Dynamic Imaging by Fluorescence Correlation Spectroscopy Identifies Diverse Populations of Polyglutamine Oligomers Formed in Vivo\textsuperscript{*}

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**Background:** Protein aggregation is implicated in numerous diseases.

**Results:** Polyglutamine oligomers maintain a heterogeneous distribution that reaches an equilibrium during aging and can be altered by genetic modifiers that suppress aggregation.

**Conclusion:** Shifts in populations of oligomers do not correlate with toxicity.

**Significance:** The dynamics of oligomerization and aggregation to inert species is essential for protein misfolding and toxicity.

Protein misfolding and aggregation are exacerbated by aging and diseases of protein conformation including neurodegeneration, metabolic diseases, and cancer. In the cellular environment, aggregates can exist as discrete entities, or heterogeneous complexes of diverse solubility and conformational state. In this study, we have examined the \textit{in vivo} dynamics of aggregation using imaging methods including fluorescence microscopy, fluorescence recovery after photobleaching (FRAP), and fluorescence correlation spectroscopy (FCS), to monitor the diverse biophysical states of expanded polyglutamine (polyQ) proteins expressed in \textit{Caenorhabditis elegans}. We show that monomers, oligomers, and aggregates co-exist at different concentrations in young and aged animals expressing different polyQ-lengths. During aging, when aggregation and toxicity are exacerbated, FCS-based burst analysis and purified single molecule FCS detected a populational shift toward an increase in the frequency of brighter and larger oligomeric species. Regardless of age or polyQ-length, oligomers were maintained in a heterogeneous distribution that spans multiple orders of magnitude in brightness. We employed genetic suppressors that prevent polyQ aggregation and observed a reduction in visible immobile species with the persistence of heterogeneous oligomers, yet our analysis did not detect the appearance of any discrete oligomeric states associated with toxicity. These studies reveal that the reversible transition from monomers to immobile aggregates is not represented by discrete oligomeric states, but rather suggests that the process of aggregation involves a more complex pattern of molecular interactions of diverse intermediate species that can appear \textit{in vivo} and contribute to aggregate formation and toxicity.

Protein misfolding and aggregation are implicated in a wide variety of diseases including Amyotrophic Lateral Sclerosis (ALS), at least nine CAG-repeat diseases, Alzheimer disease (AD), and Parkinson disease (PD) (1, 2). The basis of cellular toxicity, however, remains less well understood, in part because the relationship between aggregation, composition of aggregates, and impairment of cellular function is complex. Consequently, many models have been put forth to study the basis of cellular toxicity in these diseases, implicating different conformational and oligomeric states of mutant SOD1, TDP-43, Huntington, Ataxins, Abeta, tau, \(\alpha\)-synuclein, and parkin, respectively (3–7).

The majority of studies to elucidate the pathway of protein aggregation have employed \textit{in vitro} approaches with purified protein. This has led to the development of models of protein aggregation including nucleated polymerization, templated assembly, and nucleated conformational conversion (8). \textit{In vitro} studies suggest that more than one of these pathways contributes to the kinetics of aggregation (9–17). In addition, these pathways can be altered through interactions with other proteins, in particular molecular chaperones (18–23). A variety of methods have been employed to study aggregation, including thioflavin T binding, turbidity measurements, atomic force microscopy (AFM), electron microscopy (EM), and dynamic light scattering. Together, these methods have demonstrated the presence of a lag period, growth phase, and plateau in fibril formation, and that the lag phase involves the appearance of multiple conformational states of oligomers and soluble aggregates (17, 18, 24–30). These \textit{in vitro} approaches have also contributed to our understanding of the importance of pH, salt concentration, protein concentration, and solvent characteristics on lag-time, secondary structure, and the types of aggregate species (16).

