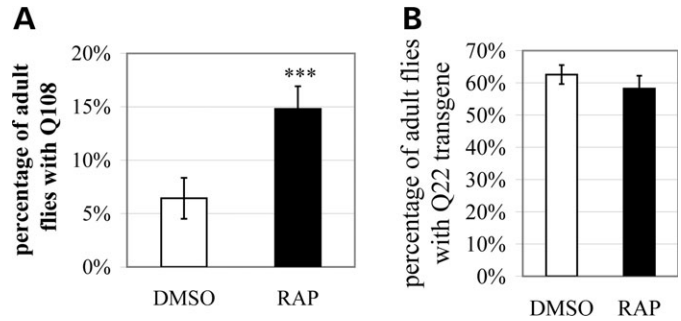
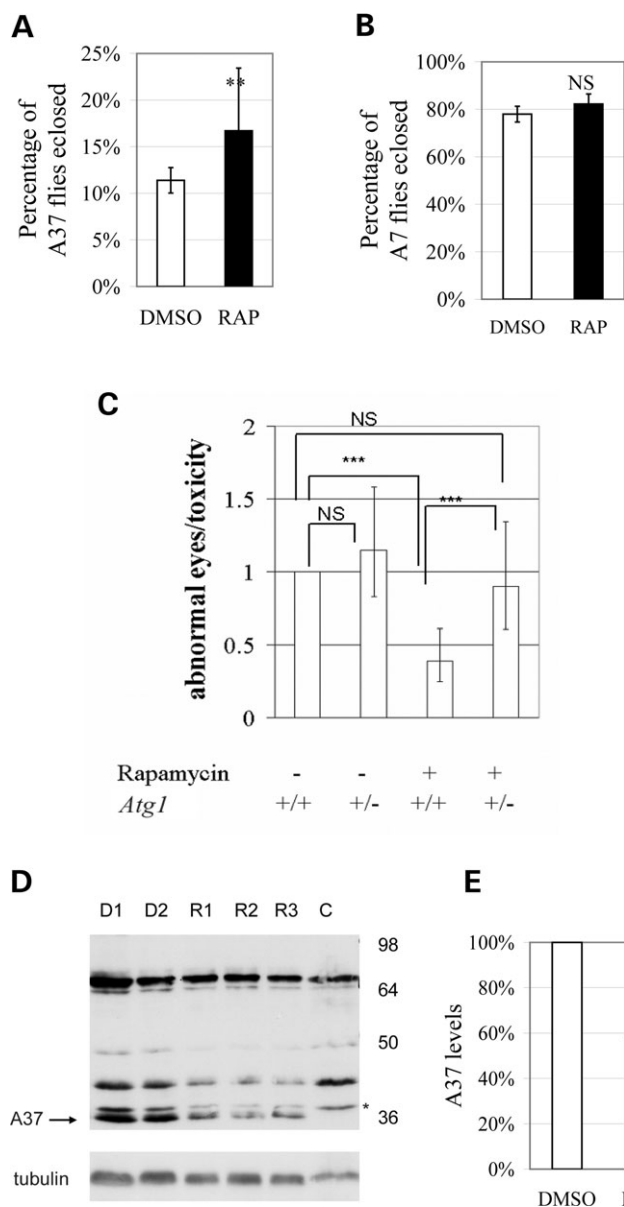


Figure 3. Rapamycin decreases polyglutamine toxicity. (A) Pre-adult lethality of flies expressing a Q108 transgene is partially rescued by rapamycin treatment. Females carrying the neuronal driver *elav-GAL4* were crossed to *Q108/TM3* and the percentage of adult progeny carrying the Q108 transgene was determined. ($P < 0.001$). We evaluated 16 bottles (approximately 1400 flies). Means \pm SEM are shown. (B) Rapamycin treatment does not increase survival of Q22 flies. Flies were crossed as described in *g* but *Q22/TM3* was used. We evaluated 10 bottles (approximately 1700 flies). Means \pm SEM are shown. NS, not significant ($P > 0.05$), *** $P < 0.001$.

