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Ubiquilin overexpression reduces GFP-polyalanine-induced protein aggregates and toxicity

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Abstract

Several human disorders are associated with an increase in a continuous stretch of alanine amino acids in proteins. These so called polyalanine expansion diseases share many similarities with polyglutamine-related disorders, including a length-dependent reiteration of amino acid induction of protein aggregation and cytotoxicity. We previously reported that overexpression of ubiquilin reduces protein aggregates and toxicity of expanded polyglutamine proteins. Here, we demonstrate a similar role for ubiquilin toward expanded polyalanine proteins. Overexpression of ubiquilin-1 in HeLa cells reduced protein aggregates and the cytotoxicity associated with expression of a transfected nuclear-targeted GFP-fusion protein containing 37-alanine repeats (GFP-A37), in a dose dependent manner. Ubiquilin coimmunoprecipitated more with GFP proteins containing a 37-polyalanine tract compared to either 7 (GFP-A7), or no alanine tract (GFP). Moreover, overexpression of ubiquilin suppressed the increased vulnerability of HeLa cell lines stably expressing the GFP-A37 fusion protein to oxidative stress-induced cell death compared to cell lines expressing GFP or GFP-A7 proteins. By contrast, siRNA knockdown of ubiquilin expression in the GFP-A37 cell line was associated with decreased cellular proliferation, and increases in GFP protein aggregates, nuclear fragmentation, and cell death. Our results suggest that boosting ubiquilin levels in cells might provide a universal and attractive strategy to prevent toxicity of proteins containing reiterative expansions of amino acids involved in many human diseases.

Introduction

Expansion of a repeating trinucleotide sequence in the genome above a certain length has been linked to the manifestation of several human disorders. These repeat disorders can be subdivided into expansions that occur in the noncoding sequence, such as introns or untranslated portions of mRNAs, or in the coding sequence [1,2]. Because amino acids are encoded by codons composed of three nucleotides, the resulting translation of a sequence with a trinucleotide repeat generates a protein with a repeating amino acid. So far most trinucleotide repeats that occur in the coding sequence are translated into a homomeric stretch of either glutamine or alanine amino acids. Expanded polyglutamine tracts have been found in nine different proteins that when mutated, cause several different neurodegenerative disorders [3, 4]. Coincidentally, expanded polyalanine tracts have been found in nine different proteins, all of which are transcription factors, with one exception, a protein that binds the polyA nucleotide that is frequently present at the end of most mRNAs (reviewed in [5-7]). Because all of the proteins containing polyalanine expansion are involved in the regulation of many important

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sets of RNAs it is perhaps not surprising that diseases associated with expanded polyalanine proteins are associated with congenital deformities of different parts of the body.

The mechanisms by which expanded polyglutamine and polyalanine proteins cause disease is still not known. Because polyalanine and polyglutamine disorders involve different amino acids it is instructive to know whether the diseases caused by the two different amino acids share any similarities. A comparison of the proteins in the pathology of expanded polyglutamine and polyalanine proteins has revealed two particular notable similarities, an amino acid length-dependent induction of protein aggregation and cell death [3,8-10]. Based on these similarities it is possible that factors that aid in the disposal of protein aggregates from cells might provide benefit in treating diseases caused by either polyglutamine and polyalanine expansions. We became interested in the possibility that ubiquitin might fit this role, because we found that overexpression of the protein was able to reduce the amount of aggregates and the cytotoxicity of the expanded polyglutamine proteins in cell and animal models [11]. Ubiquitin proteins are characterized by an N-terminal ubiquitin-like domain (UBL), a central more variable domain, and a C-terminal ubiquitin-associated domain (UBA) [12]. UBL and UBA domains are present in many proteins that are known to be involved in the regulation of the ubiquitin-proteasome system in cells. Indeed, studies of ubiquitin proteins conducted by others and us have suggested that increased ubiquitin expression can confer cytoprotection to cells, and might aid in the disposal of misfolded proteins from cells [11,13]. Here we tested whether ubiquitin can reduce protein aggregates and cytotoxicity of proteins containing expanded polyalanine tracts.

