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A bivalent Huntingtin binding peptide suppresses polyglutamine aggregation and pathogenesis in *Drosophila*

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Huntington disease is caused by the expansion of a polyglutamine repeat in the Huntingtin protein (Htt) that leads to degeneration of neurons in the central nervous system and the appearance of visible aggregates within neurons. We have developed and tested suppressor polypeptides that bind mutant Htt and interfere with the process of aggregation in cell culture. In a *Drosophila* model, the most potent suppressor inhibits both adult lethality and photoreceptor neuron degeneration. The appearance of aggregates in photoreceptor neurons correlates strongly with the occurrence of pathology, and expression of suppressor polypeptides delays and limits the appearance of aggregates and protects photoreceptor neurons. These results suggest that targeting the protein interactions leading to aggregate formation may be beneficial for the design and development of therapeutic agents for Huntington disease.

Introduction

At least eight neurodegenerative disorders, including Huntington disease, are caused by the expansion of a CAG trinucleotide repeat¹, leading to long repeats of polyglutamine within the corresponding disease protein. Huntington disease is characterized by a movement disorder, cognitive deficits and psychiatric symptoms. When the number of glutamines in the Htt protein exceeds approximately 38, late-onset disease becomes evident², but individuals with less than 35 polyglutamines are unaffected³. Other polyglutamine repeat diseases, including Kennedy disease (spinal bulbar muscular atrophy), dentatorubral pallidoluysian atrophy and spinocerebellar ataxia 1, show similar pathogenic thresholds¹ but have distinct patterns of neuronal degeneration. The age at which symptoms first appear and the severity of disease are correlated with the length of the glutamine repeat (approximately 65 or more glutamines cause juvenile-onset Huntington disease and more widespread neuronal degeneration⁴). A common feature of all of these diseases is the appearance of polyglutamine-containing inclusions in affected areas of the brain⁵. The role of expanded polyglutamine repeats in the disease process is undisputed, but the mechanism of pathology and contribution of aggregates to neurodegeneration is not clear. It is clear that Htt and other proteins with expanded polyglutamine repeats participate in protein interactions that allow aggregation. Here we ask whether synthetic polypeptides can be designed that will disrupt these protein interactions in cultured cells and, if so, whether these peptides can reduce polyglutamine-induced pathology *in vivo*.

The formation of nuclear inclusions containing expanded-polyglutamine Htt and ubiquitin was first described in a transgenic mouse model of Huntington disease⁶. Studies of brain tissues of both individuals with Huntington disease and transgenic mice^{7,8} have identified mutant Htt in intranuclear inclusions in neurons and in the neuronal processes (neuropil) of brain tissue of individuals with Huntington disease. Later studies in many model systems have reported the presence of both cytosolic and nuclear inclusions^{5,9} and have reported many cellular proteins co-localizing to the inclusions, including proteasome subunits, transcription factors and chaperone proteins^{10–18}. For almost every disease associated with polyglutamine repeats that has been investigated, nuclear aggregates have been found both in brain tissues of transgenic mouse models and in affected individuals^{5,9}.

Mutant Htt seems to participate in several aberrant binding interactions, including interactions with cellular proteins that may inhibit soluble enzyme activities and interactions between polyglutamine domains that ultimately lead to the formation of large insoluble aggregates. A simple hypothesis is that aberrant interactions involve a common binding surface generated by expansion of the polyQ domain and that targeting this binding surface might prevent aberrant binding interactions and subsequent pathology. To test this, we designed artificial polypeptides to compete for these binding interactions and tested their effects on aggregate formation and on pathology. Here we show that bivalent suppressor molecules can inhibit aggregation of Htt peptides with expanded polyglutamine repeats in cell culture. The most active suppressor protein was able to reduce both lethality and neuronal degeneration.

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Fig. 1 Suppressor design and inhibition of aggregation in cell culture. **a**, Suppressors contain coding sequence for the first 17 aa of Htt fused to 25 glutamines, a spacer region derived from helical regions (boxed) from the c-terminal region of TBP based on the published crystal structure (reprinted with permission from *Nature*), a second tract of 25 glutamines and an myc epitope tag at the c-terminus. Spacer regions are as follows: Sup1=68 aa (green), Sup2=54 aa (blue), Sup3=67 aa (red). **b**, Suppressor hypothetically interrupts aggregation by binding to one or more proteins with polyglutamine repeats. Schematic drawing of potential interactions between suppressor and polyQ, either through binding to two expanded-polyQ proteins or at two sites within a single expanded-polyQ protein. **c**, Time course of aggregation in the presence and absence of suppressor polypeptides. Plasmids were transfected at a ratio of 2 (suppressor):1 (polyQ protein). Aggregation is inhibited, but approaches that of HD103QE over a period of 96 h. Times indicated represent hours after transfection.

tion when tested in *Drosophila*. These studies indicate that pathogenesis can be reduced by targeting the binding surfaces that lead to aggregation and formation of large inclusions and raise the possibility that small molecules that interfere with this process might be beneficial for individuals with Huntington disease and other diseases associated with polyglutamine repeats.