(characterized by abnormal shape or rough surface) and loss of eyes (no eyes) (Fig. 5A). Rapamycin decreased the tau-mediated toxicity, reducing the proportion of flies without eyes, whereas increasing the proportion of flies with normal eyes (Fig. 5B). Rapamycin also led to increased survival to adulthood of flies expressing tau (Fig. 5C).

Overexpression of R406W mutant tau leads to the formation of abnormal eyes or no eyes (Fig. 6A), consistent with previous reports showing that R406W tau is more toxic than wild-type tau when expressed in *Drosophila* (31). Rapamycin also protected against toxicity caused by expression of R406W tau, manifested by a decreased proportion of 'no eyes' (Fig. 6B). Consistent with previous observations (13,14) (and ataxin1 discussed earlier), the effects of rapamycin were more pronounced with the more aggregate-prone mutant tau than with wild-type tau.

We could not detect the tau protein in the flies with the driver we used, probably because of the small proportions of cells expressing the constructs coupled with their toxicity resulting in cell death (note that tau is much more toxic than A37 with this driver; we also observed this with other highly toxic constructs, data not shown). We tested if tau could also be degraded by autophagy using cell models. A potential difficulty that may affect analyses of tau turnover is that the bulk of the tau pool is probably slowly turned over as it is bound to microtubules. This can be addressed in cell models, as it is believed that wild-type tau probably becomes aggregate-prone and exerts deleterious effects after it is phosphorylated and dissociates from microtubules. We created a pool of non-microtubule-bound, aggregate-prone tau by inducing its phosphorylation with cdk5 and its activator p25, which accumulates in the brains of AD patients (32). Rapamycin treatment decreased the total levels of tau protein in COS-7 cells transiently transfected with wild-type four repeat tau, cdk5 and p25. (Fig. 7A and B). We were not able to see any difference in the total tau levels when we transiently transfected COS-7 cells only with wild-type four repeat tau (data not shown), suggesting that rapamycin treatment led to preferential degradation of non-microtubule-bound (and aggregate-prone) forms.



We then analysed the effects of rapamycin treatment on the most common tau mutation, P301L, that has been extensively studied both in cells and in mouse transgenics (33,34) and that exhibits strong aggregation properties (35). Rapamycin treatment of COS-7 cells transfected with EGFP-P301L (without CDK5 or p25) led to decreased levels of insoluble tau but did not affect levels of the soluble form (Fig. 8A and B). Addition of 3MA to COS-7 cells transfected with P301L treated with rapamycin abolished the effect of rapamycin on insoluble tau but did not affect soluble levels (Fig. 9A–D). This suggests that these effects of rapamycin are probably mediated via autophagy. However, treatment with 3MA alone did not alter the levels of soluble or insoluble P301L tau (data not shown). Thus, our data suggest that mutant tau can be degraded by autophagy when it is induced, but that it is not predominantly degraded by autophagy under basal