The cellular crowding conditions that occur \textit{in vivo} are difficult to replicate \textit{in vitro}, and likely involve very different pathways due to the continual synthesis of monomer species and the presence of chaperones and other folding catalysts that directly interact with metastable and misfolded proteins. A plethora of...
proteins have been identified that interact with polyQ and affect its aggregation and toxicity (31–33). These proteins are involved in many cellular processes including protein folding and clearance, transcription, and cell structure. The use of dynamic imaging methods, including fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP), has provided evidence for variability in the packing structure of aggregates, as well as dynamic interactions with chaperones and transcription factors (34–36).

The biophysical imaging technique FCS provides a method that can measure changes in diffusion rate at a resolution that approaches the single molecule limit, and therefore could be useful to assess the different states of aggregation-prone proteins (37, 38). Molecular physical parameters that give rise to signal fluctuations, such as diffusion rate and concentration can be determined with precision by FCS analysis (39–42). Specifically, FCS has been used to detect changes in particle mobility such as occurs upon protein–protein interactions, and changes in local concentration of the fluorescent particle (43–47). FCS has also been used to measure aggregation events through detection of higher order state oligomers (39, 48, 49). While FCS was successfully employed to detect the change from monomer to oligomer, traditional autocorrelation fitting techniques have not yet been utilized to resolve the different oligomeric states formed.

The goal of these studies was to monitor the polyQ aggregation pathway in Caenorhabditis elegans, including the effect of genetic suppressors of aggregation, using complementary dynamic imaging techniques. Our approach was to utilize FCS based methods, coupled with FRAP and fluorescence imaging, to monitor the three main phases of aggregation: monomers, oligomers, and aggregates. We show that all three states co-exist in a C. elegans polyQ model of multiple Q-lengths and ages. Our FCS based methods show that the oligomer population is heterogeneous and not dominated by a single oligomer species, and reaches equilibrium during aging. To examine the effect of other genes on aggregation, we also tested the effect of genetic suppressors of aggregation on the oligomer distribution. For all the genes tested, oligomers were maintained in a heterogeneous state, although the size and/or brightness distribution of oligomers varied between gene modifiers. These findings show that FCS enables early detection of oligomer formation, characterization of the oligomeric population at different stages of the aggregation pathway, and correlation with different toxicity outcomes.

**EXPERIMENTAL PROCEDURES**

**C. elegans Strains and Maintenance**—Worms were maintained according to standard methods, at 20 °C on nematode growth media (NGM) with OP50 E. coli (50). The polyglutamine strains, expressing different lengths of CAG-repeats fused with YFP, have been described elsewhere: Q6 AM134 (rmiIs225[punc-54::q6::yfp]; X), Q24 AM138 (rmiIs130[punc-54::q24::yfp]); Q35 AM140 (rmiIs132[punc-54::q35::yfp]); Q37 AM470 (rmiIs225[punc-54::q37::yfp]); and Q40 AM141 (rmiIs133[punc-54::q40::yfp]). C. elegans Time Course Analysis and RNA Interference—RNA interference (RNAi) assays were performed as previously described (31). Age-synchronized L1 animals (day 1) were transferred onto NGM-OP50 bacteria plates and grown at 20 °C for different time periods (see Fig. 2). RNAi assays were performed from L1 using the commercial C. elegans RNAi library (Geneservice) (52, 53). 6-day-old animals were screened for aggregation using the stereomicroscope Leica MZ16FA equipped for epifluorescence (Leica Microsystems, Switzerland). Fluorescent microscopy images were taken using an Axiovert 200 microscope with a Hamamatsu digital camera C4742-98 (Carl Zeiss, Germany).

**Assay for Motility Defects**—Animals (6-day-old) grown on RNAi NGM plates at 20 °C were picked (20–25 animals) onto a NGM OP50-seeded plate equilibrated at 20 °C. Animal movements were digitally recorded using a Leica M205 FA microscope with a Hamamatsu digital camera C10600-10B (Orca-R2, Leica Microsystems, Switzerland), and the Hamamatsu Simple PCI Imaging software. Videos of 45 s were recorded at 2 × 2 binning and 5 frames per second, and captured frames were merged into .avi format and imported directly into ImageJ. Movie analysis was described previously (31), and the average speed of each animal was calculated by dividing the length of each track (corrected for animal body length) by the duration of the track (body length per second). The wrMTrck plugin and scripts for automated analysis are open-source and publicly available on the web. Videos were recorded for a minimum of 75 animals per experiment (n ≥ 3) and motility measurements are given as a percentage of wild type motility (% wt in empty vector L4440 RNAi).