Results

A suppressor of polyglutamine-mediated aggregation

A prominent hypothesis is that the pathology of Huntington disease and other polyglutamine-repeat diseases is caused by the aberrant interaction of extended polyglutamines (polyQs), either with other proteins (such as CREB-binding protein, CBP¹⁵) or with each other to form aggregates⁵. To design peptides, we made use of the observations that proteins with expanded polyglutamine repeats show affinity for normal proteins containing polyglutamine repeats, such as Htt with a normal number of repeats, CBP and TATA-binding protein (TBP), and are capable of sequestering these proteins into aggregates^{10,12,14,15,17,19,20}. We reasoned that molecules containing two short polyQ regions (25Q), separated by spacers of structural motifs such as α -helices, could interact with several sites in longer polyQ peptides. Binding of bivalent peptides might disrupt the formation of aberrant structures, such as postulated 'polar zippers'²¹, that may arise from the polyglutamine expansion. We tested three molecular designs based on the α -helical regions of the carboxy-terminal region of TBP (non-polyQ containing region)²². The suppressor spacers were inserted between polyQ-encoding arms (25Q) and designated Sup1, Sup2 and Sup3 (Fig. 1a). The constructs also encoded the first 17 amino-terminal residues of Htt and were epitope-tagged at the carboxy terminus with myc. If the spacers were to retain the three-dimensional conformation that they have in TBP, Sup1 would put the 25Q regions very near each other on one side of a relatively inflexible structure, Sup2 would put them on opposite sides of a highly flexible arm and Sup3 would put the two polyQ domains on the same side of the molecule, but facing towards opposite planes, and not as close as Sup1. These suppressor molecules could bind proteins associated with disease, either by interacting with two regions of one expanded-repeat molecule or by binding between two polyglutamine molecules (Fig. 1b).

Suppressor polypeptides disrupt aggregation

To test the ability of the suppressor peptides to alter polyQ aggregation in cell culture, COS-1 cells were transiently transfected with plasmids encoding a protein with an expanded polyglutamine repeat, HD103QE (containing a highly truncated Htt sequence and a c-terminal enhanced green fluorescent protein (EGFP) tag), and either Sup1, 2 or 3 under the control of a cytomegalovirus promoter. Aggregation was monitored beginning 24 hours after transfection, when expression becomes apparent, and followed until the percentage of cells with visible aggregates stabilized (Fig. 1c). These results show that the proportion of cells with aggregates increases with time, but is reduced and delayed by Sup2 and Sup3, with Sup3 showing the greatest suppression of aggregation over time. At 48 hours, when HD103QE forms aggregates in the majority of cells¹⁰, Sup1 results in a reduction of 11%, Sup2 results in a reduction of 24% and Sup3 results in a reduction of 36%, compared with HD103QE in the absence of suppressor. By 96 hours, aggregation in the presence of suppressor polypeptides approaches that of HD103QE alone, although Sup3 continues to inhibit aggregation to a greater extent than the other suppressors.

Suppression of aggregation requires a bivalent molecule

As proteins with short polyQ repeats can be found in inclusions but do not seem to disrupt them, we sought to test whether the bivalent structure of the inhibitors (that is, two polyQ domains on either side of a spacer) is essential for inhibition of aggregate formation. The bivalent nature of these molecules might promote cooperative interactions that are essential for inhibiting aggregation. We used the most effective suppressor as our base line and tested various derivatives of Sup3 for their ability to reduce aggregate formation in cultured cells (Fig. 2a). We compared 25Q alone, spacer alone and monovalent 25Q plus spacer with the bivalent 25Q-spacer-25Q (Sup3). Aggregate formation, as determined by GFP fluorescence, was monitored 48 hours after co-transfection with HD103QE and the different suppressor derivatives (Fig. 2b). Only the bivalent polypeptide, containing polyQ at both ends, with the spacer in the middle, significantly suppressed aggregation (37.6% reduction from the level of aggregates seen with HD103QE alone). The 25Q repeat

Fig. 2 A bivalent suppressor is required for inhibition of aggregation. **a**, Design of Sup3-derived suppressors. The black box represents the first 17 aa of Htt, the hatched box, the 25Q polyglutamine repeat; the gray box, spacer 3; and the white box, the myc epitope tag. **b**, Requirement of a bivalent suppressor. COS-1 cells were transiently transfected for 48 h with HD103QE alone or with Sup3-derived suppressors, and percentage of aggregation was determined by comparing with the number of cells with visible aggregates in HD103QE transfections, normalized to 100%. Only the bivalent Sup3 showed significant inhibition of aggregation, compared with the control.

alone, spacer alone, or a monovalent polypeptide with 25Q plus spacer did not suppress aggregation.

Suppressors interact directly with target proteins

Suppressor polypeptides are presumed to inhibit aggregation via direct protein–protein interactions with the expanded polyQ target. To confirm this directly, we tested for physical interactions between the suppressors and polyQ proteins using glutathione-S-transferase (GST) pull-down assays. The effectiveness of aggregate suppression correlates with binding to Htt, with Sup1 showing the lowest levels of binding and Sup3 showing the highest levels of binding (data not shown). These results are consistent with the hypothesis that direct binding of suppressor polypeptides to Htt with expanded repeats competes for other protein–protein interactions, including those leading to aggregation.

Suppressors co-localize with polyQs and alter aggregate structure

The physical appearance of visible aggregates seen by fluorescence microscopy can give insight into the chemical processes that underlie their formation. Aggregates of extended polyQ are highly stable structures that are resistant to boiling in SDS¹⁰. We hoped that suppressor peptides would disrupt this structure

when co-expressed with expanded-polyQ polypeptides. Approximately 96% of cells expressing polyQ alone (HD103QE) have a single large inclusion in the cytoplasm, whereas the remaining 4% of cells have multiple aggregates. Cells co-expressing HD103QE and Sup2 show a significant decrease in the overall number of cells containing aggregates (Fig. 1c). The fraction of cells with multiple aggregates versus single aggregates increases by approximately tenfold (40% versus 4%; Fig. 3a). Sup3 also has