Figure 4. Rapamycin effect *in vivo* is due to autophagy induction. **(A)** Rapamycin increased the survival of A37 flies to adulthood. Flies carrying *ey-GAL4* were crossed to *UAS-NLS-A37/TM6B* and the percentage of adult flies with both A37 and *ey-GAL4* transgenes was evaluated. Flies were raised at 29°C. ($P = 0.006$; OR = 1.660; 95% CI: 1.159–2.376). We evaluated six bottles (approximately 600 flies). Means \pm SEM are shown. **(B)** Rapamycin treatment does not affect the survival of flies carrying *TM6B*. Flies were crossed as in *i* but *UAS-NLS-A7/TM6B* was used. Flies were raised at 29°C in identical conditions as in *i* ($P = 0.06$; OR = 1.3186; 95% CI: 0.989–1.758). We evaluated eight bottles (approximately 1300 flies). Means \pm SEM are shown. Note that 80% of adult flies carry the A7 transgene due to toxicity of the *TM6B* balancer. **(C)** Flies of genotype *ey-GAL4/CyO; UAS-NLS-A37/TM6B* were crossed to wild-type or to +; *Atg1^{Δ3D}/TM6B* and the percentage of flies with abnormal eyes were evaluated in their progeny of genotype *ey-GAL4/+; UAS-NLS-A37/+* (cross to wild-type) or *ey-GAL4/+; UAS-NLS-A37/Atg1^{Δ3D}* (cross to *Atg1* mutant). Flies were treated during pre-adult stage with DMSO or rapamycin. We evaluated eight bottles in each cross under each condition (eight DMSO and eight rapamycin treated, at least 310 flies were analysed per cross under each condition). Data are expressed as odds ratios to enable easy comparison—frequency of abnormal flies was set to 1 in DMSO treated *ey-GAL4/+; UAS-NLS-A37/+* flies. Error bars represent 95% confidence intervals. Flies were raised at 25°C. **(D)** Rapamycin treatment decreases the levels of A37. Arrow points to the GFP-A37, asterisk marks a non-specific band. Samples D1, D2 represent fly extracts from DMSO treated A37 flies, R1–R3 from rapamycin A37 treated flies, C shows extracts from non-transgenic controls. A representative western blot is shown. The experiment was done as in (A). **(E)** Quantification of fly extracts from (B). Unpaired *t*-test ($P = 0.011$) is based on nine different fly extracts in each group (DMSO or rapamycin treated). NS, not significant ($P > 0.05$), *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

(non-rapamycin conditions), similar to what we previously observed with wild-type α -synuclein (13).

DISCUSSION

In summary, we show that rapamycin may be beneficial in a range of diseases caused by toxic gain-of-function mutations that make intracellular proteins aggregate-prone. These include different disease-associated polyglutamine mutations, a model aggregate-prone protein with a polyalanine expansion and wild-type and mutant forms of tau. We also demonstrate that the effects of rapamycin both in cells and *in vivo* are likely to proceed via autophagy. Our results are thus in agreement with previous studies (13,14) and further broaden the scope of rapamycin as a potential therapeutic for other diseases associated with intracellular aggregation. Our results with cell lines of ataxin3, which do not form obvious inclusions, support our hypothesis that autophagy is clearing monomeric or oligomeric species, rather than the large inclusions themselves.

Most importantly, we demonstrate that rapamycin can decrease the toxicity of these aggregate-prone proteins *in vivo* using *Drosophila* models. We also show *in vivo* that the effect of rapamycin is likely to proceed via autophagy. Further tests in mouse models of the disorders associated with these aggregate-prone proteins (34,36,37) will be necessary to confirm the beneficial effects of rapamycin or its analogues. However, previous studies' treatment trials in *Drosophila* models of diseases associated with aggregate-prone proteins were highly predictive of success in subsequent mouse studies (17,38).

