Suppression of polyglutamine-induced toxicity in cell and animal models of Huntington’s disease by ubiquilin

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Expanded polyglutamine (polyQ) tracts are associated with the induction of protein aggregation and cause cytotoxicity in nine different neurodegenerative disorders. Here, we report that ubiquilin suppresses polyQ-induced protein aggregation and toxicity in cells and in an animal model of Huntington’s disease. Overexpression of ubiquilin in HeLa cells and primary neurons reduced aggregation of polyQ-containing proteins and cell death induced by overexpression of a green fluorescent protein (GFP)–huntingtin fusion protein containing 74 polyQ repeats [GFP–Htt(Q74)], in a dose-dependent manner. Moreover, overexpression of ubiquilin suppressed oxidative stress-induced cell death in HeLa cell lines stably expressing GFP–Htt(Q74). In contrast, knockdown of ubiquilin expression in these cell lines was associated with increases in DNA fragmentation, caspase activation, GFP-fusion protein aggregation, and cell death. Caenorhabditis elegans lines expressing GFP–Htt fusion proteins in body wall muscle displayed a polyQ repeat length-dependent decrease in body movement compared with wild-type animals. RNA interference of the C. elegans ubiquilin gene exacerbated the motility defect, whereas overexpression of ubiquilin prevented, and could rescue, loss of worm movement induced by overexpression of GFP–Htt(Q55). These results suggest that ubiquilin might be a novel therapeutic target for treating polyQ diseases.

INTRODUCTION

Huntington’s disease (HD) is an age-related neurological illness that is inherited in an autosomal dominant fashion and is characterized by choreic movements, severe behavioral and emotional disturbances and cognitive decline. The duration of the disease is usually 15–20 years and HD ultimately results in death (1). Currently, there is no effective way to prevent or cure HD.

HD is caused by the expansion of a trinucleotide sequence (CAG) that resides in exon 1 of the gene encoding huntingtin (htt) protein (2). The expanded CAG repeats are translated into tracts of polyglutamine (polyQ) residues that in non-affected individuals ranges in length from 14 to 34 and in individuals afflicted with HD consist of more than 40 residues. To date, at least eight other neurological disorders are known to be caused by an expansion of polyQ tracts; these disorders include dentatorubral-pallidoluysian atrophy, spinal and bulbar muscular atrophy and spinocerebellar ataxias 1, 2, 3, 6, 7 and 17. The translated proteins that contain the expanded polyQ tract in these nine disorders are not related to each other in sequence, strongly suggesting that the expanded polyQ tract is the cause of the disease.

Several theories have been proposed for the mechanism by which expanded polyQ tracts cause disease (reviewed in 3–7). Chief among these is that proteins that contain longer polyQ repeats are more likely to aggregate, and it is the aggregates, through a gain-of-function property, that are toxic. However, others have argued that such aggregates are not toxic, but might, in fact, be protective (8,9 and references therein).

Regardless of the exact mechanism by which polyQ repeats cause disease, it is clear that for most polyQ repeat-associated
diseases, there is an inverse correlation between the number of polyQ repeats and the age of disease onset and the severity of the disease. Interestingly, the age of onset of HD in individuals who express Htt proteins with expanded polyQ tracts that are 40 repeats or longer can vary by as much as 35 years, leading to the suggestion that unknown genetic and environmental factors influence the age of onset of HD (10).

We became interested in the possibility that ubiquilin, a protein we had identified as an interactor of the Alzheimer-associated presenilin proteins (11), might be involved in regulating HD pathogenesis because ubiquilin proteins have been reported to bind and co-localize with polyQ-containing proteins (12,13). Here, we investigated the effects of overexpression and underexpression of ubiquilin-1 on polyQ-induced cell death and/or toxicity in cells and in a Caenorhabditis elegans animal model of HD. Our results indicate that an increase in ubiquilin expression protects against GFP–Htt–polyQ-induced cell death and toxicity.

RESULTS
Overexpression of ubiquilin reduces cell death in HeLa cells and primary neurons
To study the toxicity of a polyQ-containing protein, we transfected HeLa cells with expression constructs encoding Htt exon-1 fragment containing different numbers of polyQ repeats tagged with GFP protein and examined their effects on cell viability. As was previously reported (14,15), transient expression of GFP–Htt-exon-1 fusion protein containing either 55 or 74 polyQ repeats [GFP–Htt(Q55) and GFP–Htt(Q74), respectively] induced an increase in cell death compared with cells expressing GFP–Htt(Q28) or GFP alone (Fig. 1B). Immunoblot analysis using an anti-GFP antibody confirmed that proteins of the expected size were expressed in every case (Fig. 1A).

To examine the effect of co-overexpression of ubiquilin-1 on cell death, we concentrated on the GFP–Htt(Q74) construct because it produced the most robust effects on cell death. Interestingly, co-transfection of increasing amounts of full-length untagged human ubiquilin-1 cDNA expression construct with a constant amount of the GFP–Htt(Q74) construct resulted in dose-dependent increase in accumulation of GFP–Htt(Q74) fusion protein (Fig. 2A). We hypothesized that the increase in GFP–Htt(Q74) fusion protein accumulation induced by ubiquilin-1 overexpression might have resulted from increased survival of cells co-transfected with ubiquilin-1. To determine this possibility, we co-transfected HeLa cells with constant amounts of GFP–Htt(Q74) and monomeric red fluorescent protein (mRFP) expression constructs and increasing amounts of ubiquilin-1 cDNA and analyzed the levels of the different expressed proteins by immunoblotting. As shown in Figure 2B and C, consistent with our previous findings overexpression of ubiquilin resulted in a dose-dependent increase in GFP–Htt(Q74) accumulation. In contrast, levels of mRFP, which we used as a control to monitor transfection efficiency/cell survival, increased with a slightly different profile, but importantly, its levels too rose steeply in the cells co-transfected with the highest concentration of ubiquilin-1 cDNA, suggesting that high levels of ubiquilin-1 overexpression might promote increased survival of the co-transfected cells. More direct evidence illustrating that ubiquilin overexpression is cytoprotective was that cells co-transfected with a constant amount of GFP–Htt(Q74) and increasing amounts of ubiquilin-1 cDNA expression constructs displayed a dose-dependent decrease in cell death compared with cells transfected with GFP–Htt(Q74) alone (Fig. 2D).

We next investigated whether the modulation of polyQ toxicity by ubiquilin-1 overexpression occurs in neuronal cells. We therefore repeated these co-transfection experiments using primary mouse neuronal cultures. As shown in Figure 3A and B, the results we obtained were similar to those obtained using HeLa cells, namely ubiquilin-1 overexpression decreased GFP–Htt(Q74)-induced neuronal cell death. Interestingly, immunofluorescence examination of these transfected neuronal cells revealed a dose-dependent decrease in the number of GFP-positive inclusions (Fig. 3C),
suggesting that ubiquilin overexpression might prevent or rid cells of Htt protein aggregation.