Fig. 3 Suppressors decrease aggregation and co-localize with expanded polyQ proteins. **a**, Representative fields showing immunofluorescence of transiently transfected COS-1 cells and altered aggregation in the presence of Sup2 and Sup3. Green fluorescence indicates cells transfected with HD103QE. Arrows indicate aggregates. Scale bars=100 μ m. **b**, High-magnification immunofluorescence of aggregates in COS-1 cells transiently transfected with, from left to right, HD103QE, HD103QE + Sup2, HD103QE + Sup3 and HD103QE + Sup3-RFP. Cells were fixed and suppressors visualized using myc antibody or through intrinsic fluorescence of the RFP epitope tag. In cells where aggregates are observed, Sup2 results in the formation of multiple aggregates with co-localization of suppressor + HD103QE, whereas Sup3 results in the formation of single inclusions with co-localization of suppressor + HD103QE. Green color indicates EGFP, red indicates anti-myc or RFP and blue indicates DAPI. Scale bars=10 μ m. **c**, Aggregation is a dynamic process: video microscopy of aggregate formation in the presence and absence of suppressor polypeptides. Once seeding is observed, HD103QE coalesces into a single aggregate over a period of 25 min. **d**, Suppressor reduces and delays aggregation. In the presence of Sup3, HD103QE remains a series of smaller foci with diffuse cytosolic GFP fluorescence that do not coalesce during the filming period. After an extended period of time, small foci coalesce into a single large, amorphous aggregate; an example is shown in the last frame.

a distinctive effect on both the number and physical characteristics of aggregates. Sup3 co-expressed with HD103QE leads to a reduction in the number of transfected cells with large spherical aggregates and a significant increase in the fraction of cells with diffuse cytosolic EGFP staining (Fig. 3a).

Suppressor polypeptides were found to co-localize with HD103QE polypeptides when co-expressed, regardless of their ability to reduce the number of cells containing aggregates (Fig. 3b). For instance, Sup1 does not have a demonstrable effect on aggregation but was found to co-localize with proteins with expanded repeats (data not shown). Sup2 co-localized with small aggregates. Sup3 also co-localized with aggregates. We designed peptides to interact with the polyQ peptides; they do so in GST pull-down assays. In the cell, however, it is possible that they might interact simply by being drawn to expanding aggregates and interacting at the surface, as opposed to binding and associating with the polyQ peptides as the aggregation process proceeds. To understand the basis of the interactions between suppressors and the polyQ proteins they aggregate with, we compared the location of antibody-stained suppressor with intrinsically fluorescent suppressor peptides. For example, when detected by anti-myc antibody, Sup3 staining appears only on the surface of the aggregates, indicating the 'tight' nature of the aggregate and its ability to prevent antibody from penetrating to the internal protein. However, when a red fluorescent protein (RFP) tag was placed at the c-terminal end of suppressor polypeptides, it became apparent that the suppressor peptides (such as Sup3; Fig. 3b) were distributed throughout the aggregates, again suggesting a direct interaction with expanded polyQs in the cellular environment. Thus, suppressor peptides interact with polyQ in aggregates and alter aggregate structure.

Suppressor peptides delay aggregate formation in cultured cells

In the mammalian cell system we have developed, the time course of aggregation is condensed compared with the presumed kinetics of this process in the human brain or in the brain of experimental animals. Insight into the aggregation process and the impact of suppressors upon it can be gained from high-resolution video microscopy. We therefore used this technique to examine the kinetics of aggregation in the presence and

absence of suppressor proteins. Filming of live cells reveals a two-step process for polyglutamine aggregation. In cells transiently expressing extended-polyQ peptides, aggregation involves a slow nucleation step that is most apparent at approximately 16 hours, when diffuse polyQ protein forms a single 'seed'. Once seeding is evident, further growth of the aggregate is very rapid, typically taking place within about 20 minutes (Fig. 3c). These results are consistent with studies suggesting that nucleation is the rate-limiting step, followed by rapid growth of the aggregate, which is dependent upon protein concentration and length of polyglutamine repeat^{23,24}. When suppressors Sup2 or Sup3 are co-expressed with HD103QE, the initial nucleation event is delayed by approximately 20 hours, so that seeds are most notable at around 36 hours rather than the typical 16 hours, and more diffuse GFP fluorescence is observed. Even when some seeds (5–6) do form in the presence of these suppressors, extended polyQs do not proceed to coalesce into large inclusions during the monitoring period. For example, in the presence of Sup3, nucleation was delayed, and several small and barely visible seeds were seen (Fig. 3d) that did not progress to form a single large aggregate over the 50 minutes of filming. The disruption of aggregation in this manner by chaperones has been described previously²⁵. Eventually (10–12 hours later), single aggregates emerged (last frame of Fig. 3d).

Suppressor rescues lethality in *Drosophila*

If the binding interactions that allow aggregates to form contribute to polyQ-dependent neuropathology, then slowing and reducing the aggregation process by artificial peptides should be accompanied by a reduction of pathology *in vivo*. We have shown that expanded polyQ chains alone (Fig. 4a) are intrinsically cytotoxic and that they produce aggregates (Fig. 6a) and cause neuronal degeneration and early adult death when expressed in neurons of *Drosophila*²⁶. As Sup3 has the most pronounced effect on aggregate formation in cultured cells, it was chosen for *in vivo* analysis (Fig. 4b). Transgenic *Drosophila* expressing single copies of Sup3 in neurons (*elav*-GAL4) in the absence of polyQs show no effect on viability, demonstrating that the synthetic peptide is relatively inert biologically.