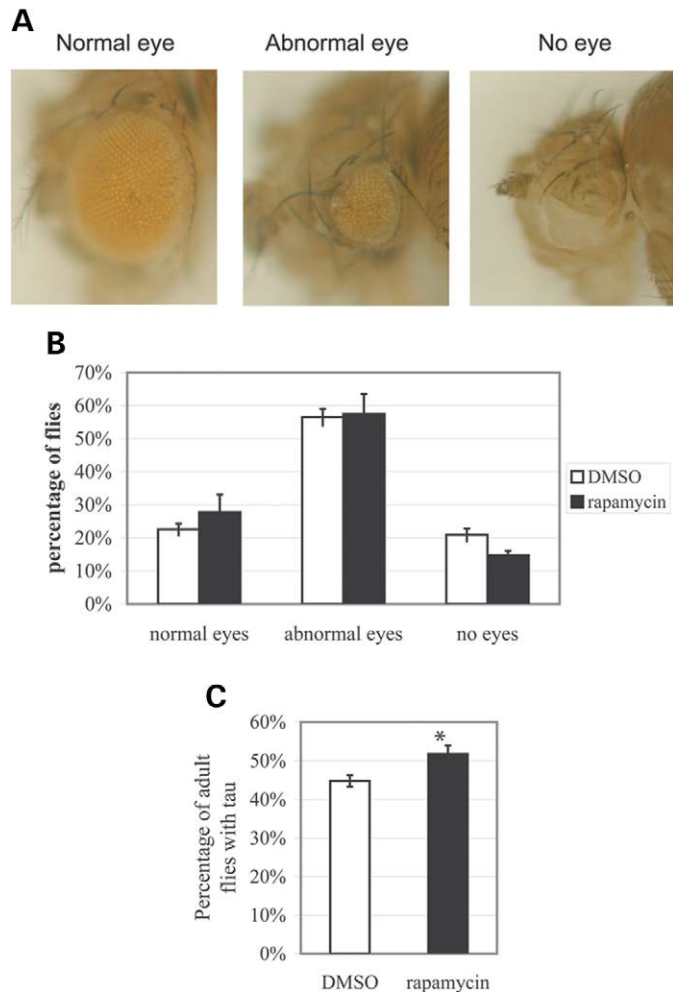


Figure 5. Rapamycin reduces toxicity of wild-type tau. (A) Expression of wild-type tau in *Drosophila* eyes under the control of ey-GAL4 leads to three major classes of phenotype: loss of eyes, abnormal eyes or normal looking eyes. Flies were raised at 23°C. (B) Rapamycin decreases toxicity of wild-type tau. Flies of genotype *w; +; UAS-tau/TM3* were crossed to ey-GAL4 and the eyes were evaluated in the progeny carrying both UAS-tau and ey-GAL4. Rapamycin decreased the proportion of flies with no eyes while increasing the percentage with normal eyes. Flies with eyes of two categories (e.g. one eye absent and one abnormal) were classified according to the more severe phenotype. χ^2 test (2 d.f.) was used ($P = 0.001$). We evaluated six different bottles (approximately 1400 flies). Flies were raised at 23°C. Means \pm SEM are shown. (C) Rapamycin increases the survival of flies with wild-type tau to the adult stage. The percentage of adult flies carrying the *UAS-tau* transgene was evaluated in the experiment described in (B). We evaluated six bottles (approximately 2200 flies). ($P = 0.018$; OR = 1.279; 95% CI: 1.043–1.570). Note that the effect is not due to rapamycin affecting the survival of flies carrying TM3 balancer (see Fig. 3B). Means \pm SEM are shown. * $P < 0.05$.

Our data with predominantly nuclear proteins (like ataxin1 and A37) are compatible with the cell line data (39) (published while this manuscript was in preparation) showing that mutant ataxin1 aggregation doubled when autophagy was inhibited. These effects may have been non-significant in the previous study (39) because of the low aggregation rates observed, and levels of soluble protein were not assessed. We agree that cytoplasmic proteins may be more accessible to autophagic