**Generation of stable cell lines expressing GFP–Htt–polyQ fusion proteins: demonstration that ubiquilin protects against sensitivity of the cells to oxidative stress induced by expanded polyQ proteins**

To obtain further evidence that ubiquilin protects cells against polyQ-induced protein aggregation and cytotoxicity, we established HeLa cell lines that stably expressed either GFP alone or GFP–Htt(Q28), GFP–Htt(Q55) or GFP–Htt(Q74) fusion proteins. The stable lines, unlike transiently transfected cells, express GFP-fusion proteins more uniformly and therefore are more reliable for assessing the effects of the different polyQ proteins. Figure 4A shows an immunoblot of the relative expression levels of the GFP proteins in different cell lines. We used two of these cell lines, GFP–Htt(Q74-2) and GFP–Htt(Q74-3), to examine first whether the length of polyQ tract affects the sensitivity of cells to different stress-inducing agents, and secondly, whether ubiquilin expression could protect against the cytotoxic effects of these agents. As shown in Figure 4B, although the stable GFP–Htt(Q28-2) and GFP–Htt(Q74-3) cell lines exhibited strong GFP fluorescence in both the cytoplasm and the nucleus, foci of fluorescence (i.e. inclusions) were especially noticeable at longer times after transfection (data not shown). The formation of foci in the GFP–Htt(Q74-3) cell line clearly demonstrates that inclusions, by themselves, are not
sufficient to induce cytotoxicity, otherwise the cell lines would not be viable. Upon further characterization, we found that the GFP–Htt(Q28-2) and GFP–Htt(Q74-3) cell lines were differentially sensitive to oxidative stress as assessed by exposure to H2O2 and serum starvation (16–21).

In initial studies, we established that HeLa cells are relatively insensitive to 5 h of exposure to H2O2 at concentrations of 200 μM (data not shown). The GFP–Htt(Q28-2) cell line was also insensitive to H2O2 concentrations within this same range, whereas the GFP–Htt(Q74-3) cell line was sensitive, with ~50% of cells dying after exposure to 200 μM of H2O2 (Fig. 4C and D), suggesting that expression of the GFP–Htt protein containing 74 polyQ repeats sensitized cells to oxidative stress. To examine whether ubiquilin exerts any protective effect on H2O2-mediated cell death, the GFP–Htt(Q28-2) and GFP–Htt(Q74-3) stable cell lines were transiently transfected with a ubiquilin-1 expression plasmid and 20 h later were exposed for 5 h to 100 μM H2O2. As shown in Figure 4E and F, the rate of cell death was very low and was unaltered either after mock transfection or after ubiquilin-1 transfection of the GFP–Htt(Q28-2) cell line, following the H2O2 treatment. In contrast, cell death caused by exposure of the GFP–Htt(Q74-3) cell line to 100 μM H2O2 was reduced by ~80% in cells transfected with ubiquilin-1 cDNA compared with mock-transfected cells, suggesting that ubiquilin-1 overexpression protects against the hypersensitivity of cells to oxidative stress caused by expanded polyQ expression (Fig. 4E).

We confirmed the protective role of ubiquilin using a different method to induce oxidative stress, namely culturing cells in the absence of serum for 5 h (21). As shown in Figure 4G, there was no significant difference in cell death between the mock-transfected and ubiquilin-1-transfected GFP–HttQ28-2 cell line following this culturing protocol. In contrast, the same treatment induced significant cell death in the GFP–HttQ74-3 cell line, and transient transfection of ubiquilin-1 reduced cell death by ~50% (Fig. 4G). Moreover, transfection of the GFP–HttQ74-3 cell line with increasing amounts of ubiquilin-1 expression plasmid resulted in progressively less cell death when the cells were cultured under the same conditions.