Materials and Methods

Cell culture, DNA transfection, establishment of stable cell lines, and fluorescent microscopy

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were transfected with plasmid DNAs using the calcium phosphate coprecipitation method. Stable cell lines expressing different GFP proteins were isolated by co-transfecting HeLa cells with pNeo together with pEGFP, or with pEGFP-A7, or with pEGFP-A37 expression plasmids at a 1:10 ratio of the plasmids, respectively. After several days of selection with G418 (700 µg/ml) individual clones with GFP fluorescence were identified and expanded. Fluorescent images of fixed or live cells were captured using either a LSM510 laser scanning confocal microscope (Zeiss) equipped with an argon and two HeNe lasers or an inverted Zeiss Axiovert 200 fluorescence microscope connected to a Hamamatsu Orca camera. The fluorescent images were analyzed for colocalization of GFP and ubiquitin using IPLab software. By this analysis we found ubiquitin was colocalized with expanded polyalanine proteins, using both microscopes, but due to space limitations only representative examples of these images are presented.

Plasmid constructs, SDS-PAGE, filter trap assay, immunoblotting, and antibodies

GFP-A7 and GFP-A37 expression plasmids, which contain a nuclear localization signal (NLS), were kindly provided by Dr. David C. Rubinsztein (University of Cambridge, UK). The construction of the ubiquitin-1 expression cDNA plasmid was described previously [12]. The protocols for SDS-PAGE, immunoblotting and the filter trap assay were described before [11]. The GFP polyclonal and ubiquitin monoclonal antibodies used in this study were also described previously [11,14].

Quantification of cell death

Cell death was quantified in the cultures by counting the proportion of GFP-fluorescent cells that exhibited an abnormal nuclear morphology under the microscope after staining of the cells with the DNA dye Hoechst 33342 (1 µg/ml). Alternatively, cell death was quantified by

counting the number of cells whose membrane permeability barrier was broken, by their positive staining for propidium iodide (PI) following addition of 3 μ M of the dye to the medium. Sensitivity of cells to H₂O₂ was performed as described previously [11]. At least 500 cells were counted for each experiment, which was done at least three times.

Knockdown of ubiquilin expression by RNA interference

Expression of ubiquilin proteins were knocked down by transfecting cells with a 10 nM mixture of SMARTpool siRNAs directed specifically against human ubiquilin-1 and ubiquilin-2 sequences using the protocol described previously [11,14]. In brief, the stable lines expressing the various GFP proteins were plated in 24-well plates (Costar) and 24 hours after the plating, the cultures were transfected with SMARTpools of siRNAs against either ubiquilin-1 and -2, or with control siRNAs that have no known target, or were mock transfected with the transfection reagent alone. The cultures were maintained for 4 days in the transfection medium and then cell death was quantified as described above or the cells were lysed and analyzed for either ubiquilin expression or for the presence of GFP protein aggregates by immunoblotting.

Statistical analysis

For statistical analysis, one-way analysis of variance (ANOVA) was applied. Significant variance between groups was determined using the t-test. Data are shown as mean $\pm$ SDM and $p < 0.05$ was considered statistically significant.

Results

Ubiquilin binds and colocalizes with GFP-proteins containing expanded polyaniline tracts

To test whether ubiquilin can reduce polyaniline-induced protein aggregates and toxicity we utilized two previously characterized expression constructs that have been used to model polyaniline protein-aggregation and toxicity in cells and organisms [8,10,15,16]. The constructs encode GFP fused with either 7 or 37 consecutive alanine amino acids, plus a SV40 nuclear localization signal, henceforth referred to as GFP-A7 and GFP-A37, respectively. The NLS was incorporated in the constructs because all of the human proteins containing polyaniline expansions are thought to localize and function in the nucleus. Previous studies had shown that expression of the GFP-A37-fusion protein in cells and organisms leads to a dose-dependent increase in GFP protein aggregation as well as an increase in cell death compared to expression of the GFP-A7 fusion protein [15].

Because we wanted to study how alterations in ubiquilin protein levels affects aggregation and toxicity of polyaniline-containing proteins we expressed the GFP-A7 and GFP-A37 expression constructs in HeLa cells, together with or without a human ubiquilin-1 cDNA expression plasmid. We utilized HeLa cells because they are of human origin and because we had established protocols for both overexpression as well as knockdown of human ubiquilin proteins [11]. As shown in Fig. 1A, immunofluorescence microscopy of HeLa cells that were transfected with either the GFP-A7 expression plasmid alone (first row) or cotransfected with ubiquilin-1 expression plasmid (second row) revealed strong and almost uniform anti-GFP staining predominantly in the nucleus. The morphology the cells overexpressing GFP-A7, either alone or together with ubiquilin-1, were similar to that of untransfected cells. By contrast HeLa cells transfected with GFP-A37 alone displayed visible GFP-fluorescent aggregates in both the cytoplasm and nucleus, but many of the nuclei and cells had a shrunken and rounded-up morphology, respectively (Fig 1A third row and data not shown). Interestingly, the aggregates and abnormal morphologies were considerably reduced in cells cotransfected with GFP-A37 and ubiquilin-1 expression plasmids (fourth row, and data not shown), despite clear indication that the GFP-A37 protein was overexpressed, which led us to speculate that overexpression of ubiquilin might prevent manifestation of these abnormal morphologies.