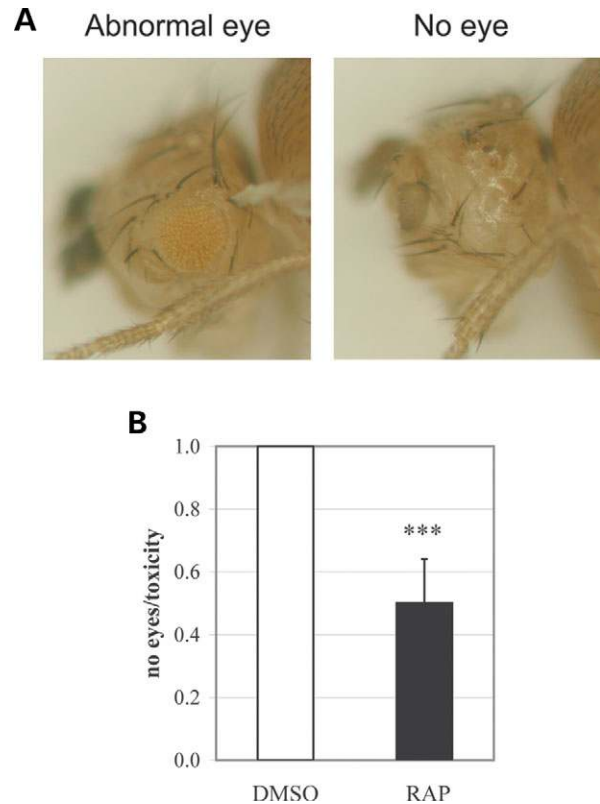


Figure 6. Rapamycin reduces toxicity of mutant tau. (A) Expression of R406W mutant tau in *Drosophila* eyes under the control of ey-GAL4 leads to two major classes of phenotype: no eyes and abnormal eyes. Flies were raised at 25°C. (B) Rapamycin decreases toxicity of R406W mutant tau, manifested by the decrease in the ‘no eyes’ ($P < 0.001$). Odds ratios with 95% confidence intervals are shown. We evaluated six different bottles in each group (approximately 1000 flies in each group). *** $P < 0.001$.

clearance compared with predominantly nuclear proteins. We believe that the dependence on autophagy will correlate with the extent to which the protein forms aggregates because the aggregate-prone species are less efficiently cleared by the proteasome—autophagy is a ‘default’ pathway.

Our data also suggest that rapamycin may be beneficial in tauopathies, diseases associated with abnormal accumulation of tau protein. Rapamycin decreased toxicity in flies expressing both wild-type tau and mutant tau that causes familial fronto-temporal dementia (40,41). Our results suggest that this protective effect can be attributed to autophagic degradation of insoluble tau, although we cannot rule out other protective effects of rapamycin. Although our tau data raise the possibility that rapamycin may have beneficial effects even in sporadic AD, some caution is needed, as there have been reports showing correlations between β -amyloid production and autophagic activity (18). Our findings showing that rapamycin can induce autophagic degradation of tau are compatible with a previous study reporting tau localization within autophagic vacuoles in a model of chloroquine myopathy (42). However, it has not been previously shown that rapamycin treatment or autophagy induction in a rapamycin-independent-manner would lead to decreased tau toxicity and decreased levels of insoluble tau.

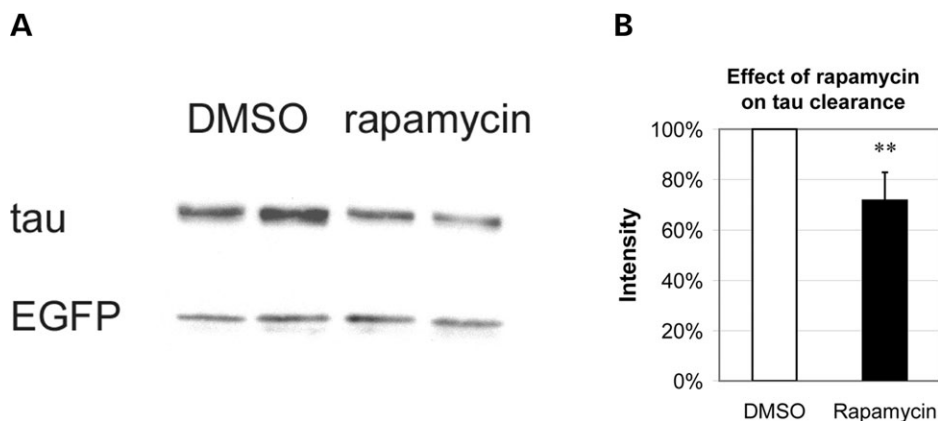


Figure 7. Rapamycin reduces the levels of wild-type tau. **(A)** Rapamycin decreases levels of tau in COS-7 cells. COS-7 cells were transfected with wild-type 1 μ g tau-EGFP, 1 μ g CDK5, 0.5 μ g p25 and 0.5 μ g EGFP (as loading control). Cells were treated for 48 h with either rapamycin or DMSO and the extracts were probed with anti-tau and anti-GFP antibodies. A representative blot is shown. Samples from two independent experiments treated with DMSO or rapamycin are shown. Tau-P301L-EGFP migrated between 64 and 98 KDa marker and EGFP runs at approximately 30 KDa (also applies to other tau blots). **(B)** Quantification of the experiment described in (A). Unpaired *t*-test ($P = 0.0035$) is based on seven samples in each group (DMSO or rapamycin treated). ** $P < 0.01$.