Figure 3. Overexpression of ubiquilin-1 reduces polyQ inclusions and cytotoxicity in mouse primary neuronal cultures. (A) Representative images of mouse neurons transfected with the plasmids indicated. Mouse cortical neurons (14 days in vitro) were transiently transfected with GFP (3 μg each well, a), or GFP–Htt(Q74) alone (3 μg each well, b), or co-transfected with both GFP–Htt(Q74) and ubiquilin-1 cDNA expression constructs with the ratio indicated [3 μg of GFP–Htt(Q74) with 0, 1.5 or 6 μg of ubiquilin-1 cDNA; b, c and d, respectively]. (B) Overexpression of ubiquilin-1 reduces GFP–Htt(Q74)-induced cell death in cultured mouse neurons. Thirty hours following transfection, the cultures were stained with both Hoechst 33342 and PI and then subjected for fluorescent microscopy analysis. PI-positively stained cells were treated as dead cells and only the green (GFP-positive) cells were included in the cell death analysis. Results are mean ± SD. (C) Overexpression of ubiquilin-1 reduces formation of GFP–Htt(Q74) inclusions in cultured mouse neurons. The results shown is the mean of the number of GFP-positive cells with eye-detectable aggregates either in the cell body or neurites ± SD.
cells were exposed to different concentrations of H$_2$O$_2$ for 5 h. Percentages of cell death calculated by determining the ratio of PI-positively stained cells to that of Hoechst-stained cells. The GFP–Htt(Q28-2) and GFP–Htt(Q74-3) stable cell lines were exposed to 200 μM concentration of H$_2$O$_2$ and after 5 h, the cells were stained with both Hoechst 33342 and PI. The cells stained by PI represent the dead cells, whereas all the nuclei are identified by Hoechst 33342 staining. The result of merging the GFP, PI and Hoechst of the same field of cells is shown on the right-hand panels. (C) Cells expressing expanded polyQ proteins are more sensitive to H$_2$O$_2$. The GFP–Htt(Q28-2) and GFP–Htt(Q74-3) stable cell lines were exposed to 200 μM concentration of H$_2$O$_2$, and after 5 h, the cells were stained with both Hoechst 33342 and PI. The cells stained by PI represent the dead cells, whereas all the nuclei are identified by Hoechst 33342 staining. The result of merging the GFP, PI and Hoechst of the same field of cells is shown on the right-hand panels. (D) Graph showing percentage of cell death after the cells were exposed to different concentrations of H$_2$O$_2$ for 5 h. Percentages of cell death calculated by determining the ratio of PI-positively stained cells to that of Hoechst-stained cells. There was a significant difference in cell death (marked with an *) upon exposure of GFP–Htt(Q28-2) and GFP–Htt(Q74-3) to 50, 100, 150 or 200 μM of H$_2$O$_2$ (P < 0.001). (E) Overexpression of ubiquilin-1 protects GFP–Htt(Q74-3) cells against H$_2$O$_2$-induced cell death. Cells stably expressing GFP–Htt(Q28) or GFP–Htt(Q74) were transiently transfected with or without (mock transfection) plasmids encoding ubiquilin-1. Twenty-four hours following the transfection, the cells were treated with 100 μM H$_2$O$_2$ for 5 h and cell death was analyzed by doubly staining the cells with Hoechst and PI. Asterisk indicates a statistically significant reduction in cell death in the GFP–Htt(Q74-3) cell line upon ubiquilin-1 transfection (P < 0.001). (F) Immunoblot of equal amounts of protein lysate from GFP–Htt(Q28-2) and GFP–Htt(Q74-3) cells transfected without (mock transfection) or with a ubiquilin-1 expression construct in a parallel experiment as described in (E). Note that the ubiquilin antibody recognizes both ubiquilin-1 (lower band) and -2 (upper band). The same membrane was rebotted for actin as a loading control. (G) Graph showing that ubiquilin-1 overexpression protects GFP–Htt(Q74-3) cell line against serum withdrawal-induced cell death. Cells stably expressing GFP–Htt(Q28) or GFP–Htt(Q74) were transiently transfected with or without (mock transfection) plasmids encoding ubiquilin-1 by the calcium phosphate co-precipitation method. Following DNA-calcium phosphate transfection, the cultures were maintained in serum-free medium for 5 h and then in medium containing serum for the remainder of the experiment. Cell death was quantified as described in (D). Asterisk indicates a statistically significant reduction in cell death in the GFP–Htt(Q74-3) cell line upon ubiquilin-1 transfection (P < 0.005). (H) Representative images showing that ubiquilin-1 overexpression protects GFP–Htt(Q74-3) expressing cells against serum withdrawal-induced cell death in a dose-dependent manner. Cells stably expressing GFP–Htt(Q74) were transiently transfected without (a, mock transfection) or with an increasing amount of a cDNA encoding ubiquilin-1 (1, 2, 3 and 6 μg; b, c, d and e, respectively). After 5 h of serum withdrawal and 30 h following the transfection, the amount of cell death was analyzed by staining cells with both PI and Hoechst 33342. (I) Graph showing quantification of cell death in the experiments described in (H). Asterisk indicates a statistically significant reduction in cell death in the GFP–Htt(Q74) line upon ubiquilin-1 transfection compared with ubiquilin-1 untransfected cells. (J) Immunoblot showing that overexpression of increasing amounts of ubiquilin-1 cDNA in the GFP–Htt(Q74-3) cell line reduces GFP-aggregates trapped in the stacking gel after SDS–PAGE. The amount of ubiquilin-1 cDNA transfected is shown. Please note that both anti-ubiquilin and anti-GFP immunoreactivity is found in the stacking gel [probably because of binding of ubiquilin and GFP–Htt(Q74) proteins] and that immunoreactivity to both antibodies decreases with increasing ubiquilin-1 expression. (K) A filter retardation assay also demonstrates that overexpression of increasing amounts of ubiquilin-1 cDNA in the GFP–Htt(Q74-3) cell line reduces GFP-aggregates. Top panel: 20 μg of cell lysates from either GFP–Htt(Q28-2) cells which do not form aggregates, used as a control, or GFP–Htt(Q74-3) cells after either mock transfection or transfection of increasing amounts of ubiquilin-1 expression construct as indicated. The lysates were filtered through a 0.2 μm pore cellulose acetate membrane and then with anti-GFP and anti-UBQLN antibodies. Bottom panel: quantification of the anti-GFP spot intensity determined in three independent experiments. The spot intensity from the 3, and 6 μg of ubiquilin-transfected GFP–HttPolyQ(74)-3 cells are significantly lower (P < 0.05) than that from corresponding cells mock transfected or transfected with 1 μg of ubiquilin.
conditions (Fig. 4H and I). Immunoblot analysis of protein lysates prepared from these transfected cells revealed that overexpression of ubiquilin-1 was associated with a dose-dependent decrease in aggregated ubiquilin and GFP–Htt(Q74) fusion proteins as revealed by the amount of the proteins that was either trapped in the stacking gel after SDS/PAGE (Fig. 4J) or retained on filters following filtration of the lysates (Fig. 4K) (22). These results, taken together, suggest that overexpression of ubiquilin-1 reduces polyQ protein aggregation and the sensitivity of cells to cytotoxic stress induced by proteins containing expanded tracts of polyQ.

We next examined what effect reducing the levels of endogenous ubiquilin protein has on cell survival of the GFP–Htt(Q74-3) cell line. Because HeLa cells express both ubiquilin-1 and ubiquilin-2 proteins, we knocked down expression of both ubiquilin proteins simultaneously by transfecting the GFP–Htt(Q74-3) cell line with siRNA SMART-pools (23). Two days after siRNA transfection, both

Figure 4. Continued.
ubiquilin-1 and -2 protein levels were reduced by >90% compared with cells transfected with control siRNAs (i.e. one with no homology to any human mRNA) or mock-transfected cells, and this low level of ubiquilin was maintained for at least 4 days after transfection when cultured in the presence of ubiquilin siRNAs (Fig. 5A). During the first 2 days after transfection, we observed no differences in growth or morphology between mock-transfected, control siRNA-transfected and ubiquilin siRNA-transfected cells. However, by day 3 after transfection, the cells transfected with ubiquilin siRNA stopped proliferating, whereas mock- and control siRNA-transfected cells continued to proliferate (Fig. 5B). Moreover, 4 days after ubiquilin siRNA transfection, we observed a statistically significant increase in cell death in the ubiquilin siRNA-transfected cells compared with mock and control siRNA-transfected GFP–Htt(Q74-3) cells, as detected by a nuclear fragmentation/condensation assay (Fig. 5C and D), terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining (Fig. 5E and F), propidium iodide (PI) staining (Fig. 5G) and activation of caspase-3 (Fig. 5H and I). In addition, a filter-trap assay demonstrated that siRNA knockdown of ubiquilin levels led to increased accumulation of expanded GFP-immunoreactive aggregates (Fig. 5J and K). These results demonstrate that a decrease in ubiquilin levels halts cell proliferation and triggers cell death and the accumulation of polyQ aggregates in the GFP–Htt(Q74-3) expressing cell line.

To ensure that the protective effects exerted by ubiquilin were not unique to the two cell lines we had utilized, we repeated many of the experiments using two independent cell lines, GFP–Htt(Q28-5) and GFP–Htt(Q74-6), expressing GFP–Htt(Q28) and GFP–Htt(Q74) fusion proteins and found similar phenomena (Supplementary Material, Figs S1–S3).

**C. elegans** lines stably expressing GFP–Htt–polyQ fusion proteins containing expanded polyQ repeats in body wall muscle exhibit a motility defect

We next sought to determine the biologic relevance of our findings by examining whether ubiquilin is capable of suppressing polyQ-induced protein toxicity in an animal model. To investigate this possibility, we utilized the nematode, *C. elegans*, to model the effects of polyQ protein aggregation and toxicity. Previous studies have shown that muscle-specific expression of GFP–polyQ fusion proteins in *C. elegans* induces polyQ length-dependent protein aggregation and toxicity that are associated with decreased worm motility (24–27).