**FRAP Analysis**—Animals were mounted on a 3% (w/v) agar pad on a glass slide and immobilized in 2 mM levamisole. Immobilized animals were subjected to FRAP analysis using the Zeiss LSM510 confocal microscope (Carl Zeiss), and the 63X objective lens at 5× zoom power, with the 514 nm line for excitation. An area of 0.623 μm² was bleached for 35 iterations at 100% transmission, after which time an image was collected every 123.35 ms. Relative fluorescence intensity (RFI) was determined by using RFI = (Tf/Cf)/(T0/C0); where T0 and C0 represent the intensity of the bleached and control unbleached regions, respectively, prior to bleaching, and Tt and Ct represent the intensity at time t after photobleaching for the bleached and unbleached region, respectively (54).

**FCS Sample Preparation and Data Collection**—Six-day-old animals (or at the time points indicated in Fig. 2D) grown on RNAi or OP50 bacteria were collected, washed with M9, and re-suspended in native-lysis buffer (50 mM Tris pH 7.4, 5 mM MgCl₂, 0.5% Triton X-100, 0.2 mM PMSF, 1 μg/ml leupeptin, protease inhibitor mixture tablet). Lysis was achieved with 4 cycles of freeze-thaw, followed by grinding with a motorized pestle. The lysates were spun in the Eppendorf 5417C micro-centrifuge and F45-30-11 rotor (Eppendorf, Germany) at 1,000 × g for 3 min, and total protein concentration was determined using the Bradford assay (Bio-Rad 500-0006). Centrifugation conditions were determined based on an approximation of the sedimentation coefficient for the large visible aggregates
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of 150,000 S, so that these aggregates were removed from the fraction analyzed by FCS. Protein lysates were aliquoted and only thawed once, to ensure minimal effects on oligomer stability. FCS measurements were collected on the Confocor3 system (Carl Zeiss) using the Apochromat 40 × 1.2 NA water immersion objective lens. Each sample was loaded into 3 wells of a Nunc 1 coverslip 8-chamber slide (Lab-Tek Chambered Coverglass w/CVR 155411, Nalge Nunc International) at a total protein concentration of 0.5 μg/300 μL. YFP was excited with the 514 nm laser line and emission signal was detected by the BP 530–610 IR filter. Each well was sampled for a total of 1500 s (either 5 × 300 s, or 50 × 30 s). Each set of measurements was performed twice and each experiment was done in duplicate (at least). This gives a minimum of 5 total hours of FCS data collection per sample, necessary to obtain a large statistical sampling of oligomers present in small relative concentrations.

**Autocorrelation Curves and Fitting**—Autocorrelation curves were generated by the Confocor3 software (Carl Zeiss) (55), and averaged over the different data collections to generate the final plot. In FCS, the autocorrelation function, \( G(\tau) \), measures the self-similarity of the fluorescent signal after a lag time (\( \tau \)) (40, 55, 56). Initially, attempts were made to fit the data using the autocorrelation function, \( G(\tau) \) (Equation 1), and fitting software provided by Zeiss. Where, \( N \) represents the total number of particles in the confocal volume at one time, \( b_i \) is the brightness of component \( i \), \( t/\tau \) is a structural parameter based on the dimensions of the confocal volume and \( \tau_DN \) is the diffusion time of component \( i \).

\[
G(\tau) = \frac{1}{N} \sum_{i=1}^{n} b_i \left( \frac{1}{1 + \frac{\tau}{\tau_DN}} \right) \left( \frac{1}{1 + \left( \frac{\tau}{\tau_DN} \right)^{t/\tau}} \right) \frac{1}{2} \quad (\text{Eq. 1})
\]