Upon rapamycin treatment, we observed reductions of both inclusions and soluble species in our experiments with different polyglutamine constructs. Interestingly, we only observed reductions in insoluble tau upon rapamycin treatment, although no difference was detected in the soluble fraction. These differences may simply represent the different procedures used to extract soluble and insoluble species—tau appears to aggregate much less in cell models than the various polyglutamine proteins. An alternative explanation is that soluble tau is bound to microtubules and dispersed throughout the cells and therefore not readily accessible to autophagic degradation.

In summary, our studies suggest that the scope for rapamycin as a potential therapeutic in aggregate diseases may be much broader than HD or even polyglutamine diseases. Rapamycin was approved as an immunosuppressant drug in 1999 and its water-soluble analogues, such as CCI-779 and RAD001, are currently in phase III (CCI-779) and phase II (RAD001) clinical trials for cancer (43,44). Because rapamycin is designed for long-term use and crosses the blood–brain barrier (17), it merits consideration for further studies in a range of neuroprotective contexts. Also, new components of the TOR signalling network may provide new targets for autophagy induction (45,46) and there may be suitable mTOR-independent autophagy enhancers that may have similar beneficial effects (47).

MATERIALS AND METHODS

Mammalian cell culture and transfection

COS-7 and CSM-14 cells used for the experiments were cultured using standard protocols. The cells were pre-treated with 0.2 μ g/ml rapamycin (LC Laboratories) or 10 mM 3MA (Sigma) for 72 and 48 h, respectively. We have used stable Tet-ON cell lines (HEK293 cells) expressing full-length human ataxin3 with 70 glutamine repeats tagged at the N-terminus with HA. The cells were cultured using standard protocols in the presence of 100 μ g/ml G418 and 25 μ g/ml

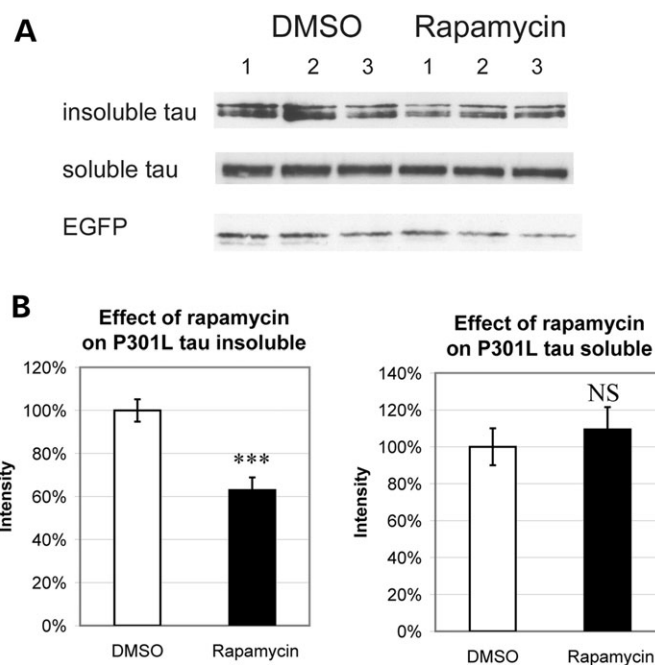


Figure 8. Rapamycin reduces levels of mutant tau. **(A)** Cells were transfected with 1 μ g P301L-EGFP and 0.5 μ g EGFP, and treated with DMSO or rapamycin for 48 h. Cells were then collected, and soluble and insoluble fractions were analysed on the western blot. Rapamycin decreases the levels of insoluble P301L but does not affect the levels in the soluble fraction. A representative blot is shown. **(B)** Quantification of the experiment described in (A). Insoluble fraction: $P = 0.0005$, $n = 14$; soluble fraction: $P = 0.6$, $n = 13$. Unpaired *t*-test was used. NS, not significant ($P > 0.05$), *** $P < 0.001$.

hygromycin B. Transfection was performed using lipofectamine reagent (Invitrogen). Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI, 3 mg/ml, Sigma). The following concentrations of drugs were used: 0.2 μ g/ml rapamycin, 10 mM 3MA, 200 nM Baf A1. Plasmids used were ataxin1 constructs (48), wild-type four repeat tau and mutant P301L tau both fused to EGFP (49); p25 and Cdk5 constructs (50).