Examination of GFP and anti-ubiquitin staining by double immunofluorescence microscopy in the cells cotransfected with GFP-A7 and ubiquitin-1 or GFP-A37 and ubiquitin-1 expression constructs revealed a clear increase in ubiquitin immunoreactivity compared to the presumably non-transfected cells. The increase in ubiquitin staining in these cotransfected cells was present throughout the cytoplasm and nucleus with the exception of oval structures in the nucleus, which we presume are nucleoli (Fig 1B). Interestingly in the GFP-A37 overexpressing cells, the patterns of ubiquitin and GFP staining in the cytoplasm and nucleus colocalized well with one another, by examination by confocal microscopy, suggesting possible interaction of the proteins.

To determine if ubiquitin interacts with GFP-A7 or GFP-A37 fusion proteins we immunoprecipitated GFP expressed proteins from cells that were either singly transfected with GFP, or GFP-A7, or GFP-A37, expression constructs and immunoblotted them for ubiquitin. As shown in Figure 1C more ubiquitin coimmunoprecipitated with GFP-A37 than with either GFP-A7 or GFP proteins. Because ubiquitin is known to bind polyubiquitinated proteins we hypothesized that more ubiquitin coimmunoprecipitated with GFP-A37 than GFP-A7 protein because the former is more prone to aggregate and to be ubiquitinated. In accord with this hypothesis we detected more anti-ubiquitin immunoreactivity in the GFP-A37 immunoprecipitated proteins than with the GFP-A7 or GFP proteins (Fig 1C). Furthermore, a filter trap assay used to measure protein aggregates in cell lysates revealed that cells transfected with GFP-A37 contained more GFP- and ubiquitin-immunoreactive aggregates than cells transfected with the GFP-A7 construct (Fig 1D). These results suggest that ubiquitin might interact more strongly with GFP-expressed proteins with longer polyaniline tracts.

Overexpression of ubiquitin in HeLa cells reduces the amount of GFP-polyalanine aggregates and cytotoxicity

To compare the cytotoxic properties of GFP-A7 and GFP-A37 fusion proteins we measured nuclear fragmentation and cell death of HeLa cells transfected with the two expression constructs. As shown in Figure 2A, expression of the GFP-A37 construct correlated with a higher percentage of GFP-expressing cells that exhibited nuclear fragmentation and death compared to cells expressing either GFP-A7, or GFP alone. The differential cytotoxic property of the two constructs in HeLa cells is in accord with the greater cytotoxic properties of the GFP-A37 found in other cell types [15]. We next examined if overexpression of ubiquitin-1 might suppress the toxicity induced by the polyaniline proteins. As shown in Figure 2B, coexpression of ubiquitin-1 cDNA with GFP-A37 reduced the GFP-A37-induced cell death in a dose-dependent manner. By contrast there was negligible, if any, reduction, in the extent of nuclear fragmentation and cell death in cells cotransfected with ubiquitin-1 cDNA and GFP-A7 (Fig 2A and results now shown). These results suggest that ubiquitin overexpression might selectively suppress the toxicity of expanded polyaniline containing proteins.

We next examined if the protective effect of ubiquitin toward GFP-A37-induced cytotoxicity correlated with a change in polyaniline protein aggregation. To examine this possibility we immunoblotted cell lysates prepared from cells transfected with either GFP-A7 or -A37 expression constructs alone, or together with ubiquitin-1 cDNA, for the presence of GFP aggregates trapped on filters (Fig 3A). By this assay we only detected GFP-immunoreactive protein aggregates in the cells that were singly transfected with GFP-A37 but not GFP-A7, in accord with our earlier findings. Importantly, the amount of these GFP-containing aggregates was reduced in cells that were cotransfected with GFP-A37 and ubiquitin-1 constructs (Fig 3A). The reduction of protein aggregates scored by this biochemical approach correlated well with a reduction in visible GFP-fluorescent aggregates seen in cells cotransfected with ubiquitin-1 and GFP-A37 compared to cells transfected with GFP-A37 alone (Fig 3B). Furthermore, the reduction in GFP-A37 protein aggregation modulated by ubiquitin-1