**Brightness Histograms**—To generate the brightness histograms we used a program written in Python. Briefly, raw FCS data is examined for local maxima greater than 150 kHz. All peaks are output for further statistical analysis, performed using the Graphpad Prism software (GraphPad Software). The \( \log_{10} \) of the brightness of the peak was taken, as there are log-fold differences in peak brightness. These values were then placed in bins of width 0.2 on the \( \log_{10} \) scale to generate the final brightness histogram. The x axis represents the bin center which relates to the \( \log_{10} \) (brightness), where brightness is photon counts per second. The y axis is the fraction of oligomers (out of 1), of a given brightness range. Statistical analysis and significance between values was determined using the Bonferroni t test (\( p < 0.05 \)).

**Purified Single Molecule FCS**—To determine the hydrodynamic size of the oligomers, data were analyzed using the purified single molecule FCS method, previously described (57). This technique uses single molecule fluorescence burst analysis. Specifically, bursts of fluorescence (signal greater than 150 kHz) are found along the time-scale over which the data were collected. The region to be analyzed is expanded around the burst by 10-fold the time-width of the burst on either side. By being restrictive in the data set analyzed, the method excludes other bursts within the region that would complicate analysis. Once the specific region has been extracted, the data can be fit with a derivation of Equation 1, yielding information on the diffusion rate of the bright species (57). Results from every burst are plotted in a diffusion rate histogram, allowing comparison of the distribution of diffusion rates for Qn-YFP in different backgrounds.

**RESULTS**

**Multiple Aggregation States Co-exist Across Diverse PolyQ Lengths and Ages**—Transgenic *C. elegans* lines expressing different polyQ-lengths (Q0 to Q40) fused to YFP, exhibit age-dependent aggregation (51) that was monitored by fluorescence microscopy and FRAP analysis. These methods provided visual and biophysical analyses at the cellular level in living animals that was complemented with single molecule FCS analysis of extracts from animals of different ages and polyQ-lengths. PolyQ aggregates are observed in Q35, Q37, and Q40 adult animals but not in Q0 and Q24 animals (Fig. 1A and Ref. 51). For Q35 animals, the appearance of visible aggregates is age-dependent, with the polyQ protein remaining diffuse by FRAP until day 4, and with the number of visible aggregates detected by fluorescence microscopy increasing during adulthood (Fig. 1, D and E and Ref. 51). The mobile polyQ protein detected by FRAP analysis in Q35 and Q37 animals was indistinguishable from mobile protein in Q0 and Q24 animals (Fig. 1, B and E). In contrast, only immobile aggregates are detected by FRAP analysis of Q40 animals (Fig. 1B). These observations demonstrate that the polyQ transgenic lines expressing different Q-lengths provide a useful tool to investigate the dynamics of protein aggregation.

**Characterization of Mobile PolyQ Species by FCS**—By FRAP analysis, the diffuse polyQ protein in Q35 and Q37 animals was indistinguishable from the protein expressed in Q24 animals, leading us to ask whether these diffuse species are indeed equivalent. To address this, we employed FCS, a small ensemble biophysical approach that would provide a quantitative measure of diffusion rate, brightness, and concentration of the diffuse polyQ species, *ex vivo*. Moreover, the single molecule sensitivity of FCS would reveal the distribution of the oligomeric species and would detect sub-populations of different species, rather than reporting solely on the average characteristics of the population that are measured by FRAP.