We used the muscle-specific unc-54 promoter (28) to drive expression of the different GFP–Htt fusion constructs in *C. elegans* and established several stable lines of worms that expressed each of the different proteins. An immunoblot of protein extracts prepared from the animals confirmed that a GFP–Htt fusion protein of the appropriate size was expressed in each line (Fig. 6A). Expression of the different GFP–Htt fusion proteins resulted in polyQ length-dependent changes in the GFP fluorescence pattern in the muscle cells of the animals. Specifically, the pattern of fluorescence changed from a diffuse fluorescence pattern in worms expressing GFP to a rope-like pattern in worms expressing GFP–Htt(Q28) and to a pattern characterized by more compact foci of fluorescence in worms expressing GFP–Htt(Q55) and GFP–Htt(Q74) (Fig. 6B and C). We examined whether muscle function was altered in these animals by counting the number of body bends flexed by the worms over a 1 min interval of continuous movement, a parameter that correlates well with *C. elegans* motility (25). Consistent with a previous report (25), we found that 1-day-old adult worms that expressed GFP–Htt–polyQ fusion proteins displayed a polyQ length-dependent decrease in motility compared with wild-type animals (Fig. 6D).

**RNA interference of ubiquilin exacerbates the motility defect in *C. elegans* lines expressing GFP–HttPolyQ fusion proteins**

We next determined the effects of reducing ubiquilin levels in the worms that stably express the GFP–Htt fusion proteins. To knockdown ubiquilin protein in the worms, we cloned the complete *C. elegans* ubiquilin cDNA (F15C11.2a) into the bacterial RNA interference (RNAi) expression vector L4440 (29,30). The L4440 vector is widely used to genetically disrupt expression of a particular *C. elegans* gene by letting worms feed on bacteria that are transformed with the plasmid containing the cDNA of the gene that is targeted for RNAi. When induced with isopropyl β-D-thiogalactoside (IPTG), the bacteria synthesize a dsRNA copy of the cDNA, which when ingested by the worms frequently induces genetic interference throughout the body of the worm. Accordingly, we fed GFP–, GFP–Htt(Q28)–, and GFP–Htt(Q74)–expressing worms bacteria that were transformed with either the L4440 vector alone or the L4440 vector containing the cloned *C. elegans* ubiquilin cDNA (L4440: ubiquilin) or a related vector containing a cloned GFP cDNA (L4417:GFP) (30), which were either exposed to IPTG or not. Two days later, we counted the number of body bends flexed by the worms during 1 min of continuous movement on agar plates.

GFP–Htt(Q28)– and GFP–Htt(Q74)-expressing adult worms fed on IPTG-exposed bacteria containing L4417:GFP plasmid had more body bends than animals fed on the same transformed bacteria not exposed to IPTG (Fig. 7A). In fact, the number of body bends in GFP–Htt(Q28)– and GFP–Htt(Q74)-expressing animals fed on IPTG-exposed bacteria containing L4417:GFP plasmid was restored to within 16 and 25%, respectively, of the level found in normal wild-type animals, suggesting that genetic interference of GFP expression in adult *C. elegans* can restore some, but not all of the crippling effects produced by expression of the GFP–Htt–polyQ fusion proteins in muscle cells. An examination of the rescued animals by fluorescence microscopy revealed almost complete loss of GFP fluorescence (data not shown), which is consistent with successful dsRNAi of GFP.

Having established that the bacterial feeding protocol was suitable for silencing GFP in the polyQ protein expressing *C. elegans* lines, we next utilized the procedure to genetically interfere with expression of endogenous ubiquilin in the different lines. Interestingly, wild-type worms that fed on IPTG-exposed bacteria containing L4440:ubiquilin plasmid
Figure 5. RNAi of ubiquilin in GFP–Htt(Q74-3) cells leads to inhibition of cell proliferation and promotes GFP–polyQ aggregation and cell death over time. (A) Immunoblots showing successful knockdown of ubiquilin protein levels by siRNAs. GFP–Htt(Q74-3) cells were either mock transfected or transfected with a combination of ubiquilin-1 and -2 SMARTpool siRNAs or transfected with a non-target control SMARTpool siRNA. Cells were harvested 2, 3 or 4 days following the transfection, and equal amounts of protein were immunoblotted for ubiquilin. Ubiquilin levels were downregulated to <10% of the normal levels 2 days after transfection and this low level of protein was maintained for at least 4 days after transfection. (B) Ubiquilin knockdown is associated with decreased cell proliferation. GFP–Htt(Q74-3) cells, cultured in a 24-well plate at a low density, were either mock transfected or transfected with a combination of ubiquilin-1 and -2 SMARTpool siRNAs or control siRNAs. The phase contrast images shown were taken just before transfection and 4 days after siRNA transfection. (C) Ubiquilin knockdown is associated in an increase in nuclear condensation/DNA fragmentation. GFP–Htt(Q74-3) cells were transfected with siRNAs as described in (A) and stained with Hoechst 33342 on day 5 following transfection. (D) Graph showing quantification of fragmented/condensed nuclei in the experiments described in (C). Asterisk indicates that there is a significant difference in nuclear condensation/fragmentation between the cells transfected with ubiquilin siRNA with either mock-transfected cells or cells transfected with control siRNA (P < 0.05). (E) TUNEL staining of GFP–Htt(Q74-3) cells transfected with reagent vesicle alone (mock transfection), ubiquilin-1 and -2 siRNAs or control siRNA. Five days following the transfection, cells were fixed and subjected to TUNEL staining. (F) Graph showing percentage of TUNEL-positive cells seen in the experiments described in (E). Asterisk indicates that there is a significant difference in TUNEL staining between the cells transfected with ubiquilin siRNA with either mock-transfected cells or cells transfected with control siRNA (P < 0.05). (G) Graph showing quantification of cell death in GFP–Htt(Q74-3) cells, 5 days after transfection with the siRNAs, as described in (B). The data shown represents the percentage of PI versus total Hoechst-positive cells. (H) Ubiquilin knockdown in GFP–Htt(Q74-3) cells is associated with increased caspase-3 activation. GFP–Htt(Q74-3) cells were transfected with siRNAs as described in (B), and 4 days after transfection, equal amounts of protein lysate were immunoblotted for the cleaved (active) form of caspase-3. The immunoblot also shows a lystate of GFP–Htt(Q74-3) cells treated with 1 μM staurosporine for 4 h as a positive control. (I) Graph showing fluorogenic measurement of caspase-3 activity after ubiquilin knockdown in the GFP–Htt(Q74-3) cell line. Experiment similar to (H), but this time caspase-3 activity was determined by measuring the cleavage of a fluorescent caspase-3 substrate. Asterisk indicates that there is a significant difference in caspase-3 activity between the cells transfected with ubiquilin siRNA with either mock-transfected cells or cells transfected with control siRNA (P < 0.05). (J) Ubiquilin knockdown increases the amount of GFP-containing aggregates formed in the GFP–Htt(Q74-3) cell line. Filter retardation assay was performed to detect GFP–Htt(Q74-3) aggregates. Equal amounts of protein lysates, diluted to different extents, prepared from cells 5 days after transfection with the siRNA described in (B) were filtered through a cellulose acetate membrane and probed with an anti-GFP antibody. (K) Graph showing measurement of spot intensity of aggregates formed in the GFP–Htt(Q74-3) cell line in three independent experiments as described in (J). Asterisk indicates that there is a significant difference in GFP staining between the cells transfected with ubiquilin siRNA with either mock-transfected cells or cells transfected with control siRNA (P < 0.05).
had ~5% fewer body bends compared with worms that fed on IPTG-unexposed bacteria containing the L4440:ubiquilin plasmid, suggesting that RNAi of ubiquilin in the absence of GFP–Htt–polyQ expression might compromise worm movement to some degree (Fig. 7B). More interestingly, GFP–Htt(Q28)- and GFP–Htt(Q74)-expressing worms that fed on IPTG-exposed bacteria containing L4440:ubiquilin plasmid had 55 and 75% fewer body bends, respectively, than siblings that had fed on IPTG-unexposed bacteria containing the L4440:ubiquilin plasmid or IPTG-exposed bacteria containing the L4440 vector alone (Fig. 7B). These results demonstrate that genetic interference of *C. elegans* ubiquilin reduces worm motility and that movement is even more compromised in animals expressing longer lengths of GFP–Htt–polyQ fusion proteins. Finally, we immunoblotted protein extracts from worms recovered from the different treatments and found that the levels of ubiquilin protein were indeed reduced ~4-fold in the worms that were fed bacteria containing L4440:ubiquilin plasmid exposed to IPTG compared with wild-type worms (Fig. 7C).