We asked whether polyQ-induced lethality²⁶ could be slowed or reduced by co-expressing the suppressor polypeptide in trans-

Fig. 4 Expression of suppressor rescues lethality in *Drosophila*. **a**, Constructs containing polyQ are expressed using the UAS-GAL4 system. Schematic of GAL4 driving expression of polyQ proteins in all neurons from embryogenesis on (*elav*-GAL4), with the sequence of flanking amino acids with or without a myc/flag epitope tag, is shown. Both Q48 and Q108 repeat-length polypeptides were used in these experiments. **b**, Structure of the UAS-driven suppressors is shown schematically. **c**, Lethality due to polyQ is reduced by co-expression of a suppressor protein in neurons. The percent survival of *Drosophila* lines expressing either Q108 or Q48tag alone in neurons is compared with co-expression of both polyQ peptides plus Sup3 derived from two independent transgenic lines, Su 3-17 and 3-14, representing different integration events. Genotypes are wildtype, w; P{w⁺mC=UAS-Q108}¹⁶/P{w⁺mC, *elav*-GAL4}, w; P{w⁺mC=UAS-Q108}¹⁶/P{w⁺mC, *elav*-GAL4}; P{w⁺mC=UAS-Su3}¹⁴/w; P{w⁺mC=UAS-Su3}¹⁷/w; P{w⁺mC=UAS-Q108}¹⁶/P{w⁺mC, *elav*-GAL4}, w; P{w⁺mC=UAS-Q48tag}¹³/P{w⁺mC, *elav*-GAL4}, w; P{w⁺mC=UAS-Q48tag}¹³/P{w⁺mC, *elav*-GAL4}, w; P{w⁺mC=UAS-Q48tag}³⁶/P{w⁺mC, *elav*-GAL4}, w; P{w⁺mC=UAS-Q48tag}³⁶/P{w⁺mC, *elav*-GAL4}. The percent survival was calculated by dividing the number of non-CyO flies by the number of CyO flies. In each case, the number of CyO flies was greater than 100.

The toxicity caused by Q108 is severe. To test whether the suppressor could improve pathology of a polyQ repeat that is more characteristic of repeat lengths of adult onset, we tested the ability of suppressors to affect survival of flies expressing Q48 peptides. Expression of suppressor increases survival of two different polyQ lines, Q48tag¹³ and Q48tag³⁶, by approximately 2.5-fold. Survival in the range of 16–24% without suppressor is increased to nearly 60% in the presence of suppressor (Fig. 4c).

As overexpression of the chaperone heat-shock proteins (hsp) hsp40 and hsp70 can also reduce pathology in *Drosophila* expressing either polyQ peptides²⁷ or ataxin 3 (refs 28,29) and can alter the overall structure of aggregates in flies^{29,30}, we tested whether suppressor transgenes were causing expression of hsp70 to increase. Western blots of larval protein from controls, Q108tag alone, suppressor alone (Su 3-14 and Su 3-17), and Q108tag plus suppressor were probed for hsp70. We saw no changes in the levels of hsp70 in larval protein (data not shown). We observed identical results in cell lysates from cells transiently expressing either HD103QE alone or in the presence of any of the three suppressor polypeptides (data not shown). Thus, suppression of lethality is not due merely to increased chaperone production in response to co-expression of suppressor polypeptide; a completely independent means of altering aggregation can reduce pathogenesis.

The photoreceptor neurons in the fly eye are arranged in a series of repeating trapezoids visible as seven rhabdomeres (subcellular light-gathering structures) within each ommatidium (Fig. 5a,c).

Similar results are obtained when Su 3-17 is co-expressed with shorter polyQ peptides, such as two different Q48 tag lines (Fig. 5*b,c*). The pathology in the shorter polyQ lines is less severe, resulting in loss of only 1–2 photoreceptor neurons and producing some gaps in the tissue. Expression of Su 3-17 with Q48 tag peptides (Fig. 5*b,c*) results in the majority of ommatidia having the normal seven rhabdomeres. Thus, co-expression of suppressor peptides can significantly reduce the extent of neuronal degeneration.

Not all neurons acquire visible aggregates and not all neurons degenerate. It is possible that suppression of aggregation and suppression of degeneration involve two distinct mechanisms and are separable events. To address this, we asked whether suppression of pathology is correlated with changes in aggregate formation *in vivo*. Suppressor protein alone does not form aggregates but appears diffuse in the cytosol (Fig. 6a). In contrast, larval brains expressing polyQ alone (Q108tag, $n=16$) show aggregates in many parts of the brain. When suppressor is co-expressed, the number of detectable aggregates is greatly reduced (Fig. 6b,c).

Examination of different cell types in the larval brain reveals different cellular responses to polyQ peptides. Because *Drosophila* neurogenesis involves distinct waves of cell division, the third-instar larval brain contains both neuroblasts (proliferating cells) and mature neurons and cells in intermediate stages³¹. In neuroblasts, diffuse polyQ appears in the cytosol and large cytosolic perinuclear aggregates are evident (Fig. 6*b,e*). In

each line. c. Toluidine blue-stained sections through the eyes of adult wildtype flies and flies containing either the Q108tag peptide, Q108tag plus suppressor (Su 3-17), Q48tag, or Q48tag + suppressor (Su 3-17). Expression was driven by *elav*-GAL4 in each case. Genotypes are wildtype, w; P{w^{mC}, *elav*-GAL4}; P{w^{mC}=UAS-Q108tag}^{2/+}, w; P{w^{mC}, *elav*-GAL4}/+; P{w^{mC}=UAS-Q108tag}^{2/2}, P{w^{mC}=UAS-Q108tag}^{2/2}, w; P{w^{mC}=UAS-Su3}¹⁴, w; P{w^{mC}=UAS-Su3}¹⁷/w/Y; P{w^{mC}, *elav*-GAL4}/+; w; P{w^{mC}=UAS-Q48tag}³⁶/P{w^{mC}, *elav*-GAL4} +; P{w^{mC}=UAS-Su3}¹⁷/w/Y; P{w^{mC}=UAS-Q48tag}³⁶/P{w^{mC}, *elav*-GAL4}.