Analysis of the soluble fraction of extracts identified the presence of oligomeric species in Q35, Q37, and Q40 animals, but not in Q24 and Q0 animals (Fig. 1C). The Q0 (YFP) and Q24 autocorrelation (AC) curves can both be fit using the single component form of Equation 1 (data not shown), and the AC curves indicate rapid diffusion compared with longer Q-lengths (Fig. 1C). For the longer Q-lengths (Q35, Q37, and Q40), the AC curves shifted toward the right, corresponding to decreased diffusion rates due to the presence of slower moving oligomeric species (Fig. 1C). This result demonstrates that FCS can distinguish between monomers and oligomers, a finding that was not possible to obtain by FRAP analysis. Furthermore, this reveals that the FRAP fast recovery highly mobile proteins, are comprised of mixed populations of mobile oligomeric species, and that in Q35, Q37, and Q40 animals both mobile oligomeric species and immobile aggregates co-exist.
FCS analysis of extracts from Q35 animals at day 2 through day 6 of life shows that the AC curves shift from primarily monomeric species to oligomeric species (Fig. 1F). These ages represent the time during which Q35 changes from diffuse to immobile, as well as from larval to adult stages of life. The AC curves of Q35 animals at day 2 to 3.5 are similar to the Q0 curve, demonstrating that Q35 is largely monomeric in animals through day 3.5 of age (Fig. 1F). Extracts from day 4 adults, however, exhibited a shift in the AC curve to the right, indicative of the formation of oligomeric species (Fig. 1F). This also corresponds to the age in adulthood when visible aggregates are detected. Continuing through day 6, we observed an increase in the size and concentration of the Q35 oligomeric species, as shown by the further rightward shift of the curves, representing lower diffusion rates (Fig. 1F). This shift in diffusion rate could be due to an increase in the size of the species or to a conformational change that leads to an increase in the effective hydrodynamic radius of the existing species.

Efforts to extract additional information from the FCS experiments revealed that we could not fit the AC curves to a 1, 2, or 3 component AC function (Equation 1) for a majority of the samples due to large residuals, oscillations in the residual curve, and diffusion rate and concentration parameters that did not coincide with known values for the monomer fraction. A possible interpretation is that there is a heterogeneous distribution of both size and brightness in the polyQ-containing species. Therefore, we introduced two complementary analyses to provide more detailed information about the oligomer distribution: brightness histograms that correlate with the number of Qn-YFP molecules per oligomer; and purified single molecule FCS analysis that reveals the hydrodynamic size distribution of the oligomers. The highest signal-to-noise light bursts from each fluorescent species transiting through the focal volume are used to determine the brightness distribution of the oligomeric species (see “Experimental Procedures”). Likewise for purified single molecule FCS, these bursts were identified, and together with the surrounding data, fit using the AC function. The fitting results were binned to generate a histogram of the TauD (τD) distribution that relates to the hydrodynamic size of the oligomers (see “Experimental Procedures”).

Through the use of brightness analysis and purified single molecule FCS, we show that diffuse polyQ, in animals expressing increasing polyQ lengths or during aging, exhibited a heterogeneous state (Fig. 2 and 3). This was confirmed by the
absence of oligomers in the Q0 and Q24 animals, and by the detection of different oligomeric states in the higher polyQ lengths, as expected from the AC curves (Fig. 2). The histograms for Q35, Q37, and Q40 showed a similar heterogeneous distribution of oligomers, revealing the absence of discrete sub-populations of species (Fig. 2). In extracts from young Q35 animals, the burst histograms identify oligomers at days 2, 3, and 3.5, which were not detected by the AC curves (Fig. 3A). We observed a higher concentration of dim species at days 2 and 3 that decreased by day 4, and a corresponding increase in the frequency of brighter species (Fig. 3A). We did not directly relate the brightness to the number of Qn-YFP present in a given species as there is a non-linear relationship between brightness and the number of YFP molecules present in an oligomer. One proposed reason for this is that hetero-FRET occurs upon oligomerization and aggregation, resulting in a decreased fluorescence lifetime (58). Taken together, the brightness and \( r_{13} \) analysis reveal that while the oligomers increase in both size and brightness as the Q35 animals age, they still maintain a heterogeneous distribution from day 2 to day 6 consistent with the conclusion that specific oligomeric species do not accumulate and that the population as a whole shifts toward larger oligomers (Fig. 3).