appeared to be dependent on the amount of ubiquilin-1 expressed, because transfection of an increasing amount of ubiquilin-1 cDNA expression plasmid resulted in a dose-dependent reduction of GFP-A37 aggregates, as scored by the filter trap assay (Fig 3C). The reduction in GFP-containing aggregates by ubiquilin was not simply due to decreased GFP-fusion protein expression, because immunoblots of equal amounts of protein from these experiments revealed that the GFP-fusion proteins were expressed to similar levels in the ubiquilin-transfected and non-transfected cells (see GFP panels in Figs 3A and 3C).

Together these results strongly suggest that ubiquilin overexpression reduces the amount of GFP-containing aggregates and the cytotoxicity induced by expression of GFP-A37 protein in HeLa cells

Overexpression of ubiquilin-1 suppresses H₂O₂-induced cell death of stable cell lines expressing expanded polyaniline proteins

To obtain further evidence in support of our finding that the amount of ubiquilin expressed in cells modulates toxicity of proteins with expanded polyaniline tracts, we isolated HeLa cell lines that stably expressed either GFP alone, or GFP-A7-, or GFP-A37-fusion proteins. Unlike transiently transfected cells where expression of the GFP-fusion proteins varied considerably, the stable cell lines expressed a constant amount of the proteins providing a more reliable system for evaluating the toxicity of the polyaniline proteins. Lines that stably expressed comparable levels of each GFP protein, determined by immunoblotting, were selected for further studies (Fig 4A). The experiments described below were repeated with other cell lines expressing the proteins and similar results to those described below were obtained. For simplicity purposes, data from only one set of these lines is presented. Similar to the pattern found in transiently transfected cells, the GFP-A7 and GFP-A37-expressing stable cell lines displayed GFP-fluorescence mainly in the nucleus, consistent with appropriate targeting of the proteins by the NLS that was incorporated into each polypeptide (Fig 4B). Interestingly, the cell line expressing GFP-A37 contained higher levels of GFP fluorescence in the cytoplasm compared to the GFP-A7-expressing line, a phenotype that was also seen in transiently transfected cells (Fig. 4B). The reason for the greater sequestration of GFP-A37 protein in the cytoplasm compared to the GFP-A7 protein is not known but may be related to differences in aggregation and/or binding properties of the proteins.

Because we found that cell lines that express proteins with expanded polyglutamine tracks are acutely more sensitive to agents that induce oxidative stress [11] than those that do not express the expanded proteins we wondered whether the GFP-A7- and GFP-A37 lines would also be differentially vulnerable to such agents. To test this possibility we treated the GFP-expressing cell lines with 100 μ M H₂O₂ for 5 hours and found that the GFP-A37 line, but not the GFP-A7 or GFP cell lines, was acutely sensitive to exposure with this dose of H₂O₂ (Fig 4C). Approximately 22% of the cells from the GFP-A37 line when exposed with H₂O₂ died (Fig 4C), while the GFP-A7 and GFP expressing cells were robust to this same treatment (Fig. 4C and results not shown). To determine if increased ubiquilin expression might protect GFP-A37 cells against the H₂O₂ insult, we measured cell death in GFP-A7 and GFP-A37 cell lines that were first transfected with either a ubiquilin-1 cDNA expression plasmid or the empty plasmid vector and then exposed to H₂O₂. The percentage of dead cells in the GFP-A7 line was low and remained unaltered in the cells transfected with either the control vector or with the ubiquilin-1 cDNA (Fig. 4C). By contrast, there were approximately 40% fewer dead cells in the GFP-A37 cell line that were transfected with ubiquilin-1 cDNA compared to the vector control (Fig 4C).

We also prepared lysates from the transfected cells to examine if GFP-protein aggregation was altered in them using the filter trap assay. As shown in Figure 4D, transfection of ubiquilin-1 cDNA, but not the empty vector, significantly reduced the amount of GFP aggregates in the

GFP-A37 cell line. A similar reduction was observed in the GFP-A7 transfected cells (Fig 4D), but this line contained significantly fewer aggregates to begin with, as expected. Further studies revealed that ubiquitin overexpression reduced GFP-polyalanine protein aggregation in the GFP-A37 cell line in a dose-dependent manner (Fig 4E).