Fig. 6 Suppressor peptide suppresses and delays polyQ aggregate formation in the central nervous system. UAS transgenes were expressed in all neurons beginning in embryogenesis. **a**, Su 3-17 alone expressed in whole brain at $\times 20$ magnification and in inset (arrow) shows diffuse cytosolic staining. Red indicates anti-myc staining. Scale bar = 50 μm . **b,c**, Q108tag (**b**) and Q108tag + Su 3-17 (**c**) expressed in whole brain shows aggregate formation reduced in the presence of suppressor. Red indicates anti-DLG and green indicates anti-flag staining. **d**, Schematic drawing of larval brain indicating central brain region (CB), ventral nerve cord (VNC) and mushroom body (MB). The mushroom body is shown by a dashed arrow, to indicate that it is deep within tissue. **e**, $\times 100$ magnification of Q108tag expression in neuroblasts from the central brain region showing single, large perinuclear aggregates (indicated by arrow) in most expressing cells. Scale bar = 15 μm . **f**, Expression of Q108tag + Su3-17 at high magnification shows a drastic decrease in aggregate formation (arrow indicates one of the few remaining small aggregates), with a concomitant decrease in staining. **g**, Expression of Q108tag in mushroom bodies produces nuclear aggregates in non-dividing neurons (indicated by arrows). **h**, Expression of Q108tag + Su 3-17 results in a significant reduction in nuclear aggregation. In both **g** and **h**, inset shows Q108tag staining alone to demonstrate the decrease in overall staining in the presence of suppressor. Genotypes of the flies are w ; $P(w^{+mC}, elav-GAL4)/+$; $P(w^{+mC}=UAS-Q108tag)^{2/+}$, w ; $P(w^{+mC}=UAS-Su3)^{17/w/Y}$; $P(w^{+mC}, elav-GAL4)/+$; $P(w^{+mC}=UAS-Q108tag)^{2/+}$. For D-H, Q108tag is indicated in green (anti-FLAG antibody); in **a-h**, blue fluorescence indicates a nucleic acid stain illuminating the nuclei.

non-dividing neurons, polyQ aggregates are also evident but are exclusively located in the nucleus. For instance, in non-proliferating Kenyon cells of the mushroom body, essential for learning and memory^{32,33}, and in neurons along the midline of the ventral nerve cord (VNC), polyQ protein is nuclear (Fig. 6f), with most cells having a single large nuclear aggregate. Cytosolic staining is not detected in most of these cells. Thus, the fate of polyQ is different between neuroblasts and neurons.

Expression of suppressor peptides inhibits the formation of aggregates, including both the perinuclear inclusions of neuroblasts and the nuclear inclusions of neurons. In addition, the presence of suppressor peptides reduces the overall accumulation of either cytoplasmic or diffuse, nuclear polyQ (Fig. 6f,h). For example, in the neuroblasts of the central zone of the brain, virtually no aggregates can be found when suppressor is present (21/23 brains) and in 2 of 23 brains that had detectable aggregates, fewer cells had aggregates and those aggregates were smaller in size (Fig. 6f). Similarly, the neuroblasts in the thoracic region of the VNC also show reduced levels of polyQ staining in the cytoplasm and an almost complete elimination of large aggregates (data not shown). In neurons such as the Kenyon cells, the suppressor reduces the number of cells with visible aggregates as well as the number of cells showing polyQ accumulation (Fig. 6h). By contrast, aggregates are clearly detected in neurons along the midline of the VNC, representative of differentiated neurons that emerged during embryogenesis (data not shown), suggesting that although suppressor delays aggregate formation, aggregates can form if given enough time to accumulate. Thus, expression of suppressor polypeptide leads to lower levels of polyQ accumulation and to a severe reduction of aggregate formation and aggregate size.

Suppression of aggregation and reduced pathology in the developing *Drosophila* eye

In the presence of suppressor, neuropathology is reduced and formation of polyQ aggregates and polyQ accumulation is delayed and reduced. How tightly are these two observations

correlated? In the eye, the course of polyQ accumulation and aggregate formation can be precisely followed and the fate of the cells observed. In the imaginal disc of the eye, a morphogenetic wave passes over a field of cells, specifying a new row of founding photoreceptor neurons roughly every 2 hours, followed by an ordered recruitment of surrounding cells to the emerging photoreceptor cluster^{34,35}. The *Elav* promoter used in these studies is activated only as specification of cell fate takes place; thus, we can observe rows of cells that have initiated expression of polyQ in increments of approximately 2 hours. When Q108 is expressed, aggregates first appear 7–15 rows behind the morphogenetic furrow (that is, approximately 14–30 hours after initiation of polyQ expression; Fig. 7a,c). It is clear that formation of aggregates is not random but highly dependent on the time that polyQ has had to accumulate. Notably, when expression of polyQ proteins is driven in all cells behind the morphogenetic furrow by *gmr-GAL4*, degeneration in the eye is most severe in the posterior portion of the eye, which is the region that has expressed polyQ and accumulated aggregates the longest (data not shown). As described for many other proteins that contain a glutamine-rich region, *Elav* tends to co-aggregate with the polyQ peptides (Fig. 7e,f). The presence of suppressor peptide sharply reduces the number and size of the aggregates that form (Fig. 7b,d), with the first appearance of aggregates delayed by about 8–14 hours (approximately 14–19 rows behind the furrow).

The delay of aggregate formation caused by the suppressor is highly correlated with protection from degeneration. For example, photoreceptor cells R7 and R8 do not degenerate and do not have visible aggregates. On the other hand, the R3 and R4 cells are among the first neurons to form inclusions and they are the first to degenerate (only 26% of ommatidia expressing Q48 retain both R3 and R4; Fig. 5). Thus, propensity to degenerate and presence of visible aggregates are strongly correlated.