Our approach of employing FRAP and FCS methods allowed the monitoring of multiple states of polyQ protein to demonstrate that populations of oligomeric species can co-exist with both polyQ aggregates and monomers. The age-dependent visualization of Q35 aggregates occurs at the same time as soluble oligomers of increased frequency and size are detected by brightness analysis. During aging and in animals expressing longer Q-lengths, the oligomer brightness distribution was unaltered consistent with an equilibrium of oligomeric protein states.

**Suppression of polyQ Aggregation Is Not Associated with Specific Alterations in Oligomeric State**—Having demonstrated the dynamic Q-length and age-dependent changes in oligomers and aggregation, we next asked whether these events were reversible. In other words, does suppression of polyQ aggregation by genetic modifiers of proteostasis affect the distribution of polyQ species? Specifically, would suppression of polyQ aggregation lead to the appearance of discrete oligomeric species or increased levels of heterogeneous species? To accomplish this, we took advantage of a genome-wide RNAi screen that identified genetic modifiers that suppressed Q35 and Q37 aggregation (31). From the Class A modifiers identified in these screens (31), we randomly selected 7 modifiers that represent multiple functional
classes, including energy and metabolism and protein folding (supplemental Table S1). We used these modifiers to ask whether distinct genetic suppressors of protein aggregation would alter the cellular environment, and therefore aggregation pathway, in the same or distinct manner.

Both fluorescence microscopy and FRAP analysis demonstrated that these modifiers suppressed the formation of large visible Q35 aggregates (Fig. 4A and supplemental Fig. S1A). Knockdown of phb-2 (mitochondrial prohibitin complex), col-61 (collagen-type protein), viln-1 (actin regulatory villin-related protein), klp-15 (kinesin-like protein), C34B2.8 (NADH-ubiquinone oxidoreductase, electron transport chain), C01G12.8 (CATP-4, Na+/K+ ATPase), and F59C6.5 (NADH-ubiquinone oxidoreductase) by feeding RNAi to Q35 animals at the L1 larval stage of development, when only diffuse Q35 protein was detected, resulted in suppression of visible aggregates (31, 51). RNAi-treated animals showed a significant reduction in Q35 foci number relative to control (Fig. 4A and supplemental Fig. S1A) (31, 51) and increased levels of diffuse Q35 (supplemental Fig. S1B). The fluorescence recovery curves indicated that the recovery rate of diffuse Q35 in RNAi-treated animals was similar to YFP alone and diffuse Q35 expressed in young animals, confirming suppression of visible aggregates by these genetic modifiers (Fig. 4A and supplemental Fig. S1B).

FCS analysis revealed heterogeneous distributions of oligomeric Q35 (Fig. 4B and supplemental Fig. S2A). While all the modifiers had the same effect on suppression of Q35 visible aggregates, we observed that the distribution of oligomers did not shift in the same direction, relative to the control curve (Fig. 4B). Some gene-modifiers led to the appearance of increased (C01G12.8) diffusion rates or decreased (phb-2) diffusion rates, while other modifiers (klp-15, viln-1, col-61, C34B2.8) had no effect on the AC curve (Fig. 4B and supplemental Fig. S2A). These Q35 AC curves contributed additional support for our earlier conclusions that polyQ protein that was diffuse by FRAP analysis reflects a mixture of monomers and higher molecular weight soluble species.
The brightness histograms indicate that each genetic modifier of aggregation caused the appearance of heterogeneous populations of Q35 oligomers (Fig. 4, C and D and supplemental Fig. S2B), as opposed to discrete oligomeric states. The resulting Q35 oligomer populations, however, are not identical. For example, phb-2 and F59C6.5 RNAi decreased the concentration of specific dim species in the bin center 2.2, whereas C01G12.8 RNAi increased the concentration of these species. Conversely, C01G12.8 RNAi decreased the concentration at bin center 3.2, whereas phb-2 and F59C6.5 increased the concentration of these species. We conclude from this analysis that different gene-modifiers do not have the same consequence on the Q35 species, even while suppressing the appearance of large immobile aggregates. Consistent with this, the majority of modifiers tested, while suppressing the formation of the large visible polyQ aggregates, maintained the polyQ oligomeric species distribution indistinguishable from the control Q35 (supplemental Fig. S2B).