**Overexpression of ubiquilin in worms can both prevent and rescue the motility defect associated with expression of GFP–Htt(Q55) fusion protein**

We next sought to determine whether overexpression of ubiquilin could prevent (rescue) the GFP–Htt–polyQ-dependent loss of movement. To investigate this possibility, we created an expression construct in which we tagged the *C. elegans* ubiquilin with the mRFP. In control experiments, we found that the mRFP–ubiquilin fusion construct, when expressed in HeLa cells, had the expected size and localization pattern as endogenous HeLa ubiquilin (Fig. 7D and E) (data not shown).

We examined whether forced expression of the mRFP–ubiquilin fusion protein in muscle cells of *C. elegans* affected the number of body bends in worms lines that stably expressed GFP–Htt(Q55). Accordingly, we established six new stable GFP–Htt(Q55)-expressing worm lines, of which three were derived by injecting plasmid DNA containing GFP–Htt(Q55) placed under the control of the unc-54 promoter
and the other three were derived by injecting a 2:1 DNA ratio of a mixture of unc-54-driven mRFP–ubiquilin and unc-54-driven GFP–Htt(Q55) plasmids, respectively. RT–PCR analysis of RNA isolated from the different worm lines indicated the presence of GFP mRNA in all six lines, whereas RFP–ubiquilin mRNA was only detected in the lines co-injected with RFP–ubiquilin (Fig. 8A). In fact, this semi-quantitative RT–PCR analysis suggested that the level of GFP–Htt(Q55) mRNA expression was approximately the same in all six worm lines, with the exception of GFP–Htt(Q55) line 1, which appeared to have slightly lower expression. Similarly, mRFP–ubiquilin mRNA expression was approximately equal in the three co-expressing lines, with indications that line 2 had the lowest expression. Importantly, as expected, all six of these worm lines displayed strong GFP fluorescence (Fig. 8B–D), whereas RFP

Figure 6. Expression of polyQ expansions in *C. elegans* muscle results in length-dependent aggregate formation and motility defect. (A) Immunoblot showing anti-GFP immunoreaction in *C. elegans* protein extracts using 3–4-day-old animals expressing different lengths of GFP–Htt–polyQ proteins: lane 1, wild-type *C. elegans*; lane 2, GFP; lane 3, GFP–Htt(Q28); lane 4, GFP–Htt(Q55); lane 5, GFP–Htt(Q74). The lower panel is the anti-actin immunoblot of the same blot shown above. (B) GFP fluorescence micrographs of young adult (3–4 days old) *C. elegans* expressing different lengths of GFP–polyQ fusion proteins. Note that GFP fluorescence is mainly localized to the body wall muscle cells. Also, note that more compact foci form with increasing number of polyQ repeats expressed. Bar, 100 μm. (C) Higher magnification showing the body wall of young adult *C. elegans* expressing different lengths of GFP–polyQ fusion proteins as described in (B). Bar, 100 μm. (D) Motility assay measured as body bends per minute in wild-type and various transgenic lines of adult *C. elegans*. Data are mean ± SD for at least 12 animals of each type. Note that the rate of movement decreases with increasing length of polyQ repeats.
Figure 7. RNAi of ubiquilin in C. elegans GFP–HttPolyQ expressing lines exacerbates the motility defect even further, whereas RNAi of GFP rescues movement. (A) Downregulation of GFP using RNAi rescues motility defect caused by GFP–Htt–polyQ proteins. Motility assay measured as body bends per minute of wild-type and transgenic lines expressing different lengths of polyQs (Q28 and Q74) grown on bacteria transformed with RNAi vectors for GFP with or without IPTG. Data are mean ± SD for at least 12 animals of each type. Asterisk indicates that there is a significant increase in worm motility in lines expressing GFP–Htt(Q28) and GFP–Htt(Q74) following conditions that induce UBQLN RNAi when compared with conditions in which RNAi is not induced (P < 0.05). (B) Downregulation of ubiquilin using RNAi exacerbates motility defect in transgenic lines. Quantification of motility index for young adult animals (wild-type, Q28, Q72) grown on bacteria transformed with RNAi vectors for ubiquilin (L4440 + ubiquilin) or empty vector, with or without IPTG as indicated. Data are mean ± SD for at least 24 animals of each type. Asterisk indicates that there is a significant decrease in worm motility in lines expressing GFP–Htt(Q28) and GFP–Htt(Q74) following conditions that induce UBQLN RNAi when compared with conditions in which RNAi is not induced (P < 0.05). (C) Immunoblot analysis of C. elegans lines showing reduction of ubiquilin protein following RNAi treatment as described in (B). Equal amounts of protein lysate either from non-RNAi treated wild-type worms (lane 1), or from RNAi-treated wild-type worms (lane 2), RNAi-treated GFP–Htt(Q28) worms or RNAi-treated GFP–Htt(Q74) worms were immunoblotted with an anti-C. elegans ubiquilin or anti-actin antibodies. (D) Representative image of HeLa cell expressing monomeric RFP–C. elegans ubiquilin fusion protein. HeLa cells were transfected with a CMV-driven mRFP–C. elegans ubiquilin expression construct and viewed under rhodamine fluorescence microscopy after 24 h. Bar, 5 μm. (E) Immunoblot of HeLa lysates showing specificity of the anti-C. elegans ubiquilin and mRFP antibodies. Panel 1 (probed with anti-C. elegans ubiquilin antibody) and panel 2 (probed with anti-mRFP antibody): lane 1, untransfected control; lane 2, transfected with an mRFP–C. elegans ubiquilin plasmid construct. Note that both antibodies detected an 80 kDa band corresponding to mRFP–C. elegans ubiquilin fusion polypeptide.
fluorescence was only detected in the lines injected with RFP–ubiquilin plasmid DNA.