Discussion

It is clear that Htt and other proteins with expanded polyglutamine repeats have unique binding properties that can inhibit the function of cellular proteins or lead to the formation of aggregates and, ultimately, to large inclusions. If a common binding surface is responsible for these phenomena, then targeting that surface with peptides that could sterically interfere with the binding interactions that produce disease should limit aggregation and reduce pathology. We have shown that a synthetic polypeptide that binds to expanded polyglutamines and inhibits

Fig. 7 Aggregation in the developing *Drosophila* eye imaginal disc is delayed and inhibited by suppressor peptide. **a,b**, $\times 60$ magnification shows the overall decrease in aggregation of Q108tag in the presence of Sup3 (Su3-17). More aggregates are formed at the posterior edge of the disc Q108tag (**a**) and Q108tag + Su 3-17 (**b**). In eye discs expressing Q108 alone, the first aggregates appear in different discs 7–15 rows behind the morphogenetic furrow (14–30 h after expression begins). In the presence of suppressor, a delay of approximately 8–14 h was observed, with the first discernable aggregates occurring approximately 14–19 rows behind the furrow. Scale bar=15 μ m. **c,d**, At higher magnification ($\times 100$), a decrease in the number of photoreceptors with aggregates per ommatidium, a decreased size of aggregates and preferential aggregate formation in R3 and R4 cells (arrows) is observed. More aggregates are formed along the equator (shown as a dashed line in **c**). Posterior of the disc is to the left and anterior is to the right. Green staining indicates polyQ (anti-Flag) and red indicates Elav (anti-Elav), a nuclear protein. Merged images are orange. In addition, aggregates are observed in photoreceptors facing lateral disc edges and not the equator (brackets with large arrowheads). For polyQ alone, 16 discs were analyzed, and, on average, aggregation starts at row 12. Twelve discs were examined in the presence of Sup3, with an average initiation of aggregation beginning in row 16. **e,f**, Staining with polyQ (**e**) and Elav (**f**) alone showing co-localization of Elav with polyQ nuclear staining and aggregation.

aggregation of polypeptides with expanded polyglutamine tracts in cell culture both inhibits aggregation and suppresses pathology in a *Drosophila* model. The ability to inhibit aggregation in cell culture correlates strongly with the ability of the suppressor polypeptide to bind to expanded polyglutamines. These results suggest that the binding activity that leads to aggregation contributes to pathogenesis and that molecules designed to interfere with these binding activities may be good candidates for therapeutic intervention.

Precedence for this approach comes from using β -sheet breaking peptides that inhibit cerebral amyloid β -protein deposition and prevent neuronal shrinkage in rat brain models of amyloidosis^{36,37} and from expression of peptides that inhibit aggregation in *in vitro* and polyglutamine-induced cell death in culture³⁸. Although the role of protein aggregates in the pathogenic process is not clear, protein aggregation itself is a hallmark of the majority of neurodegenerative diseases, including the diseases associated with polyglutamine repeats, Alzheimer disease, amyotrophic lateral sclerosis (ALS), prion diseases and some forms of Parkinson disease³⁹. Amyloid-like structures in brain of individuals with Huntington disease⁴⁰ suggest parallels to other amyloid-associated diseases, such as Alzheimer and prion diseases. A potential causal role for aggregation in ALS has been suggested by the inability of increased expression of wildtype *Sod1* in a mouse ALS model to slow disease progression or pathology⁴¹. Evidence that aggregates have a pathogenic role in Huntington disease includes the observations that the appearance of aggregates typically precedes onset of symptoms in transgenic mice⁴², that aggregates are associated with axonal degeneration as an early pathological event in mice with Huntington disease⁴³ and that in a model of tetracycline-inducible Huntington disease, both the disease phenotype and presence of aggregates are reversed when transgene expression is stopped⁴⁴. Expression of peptides that inhibit aggregation in an *in vitro* assay was also found to inhibit polyglutamine-induced cell death in culture³⁸. Finally, overexpression of chaperone proteins in cell culture reduces aggregation and suppresses toxicity^{13,45}, and, in *Drosophila*, reverses dominant, polyglutamine-mediated eye phenotypes^{26,27,29}, leading to increased solubility of polyglutamine-containing proteins or altered overall structure of aggregates^{27,29}. Other effects of excess chaperones that may be protective, however, are difficult to rule out.

Several observations argue against a causative role for aggregates per se in the pathogenic process. For instance, whereas nuclear localization was found to be required for polyglutamine toxicity, aggregation was not required for either cell death in culture⁴⁶ or

initiation of disease in ataxin-1 transgenic mice⁴⁷. In this mouse model, however, inclusion formation seems to be more important for disease progression than for disease initiation⁴⁸. It is also possible that the size of the aggregates—that is, smaller aggregates as opposed to large nuclear inclusions—may contribute to pathogenesis in ways that are still unclear. It has been proposed that smaller aggregates are more efficient at recruiting polyQ-containing proteins than very large aggregates²⁴, presumably owing to surface-area considerations. These studies leave open the question of whether aberrant protein interactions, including those that can lead to aggregation, can be toxic.

Our observations demonstrate a strong correlation between accumulation and aggregation of polyglutamine protein and subsequent degeneration and show that synthetic peptides that bind polyQ proteins can delay and reduce the formation of aggregates and reduce pathology. One of the most notable findings reported here is the temporal correlation between the appearance of aggregates in photoreceptor neurons during eye development and the loss of these same neurons during progression of pathology. The developmental status of a photoreceptor neuron seems to influence as well whether a particular neuron is prone to aggregation and subsequent degeneration. R8 is specified before all other photoreceptors and R7 is specified last. However, these two neurons neither degenerate nor have visible aggregates. In contrast, R3 and R4 are specified relatively early and do form aggregates and degenerate. What may be critical to this process is whether the photoreceptor cell is terminally differentiated. It has been shown⁴⁹ that a two-step process is required for the formation of photoreceptor neurons in *Drosophila*, with R3 and R4 among the first to terminally differentiate and R7 and R8 the last. Thus, formation of nuclear inclusions and neurodegeneration may be dependent upon both cell type (specific neurons) and differentiation state. This would be consistent with

mutant Htt causing aggregation and loss of neurons only in the brain, although the protein is widely expressed.