Together these results suggest that overexpression of ubiquitin-1 can protect cell lines that express expanded polyalanine proteins from an increase in susceptibility to oxidative stress, which correlates with a reduction in accumulation of GFP-polyalanine-containing protein aggregates.

Reduction of ubiquitin protein expression in GFP-A37 cells leads to an arrest in cellular proliferation and correlates with increases in GFP protein aggregates, nuclear fragmentation and induction of cell death

To confirm the role of ubiquitin in protecting cells against polyalanine toxicity, we used RNA interference (RNAi) to reduced ubiquitin protein levels in the GFP-A37 HeLa cell line to examine if reduction of its expression would increase polyalanine-induced protein aggregates and cell death. Because HeLa cells express two predominant ubiquitin isoforms, ubiquitin-1 and ubiquitin-2 [14], we transfected the GFP-A37 cells with a combination of siRNAs to specifically knockdown expression of both proteins. An immunoblot confirmed that both ubiquitin 1 and 2 proteins were indeed reduced by approximately 80 to 90%, respectively, compared to untransfected or mock-transfected cells (Fig 5A). Knockdown of the ubiquitin expression in the GFP-A37 cells resulted in a dramatic arrest in cellular proliferation, which correlated with a high rate of nuclear fragmentation and cell death (Fig 5B-E). Almost none of these phenotypes were observed in GFP-A37 cells that were either mock-transfected or transfected with control siRNAs that were designed not to induce genetic interference of any known gene.

Finally, we examined if RNAi of ubiquitin expression altered GFP protein aggregation in the GFP-A37 cell line. Changes in GFP aggregation were analyzed by fluorescence microscopy and by the filter trap assay. We noticed that the distribution of GFP fluorescence in the nucleus of GFP-A37 expressing cells transfected with ubiquitin siRNAs was more condensed and contained brighter foci compared to the more uniform distribution of the protein in mock and control siRNA transfected cells (Fig 6A). Furthermore, the filter trap assay revealed significantly more GFP-containing aggregates in lysates of the cells transfected with ubiquitin siRNAs compared to those in the two control transfections (Fig 6B).

Together these results indicate that a reduction in ubiquitin protein expression in cells expressing expanded polyalanine proteins increases the amount of GFP protein aggregates in cells, which correlates with an arrest in cellular proliferation and increases in nuclear fragmentation and cell death.

Discussion

In this report, we have demonstrated an inverse relationship between the amount of ubiquitin protein expressed in cells and the accumulation of protein aggregates and cytotoxicity of proteins containing expanded polyalanine tracts. Support for this conclusion is based on the evidence shown here that increased expression of ubiquitin-1 protein reduces the amount of GFP-A37 protein aggregates as well as the cytotoxicity associated with expression of the GFP-A37 fusion protein in HeLa cells. We have also demonstrated that the converse is true: a reduction of ubiquitin levels in cells by RNAi increases the amount of GFP-A37 protein aggregates, which correlates with an increase in cell death.

It is remarkable that ubiquilin is able to suppress the cytotoxicity of proteins containing either expanded polyalanine (as shown here) or polyglutamine tracts (as we have shown previously, [11]), considering that the two amino acids involved in these expansions (glutamine and alanine) are so different. A feature that we found was common to the cytoprotection of ubiquilin against proteins with expanded polyalanine and polyglutamine tracts was the inverse relationship between the amount of ubiquilin expressed in cells and the amount of aggregates formed by the expanded proteins. In both cases we found that increased expression of ubiquilin reduced the amount and number of aggregates containing the expanded proteins in cells, which correlated with a reduction in the cytotoxicity associated with expression of the expanded polyalanine and polyglutamine proteins. In both cases too, we found that a reduction of ubiquilin levels increased the amount and number of the aggregates containing the expanded proteins, which correlated with greater induction of cytotoxicity by the proteins.