Muscle-specific expression of GFP–Htt(Q55) by itself caused the worms to move with approximately 10 body bends per minute in each of the three worm lines (Fig. 8E). In contrast, all three worm lines that co-expressed GFP–Htt(Q55) and mRFP–ubiquilin were less severely affected, with line 1 displaying 28 body bends per minute, line 2 displaying 17 body bends per minute and line 3 displaying 23 body bends per minute, which represent almost ~55–120% increase in the number of body bends compared with lines that expressed GFP–Htt(Q55) alone (Fig. 8E). Closer examination of GFP fluorescence in the worms that co-expressed GFP–Htt(Q55) and mRFP–ubiquilin revealed a noticeable difference in the pattern of fluorescence in the three lines: line 1 worms displayed diffuse fluorescence that was similar to that seen in animals expressing GFP alone (compare Fig. 8Ba and Da with Fig. 6Ba and Ca, respectively), whereas line 2 contained more GFP fluorescent foci and line 3 had slightly fewer foci than line 2, but more than line 1 (compare GFP foci in Fig. 8Ba, d and g). We speculate that the number of GFP foci that formed in the animals is governed by the relative ratio of expression of the GFP- and RFP-fusion proteins. Interestingly, we noticed that the RFP fluorescence co-localized with many, if not most, of the GFP foci, particularly compact foci, in the co-expressing animals (Fig. 8Dc–e, arrows), suggesting that RFP–ubiquilin fusion protein binds to the polyQ GFP-containing aggregates in live animals. Taken together, these results indicate that loss of C. elegans ubiquilin expression exacerbates polyQ-induced toxicity in muscle cells of the worms and that overexpression of ubiquilin protein can protect the worms against this polyQ-induced loss of movement.

To obtain further evidence that modulation of ubiquilin levels can protect against polyQ toxicity, we decided to see if we could rescue worm movement of the GFP–Htt(Q55) line 1, expressing GFP–Htt(Q55) alone, by injecting the worms with mRFP–ubiquilin plasmid DNA. Following this injection, we examined their progeny, and identified four worms that expressed GFP alone (presumably that did not inherit the injected DNA) and another four that co-expressed GFP and RFP. Importantly, quantification of worm body bend movement revealed that those that co-expressed RFP and GFP exhibited at least 2-fold, or greater, number of body bends than those that expressed GFP alone (Fig. 8F). These results suggest that ubiquilin overexpression not only prevents, but can also rescue the toxic effects of expanded polyQ tracts, at least in C. elegans lines expressing Htt–polyQ fusion proteins in the muscle.

**DISCUSSION**

Here, we presented evidence that overexpression of ubiquilin suppresses polyQ-induced toxicity in a cell and an animal model of HD. Our results clearly demonstrate that overexpression of ubiquilin in cells decreases both aggregation and cytotoxicity of GFP–Htt fusion proteins containing expanded polyQ repeats, whereas a reduction in ubiquilin levels by RNAi produced the opposite effects, i.e. increased aggregation and cytotoxicity. These results, together with the recently reported findings that variants in the human Ubiquilin-1 gene are genetically associated with late onset Alzheimer’s disease (AD) (31) and the previous demonstration that ubiquilin is localized to neuropathological inclusions in AD, Parkinson’s disease (11) and HD (13), lead us to speculate that ubiquilin might play a general role in a number of different neurodegenerative disorders.

Several findings support a role for ubiquilin in regulating aggregation and toxicity of polyQ proteins. First, ubiquilin proteins bind to and co-localize with polyQ aggregates both in vitro and in vivo (12,13). In accordance with this property, we found that mRFP-tagged C. elegans ubiquilin-fusion protein co-localized with GFP–Htt(Q55) fusion protein in compact foci in transgenic worm lines. The fact that this co-localization was detected in living animals suggests that