In both the central nervous system and eye discs, neurons can tolerate an accumulation of polyQ and the formation of aggregates for a period of time before degenerating. Cytosolic, perinuclear aggregates are observed in neuroblasts and nuclear aggregates are observed in neurons (such as Kenyon cells). Suppressor reduced and delayed this aggregation regardless of whether aggregates were nuclear (neurons) or cytosolic (neuroblasts). In agreement with results in cell culture, with sufficient time to accumulate polyQ protein, aggregates can form in neurons, even in the presence of suppressor. For example, aggregates in the differentiated neurons along the midline of the VNC are similar in the presence or absence of suppressor polypeptide; these neurons emerged during embryogenesis and have had a long time to accumulate protein. It is clear that appearance of aggregates does not immediately lead to degeneration. Persistent aggregates are seen in neurons of transgenic mice⁴² and in post-mortem brain samples of individuals with Huntington disease, before the loss of neurons in affected regions⁷. In the eye disc, formation of an aggregate also does not immediately cause degeneration of photoreceptor cells (mature neurons), as loss of these neurons does not become apparent until at least a few days after the appearance of visible aggregates. These data suggest that prolonged exposure to aggregates, or prolonged exposure to processes of which aggregates are indicative, is eventually deleterious.

There are several mechanisms that should be considered to explain how the unique binding properties of expanded polyglutamine polypeptides can cause pathology, including: (i) direct binding of expanded polyglutamine peptides to cellular proteins, leading to inhibition of their activities; (ii) sequestration of cellular proteins into aggregates with expanded polyglutamine peptides, reducing the levels and activities of these cellular proteins; and (iii) mechanical disruption of cellular functions by aggregates formed by pathogenic peptides, such as interfering with the spindle bodies. It is possible that all three of these mechanisms contribute to pathology. However, aggregates do not seem to affect cell division in a stably transfected PC12 cell model of Huntington disease (A.K. and B. Apostol, unpublished results), and in photoreceptor cells (mature neurons), no cell divisions occur between formation of aggregates and degeneration, suggesting that mechanical disruption of cell division is not a key consequence of aggregation.

If the binding surface interactions that inhibit the function of cellular proteins and those that lead to the formation of aggregates are the same, it may not be possible to separate the different events experimentally. However, the fact that one can relieve pathogenesis by compensating for the decreased activity of cellular factors arising from interactions with mutant Htt^{17,50} suggests that the availability of cellular proteins is key. In addition, within aggregates, enzymatic activity of sequestered protein is compromised (A.K. and B. Apostol, unpublished results). We also note that disruption of polyglutamine binding by the suppressor peptides described here reduces the total accumulation of polyQ peptide staining. Interfering with the polyglutamine binding process and formation of aggregates may thus lead to increased clearance of toxic proteins with expanded repeats and additional relief of symptoms.

We have used the appearance of visible aggregates as an assay for polyQ–polyQ interactions, as well as otherwise invisible interactions in cells and tissues, and targeted this process with suppressor polypeptides. From a therapeutic perspective, there are significant challenges to the intracellular delivery of peptides. The development of suitable delivery protocols for peptides is an

active area of research that extends into the realm of gene therapy^{51–53}. A small molecule that can inhibit the polyQ interactions described here could prove effective in treating Huntington disease. The ability of suppressor polypeptide to relieve polyglutamine pathogenesis in *Drosophila*, independent of disease-gene context, also suggests that peptides or compounds developed for one disease may be effective in other polyQ diseases through a common mechanism. Taken together with other recent results^{17,20,27,29,30,45,50,54,55}, a multipronged approach aimed at compensating for the reduction of key cellular factors while reducing additional deleterious interactions could lead to reduced polyQ accumulation and improved outcome in the treatment of Huntington disease and other related polyglutamine-repeat diseases.

Methods

Mammalian expression constructs. Htt polyglutamine expression constructs have been described previously¹⁰. By PCR, we amplified suppressor spacers derived from regions of TBP from full-length *TBP* cDNA as described¹⁰, using primers that introduced new *KpnI*, *BglII* and *HindIII* and *BamHI* sites at the 5' and 3' ends, respectively. The amino-acid sequences of suppressor polypeptides (Sup1, Sup2 and Sup3 spacers) are available upon request. We used HD25Qmyc in BlueScript (Stratagene) as the foundation for further cloning. DNA fragments encoding Sup1, Sup2 and Sup3 spacers were digested with *KpnI* and *HindIII* and subcloned amino-terminal to the 25Q repeat. A DNA fragment encoding 25Q was isolated by *KpnI* and *BamHI* digestion and ligated N-terminal to the polypeptide fusion, H/H 25Q, that was digested with *KpnI* and *BglII*. We subcloned the resulting HD 25Q H/H 25Q polypeptide fusions into the mammalian expression vector pcDNA 3.1. (Invitrogen) using *KpnI* and *XbaI*. Final clones were verified by sequencing.

For monovalent suppressors, each spacer region was subcloned into pBluescript using *KpnI* and *BamHI*. Spacer fragments isolated by *BglII* and *XbaI* digests of pBluescript constructs were ligated within HD25Qmyc in pcDNA3.1 digested with *BamHI* and *XbaI*. We used PCR amplification to introduce a Kozak box, an initiator methionine and a glycine followed by Sup3 sequence and a c-myc tag to generate a plasmid-encoding suppressor polypeptide alone. Suppressor DNA was subcloned into pcDNA 3.1 using *KpnI* and *BamHI*. We amplified RFP from pDsRed1-N1, with primers containing *BamHI* at the 5' end and *XbaI* at the 3' end, and subcloned it into *BamHI* and *XbaI* sites of pcDNA3.1, in-frame with suppressor sequences. GST-fusion proteins have been described previously¹⁵.