There is considerable, and unresolved, debate whether protein aggregates formed by expanded polyglutamine proteins are toxic or protective [3,17-21]. Our results (both those reported here and previously) are consistent with the notion that a build-up of aggregates composed of polyalanine and polyglutamine proteins are toxic to cells. In accord with the notion that aggregates are toxic, we observed that expression of GFP-A37, containing a stretch of 37 alanines, was more prone to form aggregates than GFP-A7, containing a stretch of 7 alanines, and this correlated with the more deleterious property of the GFP-A37 protein in transiently transfected and stable HeLa cell lines. Furthermore, overexpression of ubiquilin reduced the amount of polyalanine and polyglutamine aggregates that build-up in cells, and this directly correlated with a reduction in the toxicity associated with expression of the expanded polyalanine and polyglutamine proteins in cells.

Our experiments do not provide answers as to how ubiquilin protects cells against toxicity induced by expanded polyalanine and polyglutamine proteins. However, based on the properties of ubiquilin proteins discovered so far, we speculate it may function in the following way(s). One possibility is ubiquilin may escort misfolded proteins, such as those containing expanded polyalanine and polyglutamine tracts, to the proteasome for degradation. This property would be in accord with the known ability of ubiquilin to bind ubiquitinated proteins and proteasome subunits via its UBA and UBL domains, respectively [22-25]. Thus overexpression of ubiquilin may accelerate the delivery of misfolded protein to the proteasome and thereby enhance their clearance. Consistent with this theory we found that ubiquilin coimmunoprecipitated more with ubiquitinated, and the presumably the more prone to misfold, GFP-A37 fusion protein than with the GFP-A7 or GFP proteins, and that this correlated with a reduction in GFP-A37 aggregates in cells that overexpressed ubiquilin. Another possibility is that ubiquilin may enhance clearance of polyalanine and polyglutamine aggregates by autophagy. This possibility might arise because ubiquilin has been found to interact with mTor, a key regulator of autophagy [26]. Because inhibition of mTor kinase activity activates autophagy, we speculate that overexpression of ubiquilin would lead to increased binding to mTor, which might prevent the kinase from binding its normal targets, or inactivate the kinase, or that it may stimulate mTor degradation. A preliminary report suggested that ubiquilin overexpression does not alter mTor kinase activity [26]. Finally, ubiquilin might reduce polyalanine and polyglutamine-induced toxicity due to its ability to function as a molecular chaperone, probably in a complex with other proteins. In accord with this idea, Kaye FJ et al. [27] reported that ubiquilin interacts with Stch, a heat shock protein possessing an ATPase domain, which might be involved in refolding the potentially toxic misfolded proteins containing expanded polyalanine and polyglutamine tracts. In addition other studies have shown that ubiquilin can protect neurons and cells from injury induced by oxidative stress and hypoxia [11,13]. In this study we have provided additional evidence that ubiquilin can protect cells from increased vulnerability to oxidative stress caused by expression of expanded polyalanine proteins. It is conceivable that all three mechanisms, or some combination of them,

might be involved in the protective effects exerted by ubiquilin. It will be interesting in the future to determine exactly how ubiquilin suppresses cytotoxicity of expanded polyalanine and polyglutamine proteins.

The evidence emerging from our studies is that overexpression of ubiquilin serves to eliminate aggregates containing potential toxic misfolded proteins from accumulating in cells. This protective role has huge implications as a potential therapy for human diseases, especially neurodegenerative diseases that are frequently associated with an accumulation of misfolded protein aggregates in the brain. There are several reasons to suspect that ubiquilin is indeed involved in binding misfolded proteins seen in a variety of human diseases. First, others and we have shown that ubiquilin binds and colocalizes with protein aggregates composed of expanded polyglutamine tracts in brain and tissues of animal models of Huntington's disease [11,28]. Second, ubiquilin interacts with presenilins, the chief catalytic component of γ -secretase [12,29,30]. Interestingly, overexpression of ubiquilin reduces many of the essential components of the γ -secretase complex [14]. Third, ubiquilin was also localized to neurofibrillary tangles and Lewy bodies in Alzheimer's and Parkinson's disease afflicted brains, respectively [12]. Fourth, there are a number of reports that have indicated that variants in the ubiquilin-1 gene may be associated with late-onset Alzheimer's disease, although this has not been found in all of the studies [31–36]. All of these findings lead us to speculate that methods to modulate ubiquilin expression might have utility to treat diseases not only associated with expanded polyalanine and polyglutamine proteins, but also diseases associated with misfolding and aggregation of other unrelated proteins.