**Figure 8.** Co-expression of ubiquilin diminishes aggregate formation and can both prevent and rescue the motility defect caused by GFP–Htt(Q55). (A) RT–PCR analysis showing the profile of GFP–Htt(Q55) and mRFP–ubiquilin mRNA expression in different worm lines. Total RNA was isolated from three independent lines injected with GFP–Htt(Q55) expression plasmid (first three lanes) and from three additional independent lines injected with a 2:1 mixture of mRFP–ubiquilin and GFP–Htt(Q55) expression plasmids (next three lanes), or from control uninjected wild-type worms (last lane). A constant amount of RNA was utilized to RT–PCR amplify a portion of the transgenic GFP coding sequence (upper panel) or a sequence spanning the fused mRFP–ubiquilin transgenic coding sequence (middle panel) or the endogenous C. elegans ama-1 ribosomal subunit RNA (lower panel). All reactions were performed under identical conditions and a constant amount of the resultant reactions were analyzed by ethidium bromide staining after agarose gel electrophoresis. (B and C) Fluorescence micrographs of three lines of young adult (3–4 days old) C. elegans co-expressing GFP–Htt(Q55) and mRFP–ubiquilin (a–i) or GFP–Htt(Q55) only (j–l). The fluorescence micrographs for GFP and RFP in the co-expressing lines 1, 2 and 3 are shown in (a, d and g) and (b, e and h), respectively, and the result of merging the GFP and RFP images is shown in (c, f and i), respectively. Note that (a, d and g) display different extents of more diffuse pattern of GFP fluorescence compared with (j, k and l). Bar, 100 μm. (D) Fluorescence micrographs of young adult C. elegans co-expressing GFP–Htt(Q55) and mRFP–ubiquilin. (a) fluorescence micrograph for GFP fluorescence in the co-expressing line 1 and (b) corresponding fluorescence micrograph for RFP in the same animal. Note that co-expression of mRFP–ubiquilin diminishes aggregate formation of GFP–Htt(Q55). (c) fluorescence micrograph for GFP fluorescence in the co-expressing line 3; (d) corresponding fluorescence micrograph for RFP in the same animal; (e) merged image of (c) and (d). Small arrows indicate that GFP–Htt(Q55) and mRFP–ubiquilin co-localize at foci, whereas arrowheads indicate lack of co-localization of ubiquilin with the GFP–polyQ fusion protein. Bar, 100 μm. (E) Motility assay measured as body bends per minute in three lines of young adult C. elegans that were injected with GFP–Htt(Q55) alone or with GFP–Htt(Q55) and mRFP–ubiquilin expression constructs. Data are mean ± SD for at least 12 animals of three independent lines that either expressed GFP–Htt(Q55) fusion protein alone or co-expressed GFP–Htt(Q55) and mRFP–ubiquilin fusion proteins. Note that co-expression of mRFP–ubiquilin prevents to a significant extent (P < 0.05) the motility defect, to different extents, caused by GFP–Htt(Q55) compared with lines expressing GFP–Htt(Q55) alone. (F) Evidence that overexpression of mRFP–ubiquilin can rescue the motility defect in the GFP–Htt(Q55)-1 expressing worm line. The GFP–Htt(Q55)-1 worm line characterized above was injected with mRFP–ubiquilin expression plasmid and the resultant progeny from four independent worms that expressed either GFP–Htt(Q55) only or GFP–Htt(Q55) and mRFP–ubiquilin were utilized to measure the number of body bends exhibited by the worms for a 1 min interval. Data are mean ± SD for at least three trials of each type. Note that co-expression of mRFP–ubiquilin rescues to a significant extent (P < 0.05) the motility defect, to different extents, in the GFP–Htt(Q55)-1 line compared with worms expressing GFP–Htt(Q55) alone.
the previous co-localization of ubiquilin and polyQ proteins reported in mouse brain (13) was not an artifact produced by fixation and/or staining. Secondly, overexpression of ubiquilin-1 reduced GFP–polyQ protein containing inclusions and aggregation and also suppressed polyQ-induced cell death in HeLa cells and in primary cortical neurons. Thirdly, overexpression of ubiquilin-1 suppressed oxidative-stress-induced cell death in stable HeLa cell lines expressing GFP–Htt(Q74) fusion protein. In contrast, knockdown of ubiquilin expression in these same cell lines was associated with increased DNA fragmentation, caspase activation, GFP-aggregate formation and cell death. Fourthly, RNAi of the C. elegans ubiquilin gene led to a reduction in body bend movement in worm lines stably expressing GFP–Htt–polyQ fusion proteins. In contrast, co-expression of mRFP-tagged ubiquilin with GFP–Htt(Q55) fusion protein in the muscle prevented and could also rescue the motility defect seen in worm lines that expressed the polyQ fusion protein alone.

One unanswered question that emerges from our work is how does ubiquilin suppress polyQ-induced toxicity? Because the mechanism by which expanded polyQ proteins cause disease is still not understood, we can only speculate on possible answers. One possible clue is provided by our demonstration that overexpression of ubiquilin suppresses cell death induced by agents that induce oxidative stress. HeLa cells that stably expressed the GFP–Htt(Q74) were more sensitive to H₂O₂ and serum withdrawal than the HeLa cells that stably expressed GFP–Htt(Q28), and overexpression of ubiquilin-1 suppressed the vulnerability of the GFP–Htt(Q74)-expressing cells to these agents. The increase in sensitivity of the GFP–Htt(Q74) cell line to oxidative stress is consistent with numerous other reports showing that expanded polyQ repeats induce cell stress, but the exact reason for why cells elicit this stress response is still not fully understood (32–34). One possibility is that the increased tendency of proteins with expanded polyQ repeats to misfold and aggregate induces the cellular machinery that is responsible for clearing the misfolded aggregates, leading to an induction of cell stress. Two key components that are induced during cell stress are molecular chaperones and components of the ubiquitin–proteasome system (reviewed in 3,4,35). Several properties of ubiquilin suggest that it might play a dual role in both the ubiquitin–proteasome system and as a molecular chaperone. Ubiquilin proteins contain an N-terminal ubiquitin-like domain and a C-terminal ubiquitin-associated domain (UBA) (11), both of which have been shown to mediate binding to the proteasome (36). Moreover, the UBA domain of ubiquilin binds poly-ubiquitin chains (37), leading us to speculate that ubiquilin, like RAD23, with which it shares structural homology, could be a shuttle factor that binds ubiquitinated and misfolded proteins and escorts them to the proteasome or ubiquitin-regulated pathways for degradation (38–40). Other studies suggest that ubiquilin might possess chaperone-like properties because it binds heat shock proteins (41) and because overexpression of ubiquilin leads to increased synthesis of presenilin proteins (11,37).

Several observations lead us to propose that ubiquilin might rid cells of misfolded, aggregated and ubiquitinated proteins, such as those that are formed by expanded polyQ proteins. First, we found that overexpression of ubiquilin reduced the amount of GFP-tagged polyQ proteins that aggregated in cells in a dose-dependent manner. The reduction was evident both by fewer number of GFP–Htt–polyQ fusion protein inclusions that formed in neuronal cells when higher doses of ubiquilin cDNA was co-expressed compared with when it was not co-expressed and by a reduction in the amount of GFP-fusion protein that appeared to be aggregated, as revealed by the amount of protein that was trapped in the stacking gel after SDS–PAGE and retained on filters in the filter trap assay. Conversely, reduction of ubiquilin expression by RNAi led to an increase in the amount of aggregated GFP–Htt fusion protein in cell lysates. Secondly, in accordance with our hypothesis that ubiquilin binds and targets more highly misfolded polyQ proteins for degradation, we found that mRFP-tagged ubiquilin co-localized with more compact foci containing GFP–Htt(Q55) fusion protein and less so with the more filamentous and presumably less aggregated forms of the GFP–Htt(Q55) fusion protein. Thirdly, in previous studies, we found that ubiquilin co-localized with ubiquitin-positive structures in cells (37). Interestingly, Doi et al. (13) demonstrated that ubiquilin co-localizes with Htt aggregates that are ubiquitin-positive in both cells and mouse brain. These observations are consistent with our hypothesis that ubiquilin might bind and target only misfolded and ubiquitinated Htt proteins for degradation.

Finally, it is important to note the growing evidence linking ubiquilin to neurodegenerative diseases. In addition to the results reported here, prior studies have shown that high levels of ubiquilin protect neuronal cells from cell death triggered by hypoxia (42) and that ubiquilin co-localizes with neuropathological lesions in AD and Parkinson’s disease (11). In addition, ubiquilin regulates biogenesis and degradation of presenilins (11,23,37), and variants in Ubiquilin-1 have been genetically associated with late-onset AD (31). In fact, two additional independent studies have implicated variants of the Ubiquilin-1 gene in the possible age of onset of AD (43,44), but others could not document this association, at least in their sample set (45,46). Interestingly, Bertram and Tanzi recently reported that they had found additional variants in the Ubiquilin-1 gene with AD and that some of these variants were in the ubiquilin-1 promoter [see letter accompanying report by Slifer et al. (43)]. The latter finding is consistent with our view that proper regulation of ubiquilin levels might be important in preventing neurodegeneration. Our results lead us to suggest that careful genetic analysis of ubiquilin genes needs to be undertaken to determine whether variants in ubiquilin genes are involved in AD and other neurodegenerative disorders, particularly HD. Finally, it has not escaped our notice that methods to increase ubiquilin levels might provide an attractive therapeutic strategy to prevent or reduce polyQ-induced aggregation and toxicity.