Further support for these conclusions can be drawn from purified single molecule FCS where Q35 species with similar brightness distributions correspond to very different diffusion rates (Fig. 4D). For example, while both phb-2 and F59C6.5 RNAi generated Q35 oligomers with similar brightness distributions, the \( \tau_2 \) values for F59C6.5 were shifted to the left relative to phb-2. This suggests either tighter packing for F59C6.5 RNAi-generated Q35 oligomers or differences in the composition of phb-2-generated Q35-containing species. This type of comparison not only provides information regarding the molecular packing of oligomers and heterotypic interactions with other proteins, it also corroborates the finding that the RNAi-generated Q35 oligomers are heterogeneous. Taken together, the use of FCS based analyses has provided a new level of resolution for the oligomeric states adopted by polyQ in cellular environments in which large visible aggregates are suppressed. While some of these proteostasis network regulators had effects on the oligomer distribution, other regulators had no obvious effect.

**DISCUSSION**

Our use of a complement of imaging methodologies coupled with genetic tools provided important new insights into the aggregation pathway and allowed us to dissect the dynamics of aggregation. While FRAP provides information on mobile states and immobile aggregates, it is a population-based measurement of diffusion rates and does not resolve individual species. FCS can detect the presence of oligomer states, and the use of burst analysis and purified single molecule FCS provides a read-out of the oligomer distribution. Moreover, FCS-based burst analysis detected oligomeric states that could not be detected using traditional FCS methods. These methods, coupled with the ability to monitor changes in aggregation with age, and upon suppression of immobile aggregates provided new details about polyQ aggregation, including a heterogeneous oligomer distribution that reaches an equilibrium, and the ability to suppress immobile aggregates without changing the oligomer distribution.

The relationship between oligomeric states and cellular toxicity has been an area of interest, and whether particular species are associated with the suppression or enhancement of toxicity. Our results indicated an increase in the average size and brightness of oligomeric species at later ages and longer Q-lengths that correspond to enhanced motility-based toxicity. Therefore, we hypothesize that if an increase in oligomer size correlated with toxicity, then gene-modifiers that increased the size of oligomers should also increase toxicity, and conversely, those that decreased oligomer size should be linked with reduced toxicity. Expression of Q35 results in a 40% reduction in motility that can be restored to near wild-type (wt) motility by knock-down Q35 expression with sfp RNAi (supplemental Fig. S3 and Ref. 31). Of the aggregation suppressors examined in this study, we observed three distinct toxicity outcomes, decreased (klp-15 and C34B2.8), unaffected (col-61 and C01G12.8), and enhanced (phb-2, viln-1, and F59C6.5) motility defects (supplemental Fig. S3 and Ref. 31). As demonstrated earlier, C01G12.8 has the opposite effect on oligomer distribution than phb-2 and F59C6.5, however, this does not lead to the opposite effect on toxicity, as motility is unaffected upon knock-down of C01G12.8. Likewise, while viln-1 is in the same toxicity class as phb-2 and F59C6.5, it does not have similar changes in oligomer distribution. Finally, the oligomer distribution of the modifiers that decrease toxicity is indistinguishable from the control Q35 distribution. These results reveal that there is no simple relationship between soluble intermediate oligomers and toxicity based on size or brightness.