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Figure 1. Ubiquitin associates with expanded GFP-polyalanine proteins

A. Representative images of HeLa cells transfected with either a GFP-A7 or GFP-A37 expression plasmid alone or together with a ubiquilin-1 cDNA expression plasmid. 24 hours after the transfection, cells were fixed and immunostained for ubiquilin. The panels shown in each row show the ubiquilin (abbreviated as Ubqln in all subsequent figures, red), GFP (green), and the DAPI (cyan) fluorescent images taken with a 100X objective through a group of transfected cells, together with the image obtained after merging the red and green images. Bar, 5 μ m for all the panels.

B. Confocal images of the cells cotransfected with GFP-A37 and ubiquilin-1 cDNA showing extensive colocalization of ubiquilin with GFP in the cytoplasm and nucleus. Please note the brighter staining of some of the cells in the field for ubiquilin, which is consistent with overexpression of the protein by transfection. Bar, 5 μ m.

C. More ubiquilin coimmunoprecipitates with GFP-A37 than GFP-A7 or GFP proteins. HeLa cells were transiently transfected with GFP, GFP-A7, or GFP-A37 constructs and lysates were prepared from the cells and the GFP expressed proteins were immunoprecipitated from them using a polyclonal anti-GFP antibody. The immunoprecipitated complexes were separated by SDS-PAGE, the proteins transferred to nitrocellulose membranes, and then immunoblotted (IB) with monoclonal antibodies against ubiquilin (upper panel), GFP (middle panel), or ubiquitin (lower panel).

D. Ubiquilin is present with GFP-A37 aggregates trapped on filters. Cell lysates were prepared from GFP-A7- and GFP-A37-transfected HeLa cells and filtered through a cellulose acetate membrane to trap protein aggregates. The filter membrane was first immunoblotted with a anti-ubiquilin monoclonal antibody and after stripping was then re-blotted with a anti-GFP polyclonal antibody.

A **B**

Figure 2. Overexpression of ubiquilin-1 cDNA reduces GFP-polyalanine protein-induced cell death
 A. HeLa cells were transfected with 1 µg of GFP, GFP-A7, or GFP-A37 expression plasmids alone or together with a ubiquilin-1 expression plasmid. The next day, the cells were treated with 100 µM of H₂O₂ for 5 hours after which cell death was quantified by doubly staining the cells with Hoechst or propidium iodide (PI). Fragmented nuclei and PI-positive stained cells were counted as dead cells. The results show that overexpression of ubiquilin-1 reduces GFP-A37 induced cell death. * $p < 0.05$.

B. Overexpression of ubiquilin-1 cDNA reduces GFP-A37-induced cell death in a dose-dependent manner. HeLa cells were transfected with 1 µg of the GFP-A37 expression construct along with the indicated amounts of ubiquilin-1 cDNA. 20 hours after transfection, the cultures were treated with 100 µM of H₂O₂ for 5 hours, after which cell death was quantified according to the procedure described above.

A **B**

Figure 3. Overexpression of ubiquilin-1 cDNA reduces the amount of GFP-polyalanine-containing aggregates in cells

A. HeLa cells were cotransfected with GFP-A7 or GFP-A37 expression plasmids and either an empty vector plasmid or a ubiquilin-1 cDNA-expression plasmid. 24 hours after transfection, the cells were lysed and the amount of GFP-containing protein aggregates in similar amounts of protein lysate was determined by the filter trap assay (bottom panel). Meanwhile, equal portions of the lysates were immunoblotted for ubiquilin, GFP, and actin proteins.

B. HeLa cells were transfected with GFP-A37 and the ubiquilin-1 cDNA expression plasmid or GFP-A37 and the empty vector and after 20 hours the percentage of cells containing obvious GFP aggregates were determined relative to the total number of GFP-fluorescent cells. The data shows that overexpression of ubiquilin-1 significantly reduces the formation of GFP-A37 aggregates. * $p < 0.001$.

C. Biochemical analysis demonstrating overexpression of ubiquilin-1 cDNA reduces the amount of GFP-A37-containing protein aggregates in cells in a dose-dependent manner. HeLa cells were co-transfected with GFP-A37 and an increasing amount of ubiquilin-1 cDNA expression plasmid or an equivalent amount of empty vector plasmid, as indicated. 24 hours after transfection the cells were lysed and analyzed for the presence of GFP-containing aggregates or ubiquilin, GFP, or actin, proteins as described in B.