Fluorescent analyses of transfected cells. Polyglutamine aggregation was assayed in COS-1 cells. Cells were grown on coverslips to 50% confluence and transfected using lipofection for 2 h with Transfectam reagent (Promega) or LipofectaminePLUS reagent (Gibco BRL) according to the manufacturer's protocol. HD103QE, comprised of the first 17 aa of Htt, followed by 103Qs and an EGFP c-terminal epitope tag, and plasmids encoding suppressor peptide were transfected at a ratio of 1:2, respectively. We monitored polyglutamine aggregation from 12 h to 96 h after transfection, measured as a percentage of transfected cells (EGFP-positive) with aggregates. We fixed cells in 2% formaldehyde/0.1% Triton X-100 for 10 min and determined myc immunoreactivity by incubating with primary mouse monoclonal anti-c-myc (Invitrogen) antibody (1:500) and secondary FluoroLink Cy3 (Amersham Life Science) antibody (1:2,000). We carried out epifluorescent microscopy on a Zeiss Axioplan II equipped with a Quantix CCD camera (Photometrics) and Spectrum imaging software (Scanalytics). SDS solubility assays have been described previously¹⁰.

Videomicroscopy of aggregate formation. COS-1 cells were transfected with 1 µg of plasmid DNA expressing HD103QE and co-transfected with suppressor plasmids using Transfectam reagent (Promega) according to the manufacturer's directions. After 16 h (HD103QE) or 36 h (HD103QE + suppressors), cells were rinsed with PBS and switched to HEPES-based microscopy media, layered with mineral oil and subjected to time-lapse videomicroscopy using standard epifluorescence illumination⁵⁶. Images were captured with shuttered light and a SPOT2 CCD camera (Diagnostic Instruments) controlled by IP Labs software (Scanalytics).

GST pull-downs. We carried out GST pull-down experiments as described previously¹⁵ using GST, GST-83Q and GST 83QP. We synthesized [³⁵S]methionine-labeled Sup2 and Sup3 *in vitro* using rabbit reticulocyte lysate systems (Promega). We added radiolabeled proteins in approximately equal molar amounts to GST fusion proteins pre-equilibrated in buffer A (20 mM Hepes-KOH pH 7.5, 100 mM NaCl, 10% glycerol, 0.1% Triton X-100, 100 μM phenylmethylsulfonyl fluoride) containing 0.2% BSA in a final volume of 200 μl. After 30 min rocking at room temperature, the beads were recovered and washed three times at room temperature in 1 ml of buffer A. We analyzed the bound proteins using SDS-PAGE, followed by autoradiography and phosphorimager analysis.

Fly stocks and crosses. Expression of polyglutamine-containing peptides (Q108¹⁶, Q108tag², Q48^{13,36}) is driven by the bipartite UAS-GAL4 (see below) expression system in transgenic flies, as previously described²⁶. Constructs under the control of a yeast upstream activating sequence (UAS) are crossed to flies expressing the yeast GAL4 transcriptional activator driven by various tissue-specific promoters, thus driving tissue-specific expression of the transgenes. For these studies, polyQ peptides were expressed in all neurons from embryogenesis on, using an *elav*-GAL4 driver (Fig. 4a). Germline transformants of suppressors were generated as described^{57,58}, with modifications⁵⁹. GAL4 stocks were *w*; P{*w⁺mC₂;elav-GAL4*}/CyO, P{*w⁺mC₂=Act-GFP*}/JMR1 (ref. 60). Flies were grown on cornmeal molasses medium at 25 °C, 27 °C or 29 °C, depending on the experiment.

Histology. We fixed adult heads for light microscopy in 2% glutaraldehyde, 0.1 M phosphate buffer overnight at 4 °C, followed by a fix in 1% osmium tetroxide, 0.1 M PBS for 1 h at room temperature. Tissues were dehydrated through a graded series of ethanol and embedded in Durcupan (Fluka). Serial sections were cut at 2 μm, stained with 1% toluidine blue, 1% borax and mounted in DPX (Electron Microscopy Sciences).

Antibody staining. Late third-instar larvae were dissected in ice-cold PBS, fixed in 4% formaldehyde in PBS for 30 min at room temperature, washed in PBS, permeabilized in ethanol/acetic acid (20:1) for 15 min at -20 °C, washed to rehydrate in PBS and finally blocked in 3% BSA, 0.5 % NP-40, PBS for 2 h at room temperature. We used a three-antibody stacking technique to enhance the detection of peptides. Primary antibodies were mouse anti-FLAG M2 (Eastman Kodak) at 1:1,000, guinea pig anti-DLG (gift from Peter J. Bryant) at 1:1,000 and rat anti-Elav (Developmental Studies Hybridoma Bank) at 1:50. Secondary antibody was unlabeled rabbit anti-mouse IgG (Jackson Immuno Research), at 1:200. Tertiary antibodies were FITC-conjugated donkey anti-rabbit IgG (Jackson Immuno Research) and Cy3-conjugated donkey anti-guinea pig IgG (Jackson Immuno Research) at 1:100. Tissues were incubated with primary antibodies overnight at 4 °C and with secondary antibodies for 2 h at room temperature. TO-PRO-3 (Molecular Probes) was added at 1:100 to counterstain nuclei, along with the tertiary antibody, for 2 h at room temperature. We analyzed samples with a BioRad MRC 1024 scanning confocal microscope in the Optical Biology Core facility, Univ. of California, Irvine.

GenBank accession numbers. Full-length *TBP* cDNA, M55654.

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Competing interests statement

The authors declare that they have no competing financial interests.

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