An underlying assumption has been that the toxicity associated with highly aggregation-prone proteins is due to a specific oligomeric species. The search for this species has converged upon “toxic oligomers” with similar size or conformational properties that are common to proteins of diverse sequence composition and function (60–70). Conformation specific antibodies have been generated that recognize both in vitro and in vivo formed oligomers for multiple proteins, including huntingtin, \( \alpha \)-synuclein, and A\( \beta \) (59–62), these antibodies have been shown to recognize oligomers that span multiple sizes (63). Our results reveal that the polyQ oligomers are maintained as a heterogeneous population, even when immobile aggregates are genetically prevented from forming. Moreover, the heterogeneous oligomer distribution is detected both in enhanced and suppressed toxicity environments, thus supporting a conformation rather than a size-based oligomer toxicity. Furthermore, it is important to note that FCS measurements are not able to distinguish between changes in size versus conformation, as changes in both can effect the time of diffusion. Therefore, within this diverse size-based population of oligomers could be discrete species of low concentration that contain specific conformations associated with toxicity. Support of this has been provided by in vitro polyQ studies (64, 65), which suggest changes in conformation of the monomer and higher order species. In the future, separating size from conformation-based changes, perhaps through use of lifetime measurements, may allow oligomer populations to be further distinguished including an association with toxicity.

These studies show that oligomers and immobile aggregates both appear in a time dependent manner, a result that is consistent with a nucleation-dependent reaction observed in vitro for many aggregation-prone proteins (14, 27, 66, 67), and the
appearance of regions comprised of oligomers and monomers that appear diffuse in vivo (68–70). While some in vitro studies have suggested a predominant oligomer species (30, 71, 72), others indicate the appearance of a heterogeneous distribution of oligomers (63, 73, 74). Furthermore, recently both simulations and models have also predicted a heterogeneity of oligomer and aggregate species (75, 76). Studies on Htt aggregation in cells have also described an oligomer population reaching an equilibrium, suggesting that oligomers are the rate-limiting event in inclusion body formation (73, 77). Our results indicate that, in both aged animals at the threshold length, Q35, and for Q37 and Q40, an equilibrium is reached that maintains oligomers in a specific populational distribution, perhaps through interactions with cellular factors and quality control machinery, such as chaperones and clearance machines, and by the continual regeneration of monomers. Flanking sequences may also affect the aggregation pathway. In these experiments, the flanking sequence is YFP, which may stabilize the polyQ similar to the demonstrated role of GST in vitro (78), and may alter the aggregation pathway. Moreover, through use of an RNAi approach that identified genetic modifiers that suppressed the appearance of immobile aggregates, we have demonstrated that their formation is not dependent upon a shift in oligomer distribution. This suggests that these genetic suppressors could encode proteins that prevent nucleation or enhance the clearance of nucleated species and that there are multiple ways to affect proteostasis.

The use of genetic modifiers to alter the cellular environment of polyQ aggregation supports the often-contradictory observations regarding the effect of enhancement or suppression of aggregation on oligomers and toxicity (19, 79–84). Our studies, on the age-dependent change in oligomer distribution, suggest an orderly aggregation process as there is a gradual increase in size as the animals age. If this hypothesis is correct, then the aggregation suppressors should have the reciprocal effect of aging. The results with suppressors of aggregation however, suggest that the aggregation pathway is not a simple or linear process, but can be driven in multiple directions depending on the cellular environment. This suggests that there may be two or more orthogonal aggregation paths, the end-product of one such path may be large aggregates, while others may contribute to a specific oligomer conformation. Altering the cellular environment with genetic modifiers that suppress the formation of immobile aggregates resulted in a heterogeneous population of polyQ oligomers, however with different oligomeric sizes and brightness distribution. Whereas one class of genetic modifiers suppressed aggregation without affecting the distribution of oligomers, the other class of modifiers led to either increased or decreased oligomer size and brightness. The class of modifiers that affect the oligomer distribution, may cause a change in flux between the orthogonal pathways upon suppression of large aggregates, leading to substantial changes in the other pathways. The lack of correlation between suppression of visible aggregates and the appearance of specific populations of oligomers reveals an imperfect relationship between oligomerization and aggregation. Consistent with this, suppression of aggregation does not appear to be a simple reversal of age-related effects, but rather results in multiple distributions. These observations support our contention that the proteostasis network can employ diverse mechanisms to prevent or reverse the formation of immobile aggregate species.

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