C **D**

Figure 4. Overexpression of ubiquilin-1 cDNA protects HeLa cell lines stably expressing expanded polyalanine proteins against increased vulnerability to H₂O₂-induced cell death

A. A GFP immunoblot of equivalent amount of protein lysate from three stable cell lines showing the levels of expression of either GFP alone, GFP-A7 or GFP-A37 proteins, relative to actin (bottom panel).

B. Representative low and high magnification fluorescent images of the GFP-, GFP-A7-, and GFP-A37-expressing stable cell lines used in A as well as in studies described below. Bar, 5 μm.

C. The GFP-A7 and GFP-A37 cell lines were challenged with 100 μM of H₂O₂ for 5 hours and then stained with Hoechst 33343 and PI to determine the extent of cell death in the cultures. An additional set of the cultures were transfected with the ubiquilin-1 cDNA prior to the H₂O₂ treatment. The graphs show that the increased vulnerability of the GFP-A37 expressing cells to H₂O₂-induced cell death is partially attenuated by overexpression of ubiquilin-1. Cell death was quantified as described in Figure 1A. * p<0.05.

D. Biochemical analysis showing overexpression of ubiquilin-1 reduces the amount of GFP-containing aggregates in the GFP-A37 cell line. The GFP-A7 and GFP-A37 cell lines were transfected with either a ubiquilin-1 expression plasmid or the empty vector. After 24 hours, the cells were lysed and the amount of GFP-immunoreactive aggregates present in equal protein portions of the lysates was determined by the filter trap assay (bottom panel). Meanwhile, equal amounts of the protein lysates were also immunoblotted for ubiquilin, GFP, and actin (upper three panels).

E. Biochemical analysis demonstrating overexpression of ubiquilin-1 cDNA reduces the amount of GFP-A37-containing protein aggregates in cells in a dose-dependent manner. The GFP-A37 cell line was transfected with varying amounts of ubiquilin-1 cDNA expression plasmid (0 to 0.9 μg DNA), or an equivalent amount of empty vector plasmid as indicated. 24 hours after transfection the cells were lysed and analyzed for the presence of GFP-containing aggregates or ubiquilin, GFP, or actin, proteins, similar to D.

D

E

Figure 5. Reduction of ubiquilin expression by RNAi in the GFP-A37 cell line leads to a decrease in cellular proliferation and increases in DNA fragmentation and cell death

A. Ubiquilin and actin immunoblots of equal amounts of protein lysates from cultures of the GFP-A37 cell line that were transfected with a combination of siRNAs specific for ubiquilin-1 and ubiquilin-2, or with control siRNAs that do not target any known gene, or mock-transfected.

B. Representative phase contrast images showing the equivalent cell density of the three groups of cells at the beginning of a similar experiment described in A. Bar, 100 μ m.

C. Representative GFP and Hoechst fluorescence images of the GFP-A37 cultures in the experiment described in B, four days post-transfection. Note the decrease in proliferation and increase in nuclear condensation in the cells GFP-A37 cells transfected with ubiquilin siRNAs compared to the cells transfected with the control siRNA.

D. GFP, GFP-A7, and GFP-A37, cell lines were plated at equal cell density. The cultures were then transfected with either the control or the ubiquitin-specific siRNAs. Three days after transfection the percentage of cells containing fragment nuclei were measured in the cultures (in the absence of H₂O₂ treatment). The graphs show that the GFP-A37 cell line displays significantly higher (* $p < 0.0001$) nuclear fragmentation compared to the cells that were transfected with the control siRNA.

E. Quantification of cell death seen after 3 days in the experiment described in D. The graphs show that the GFP-A37 cell line displays significantly higher (* $p < 0.01$) cell death compared to the cells that were transfected with the control siRNA.

Figure 6. Reduction of ubiquilin expression by RNAi in the GFP-A37 cell line results in increased accumulation of GFP-containing aggregates in cells

A. Similar experiment as described in Figure 5 showing, representative, high magnification images of GFP fluorescence in GFP-A37 cells, four days after transfection with control and ubiquilin specific siRNAs. Note the brighter fluorescence of GFP aggregates in the cells transfected with ubiquilin siRNAs (indicated by arrows). Bar, 5 μ m.

B. A GFP immunoblot of a filter trap assay to measure protein aggregates present in equal amounts of protein lysate (10, 20 or 40 μ g) prepared from mock, ubiquilin siRNA, and control siRNA transfections of the GFP-A37 cell line. Note the increase in the amount of aggregates in the lysates from the cells that were transfected with ubiquilin siRNAs.