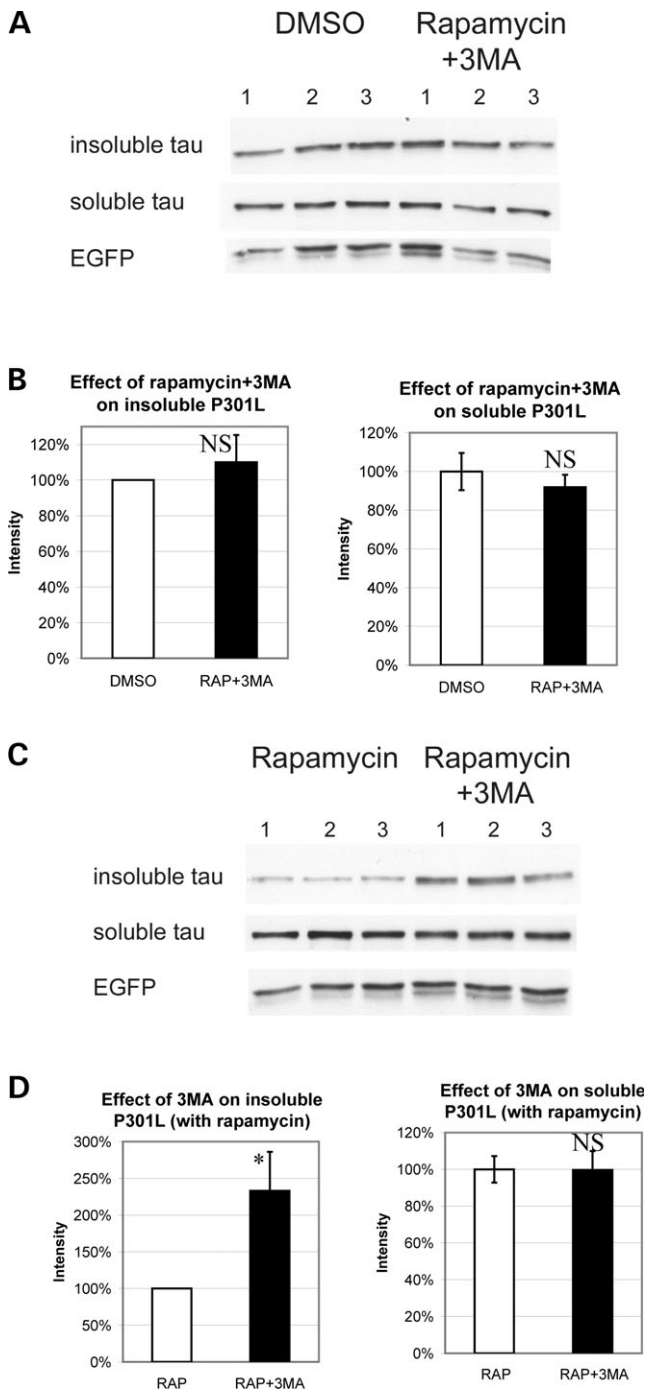


Figure 9. Rapamycin reduces levels of mutant tau by inducing autophagy. (A) Cells were transfected with P301L-EGFP (1 μ g) and EGFP (0.5 μ g), treated with DMSO or rapamycin + 3MA, cells were collected 48 h after transfection and soluble and insoluble fractions were analysed on the western blots. No difference in either soluble or insoluble fraction is seen. (B) Quantification of the experiment described in (A). Insoluble fraction: $P = 0.6$, $n = 16$; soluble fraction $P = 0.5$, $n = 16$. Unpaired t -test was used. (C) Cells were transfected as described in (A) and treated either with rapamycin or with rapamycin + 3MA. Soluble and insoluble fractions were analysed on the western blot. Treatment of cells with 3MA in the presence of rapamycin increases the levels of insoluble tau compared with rapamycin treatment alone. Soluble levels appear unaffected. A representative blot is shown. (D) Quantification of the experiment described in (C). Insoluble fraction: $P = 0.0347$, $n = 16$; soluble fraction $P = 0.9689$, $n = 16$. Unpaired t -test was used. NS, not significant ($P > 0.05$), * $P < 0.05$.

Western blots

Adult flies were homogenized in a microtube containing 1 \times SDS-PAGE sample buffer (0.0625 M Tris-HCl pH = 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromphenol blue, 1 \times cocktail of protease inhibitors, Roche). The homogenate was boiled at 95°C for 5 min, briefly centrifuged at 15 700g and the supernatant was used for western blotting. Approximately one fly equivalent was used for each lane. Our initial analysis did not reveal any difference on the western blots whether the homogenate was centrifuged or not, indicating that no GFP + protein was being left in the pellet during centrifugation (data not shown).

Cells (other than cells transfected with tau) were lysed for 15 min on ice in RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris pH 7.5 and 1 \times protease inhibitor cocktail, Roche) then centrifuged at 15 700g for 5 min at 4°C. Samples were then prepared in SDS-PAGE sample buffer as described earlier.

Total levels of tau were analysed by solubilizing the cells in RIPA buffer, soluble and insoluble tau were analysed as described previously (33). Briefly, cells were lysed in PBS with 1% Triton X-100 and a cocktail of protease inhibitors. After sonication, cells were centrifuged at 100 000g at 4°C for 30 min. The soluble tau was in the supernatant and the insoluble tau in the pellet was dissolved in 1% Triton X-100/1% SDS.