**MATERIALS AND METHODS**

**Cell culture, DNA transfection, immunofluorescence microscopy**

HeLa cells were grown in DME supplemented with 10% FBS. Primary mouse cortical neurons were grown in MEM supplemented with 10% FBS. Cells were transiently transfected...
with plasmid DNA by calcium phosphate co-precipitation. Stable HeLa cell lines were isolated by co-transfection of EGFP expression constructs with a pNeo plasmid and selection with G418. Stable cell lines were identified by GFP-fluorescence. Immunofluorescence staining and fluorescence images of fixed and live cells and animals were captured on a Zeiss Axiovert 100 microscope using a Hamamatsu camera using C-Imaging software.

Protein preparation, SDS–PAGE, filter-retardation assay and immunoblotting

Cell, worm and tissue protein lysates were prepared as described previously (37). Our standard protocol for protein separation and immunoblotting was followed (37). The filter retardation assay was performed as described elsewhere (22). Antibodies against GFP and C. elegans ubiquilin were prepared by injecting rabbits with purified GST–GFP fusion protein and with a KHL-conjugated peptide corresponding to inferred residues 7–30 of the ubiquilin open-reading frame (ORF), respectively. The anti-ubiquilin monoclonal antibody used was described previously (23).

Quantification of cell death

Cell death was monitored either through examination of the nuclear morphology observed after Hoechst 33342 (1 μg/ml) staining or through detecting the membrane selective permeability following 3 μM of PI staining. TUNEL staining was performed with DeadEnd colorimetric TUNEL staining kits purchased from Promega. For detecting caspase-3 activation, two methods were used. Cell lysates were separated by SDS–PAGE and probed with an antibody against the cleaved substrate for caspase-3. Alternatively, caspase activity was also detected by flurorogenic techniques by incubating the cell lysates with a flurorogenic caspase-3 substrate, AC-DEVAD-AMC in a 96-well plate. After incubation, the cleaved free AMC was scanned by a fluorescence multi-well plate reader (SOSTmax, Sunnyvale, CA, USA) with an excitation at 380 nm and emission at 460 nm.

RNAi studies

Stable expression GFP–Htt(Q74) cell line was plated in 24 well-plate (Costar) at a low cell density. Twenty-four hours after plating, cells were transfected with SMARTpools of siRNAs to ubiquilin-1 and -2 (23), or a RISC-free control SMARTpool siRNAs, with no known homology (23), or mock transfection by just adding the transfection reagent according to the suggested protocol by the company (Dharmacon, Inc.). For comparing cell growth, phase contrast microscopy was performed just before siRNA transfection and 4 days after the transfection. Cell death assays were performed by collecting cells 4 days after transfection to monitor caspase-3 activity, or at different times (as indicated) after transfection for analysis of PI, Hoechst 33342 or TUNEL staining.

DNA cloning

Expression of human ubiquilin-1 cDNA was described previously (23). The GFP–Htt-Exon1 mammalian expression constructs encoding different length of polyQ repeats were kindly provided by Dr David C. Rubinsztein (14). For expression in C. elegans body wall muscle, we subcloned the various GFP-tagged constructs downstream of the unc-54 myosin heavy chain promoter using the pPD3038 vector (28). Expression of C. elegans ubiquilin protein in mammalian cells was achieved by cloning the entire ORF of the C. elegans ubiquilin cDNA in-frame and downstream of mRFP under the control of the cytomegalovirus (CMV) promoter. For expression in C. elegans body wall muscle, the mRFP-tagged ubiquilin construct was subcloned in the pPD3038 vector downstream of the unc-54 promoter. RNAi construct L4440:ubiquilin was created by cloning the complete ORF of C. elegans ubiquilin between the two T7 polymerase promoters in the L4440 vector (29,30). RNAi GFP construct L4417:GFP was obtained from Dr A. Fire (30).

C. elegans methods and generation of transgenic lines

Nematodes were maintained using standard methods (47). Transgenic lines stably expressing GFP- and mRFP-tagged polyQ and ubiquilin fusion proteins, respectively, were generated by injecting plasmid DNA into the gonads of early-adult hermaphrodites (48). Transgenic F1 progeny were selected on the basis of fluorescence in muscle cells. Individual fluorescent F2 animals were picked to establish transgenic lines. Bacterial-mediated RNAi was performed essentially as described (29,30). Plasmids containing the appropriate vector or vector L4440 alone were transformed into Escherichia coli strain HT115(DE3) by using standard methods. Individual colonies were inoculated into LB broth containing ampicillin (50 μg/ml) and tetracycline (12.5 μg/ml) and grown overnight at 37°C. Cultures were diluted 1:100 and allowed to grow to OD600 ~ 0.4. IPTG was then added to 0.4 mM and the cultures were grown for a further 2–4 h at 37°C. Bacteria were then seeded onto NGM plates containing ampicillin and IPTG and allowed to dry overnight. For motility assays, individual animals were picked to fresh plates and the number of body bends was counted at 1 min intervals using a Leica dissection stereomicroscope.

For RT–PCR analysis of mRNA expression in the worms, total RNA was first isolated from worm lines using Trizol. The same amount of RNA from each line was utilized to perform RT–PCR reactions using the following combination of primers designed to specifically amplify either GFP coding sequences, 5'-ACGGCAAGCTGACCTGAAAGTTTCAT-3' and 5'-TCGATGTGTGGCGGATCTTGAAGT-3', or mRFP–ubiquilin coding sequences, 5'-TGCGCCTGGGACAC-3' and 5'-TTGGGTGTCCAGCACGCCAAGTGG-3', or the C. elegans ama-1 (resistance to α-amanitin) encoding a portion of the RNA polymerase II subunit, 5'-CAGTTGCGCTCATGTCGAGTTTCCAGA-3' and 5'-CGACCTTCTTTCCATCATCATTGGG-3'. The RT–PCR reactions generated the expected products of 377, 400 and 390 bp for the three products, respectively, which were
visualized by ethidium bromide staining after agarose gel electrophoresis.

Statistical analysis
For statistical analysis, one-way analysis of variance was applied and significant levels for comparisons between groups were determined with t-tests. Data are shown as mean ± SDM and \( P < 0.05 \) was considered statistically significant.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None of the authors have any direct conflicts of interests with the work presented here.

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5. La Spada, A.R. and Taylor, J.P. (2003) Polyglutamines placed into C. elegans yk clones. We thank an anonymous reviewer for suggesting the experiment shown in Figure 2B and C.