Western blots were done as previously described (29). Bands on the Hyperfilm ECL films were scanned and band intensities were quantified using Scion Image software version beta 4.2. The intensity of the protein band of interest was divided by the intensity of the band representing a loading control to calculate the relative amount of a protein of interest.

Antibodies

We used the following antibodies in the study: anti-Xpress (Invitrogen), anti-ataxin3 (Chemicon), anti-tau 5 (reacts with phosphorylated as well as non-phosphorylated forms of tau, Abcam), anti-GFP (Clontech), anti-tubulin and anti-actin (Sigma). Western blotting was performed using standard protocols and densitometry analysis was performed using Scion Image Beta 4.02 software.

Drosophila crosses

Flies were grown on standard fly food with 40–70% humidity and with 12/12 h light/dark cycle. Instant fly food (Philip Harris Ltd, UK) was used when rapamycin (or DMSO) was added to the medium. Flies were always kept at 25°C, unless stated otherwise in the text. Crosses with polyglutamine, polyalanine and tau flies were done as described in the main text. Survival to adulthood was tested as described previously (29). Eyes were scored as abnormal when any external abnormality was seen under the dissecting microscope. Typically, this was manifested by abnormal shape, rough surface or rarely complete absence of the eyes. Figures can be found in the work of Berger *et al.* (29).

Drug treatment

Flies were allowed to mate on normal fly food for 2–3 days and then transferred to instant fly food containing rapamycin (1 μ M rapamycin, LC laboratories) or DMSO. For testing the effects of mutants or drug treatments, crosses were always done at the same time under exactly the same conditions. Analysis was performed with the observer blinded to the identity of the flies. Unconditional logistical regression analysis with the general log-linear analysis option of SPSS Version 6.1 was used (SPSS, Chicago, IL, USA).

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Conflict of Interest statement. D.R. and B.R. are inventors and Z.B. and C.O'K. are contributors on a patent describing the use of rapamycin for the treatment of diseases caused by aggregate-prone proteins. This work was partly funded by a grant from Wyeth. MNP holds stock and is employed by Wyeth who make rapamycin.

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