Structural Mechanisms of Mutant Huntingtin Aggregation Suppression by the Synthetic Chaperonin-like CCT5 Complex Explained by Cryoelectron Tomography*§

Huntington disease, a neurodegenerative disorder characterized by functional deficits and loss of striatal neurons, is linked to an expanded and unstable CAG trinucleotide repeat in the huntingtin gene (HTT). This DNA sequence translates to a polyglutamine repeat in the protein product, leading to mutant huntingtin (mHTT) protein aggregation. The aggregation of mHTT is inhibited in vitro and in vivo by the TCP-1 ring complex (TRiC) chaperonin. Recently, a novel complex composed of a single type of TRiC subunit has been reported to inhibit mHTT aggregation. Specifically, the purified CCT5 homo-oligomer complex, when compared with TRiC, has a similar structure, ATP use, and substrate refolding activity, and, importantly, it also inhibits mHTT aggregation. Using an aggregation suppression assay and cryoelectron tomography coupled with a novel computational classification method, we uncover the interactions between the synthetic CCT5 complex (~1 MDa) and aggregates of mutant huntingtin exon 1 containing 46 glutamines (mHTTQ46-Ex1). We find that, in a similar fashion to TRiC, synthetic CCT5 complex caps mHTT fibrils at their tips and encapsulates mHTT oligomers, providing a structural description of the inhibition of mHTTQ46-Ex1 by CCT5 complex and a shared mechanism of mHTT inhibition between TRiC chaperonin and the CCT5 complex: cap and contain.

Background: Huntington disease patients show an accumulation of oligomers and fibrillar species of mutant huntingtin (mHTT).

Results: Cryoelectron tomography and subvolume averaging visualizes heterogeneous mHTT oligomeric species inside the chaperonin-like CCT5 cavity.

Conclusion: The structural basis of mHTT aggregation inhibition by CCT5 is through capping of fibrils and encapsulation of oligomers.

Significance: These structural mechanisms inspire the development of new strategies for inhibiting mHTT aggregation.

Huntington disease (HD) is an autosomal dominant disease that demonstrates genetic anticipation (1) and affects about 5–7 in every 100,000 people (1, 2). Symptoms of HD include movement abnormalities, cognitive deficits, and psychiatric disorders (2). HD is caused by the expansion of a CAG triplet repeat in the huntingtin gene, resulting in an expanded polyglutamine tract in the amino-terminal domain of the protein product, which then has a propensity to misfold and aggregate (1, 2). The huntingtin protein in unaffected individuals can have a polyglutamine tract with up to 35 repeats, whereas the disease manifests when the expansion reaches 40 or more repeats (1). The age of onset of symptoms is related to the number of glutamines, with longer stretches leading to earlier onset (2, 3). The disease is progressive and invariably results in death ~15–20 years after diagnosis (1, 2).

The wild-type huntingtin protein is a 348-kDa, largely cytoplasmic protein that is ubiquitously expressed but only found at high levels in the central nervous system and the testes (3). Aggregates of mutant huntingtin (mHTT) protein have been identified in mouse models and found in patient brains (4, 5). These aggregates contain fragments of mHTT protein, including those composed of only the first exon of Htt, the region where the polyglutamine repeat is located (6). Recent studies show that these highly toxic exon 1 fragments are created through aberrant splicing of mHTT mRNA rather than proteolytic cleavage of the full-length mHTT protein (7, 8), making the study of exon 1 protein fragments increasingly important. In cell models, large insoluble aggregates have been found as part of inclusion bodies, whereas smaller, soluble aggregates are ubiquitous throughout the cell (3). Preventing the accumulation and aggregation of mHTT protein could relieve the disease.

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3 The abbreviations used are: HD, Huntington disease; mHTT, mutant huntingtin; TRiC, TCP-1 ring complex; TRX, thioredoxin; cryoEM, cryoelectron microscopy.
burden of the cell, potentially allowing the clearance of these misfolded proteins (6, 9–11).

Molecular chaperones suppress neurodegeneration in several animal models of protein misfolding diseases, including HD (12–15). Specifically, TCP-1 ring complex (TRiC) chaperonin has been shown previously to decrease mHTT aggregation in vitro, in yeast cells, and in cell culture (16, 17). TRiC (~1 MDa) is a back-to-back double-ring complex composed of eight distinct subunits per ring (CCT1–8) that interacts with ~9–15% of all nascent peptides, including actin and tubulin (18, 19). Each chaperonin subunit is a single polypeptide conceptually divided into three domains, each with different functions. The equatorial domain contains the site of ATP-binding and subunit-subunit contacts, the intermediate domain acts as a hinge region, and, finally, the apical domain recognizes and binds substrates (20). By cryoelectron tomography, TRiC has been shown to interact at its apical domains with mHTTQ51-Ex1 fibrils and encapsulate oligomers through a “cap and contain” mechanism (16).

TRiC chaperonin, because of its inhibition of mHTT aggregation, is an intriguing potential candidate for the treatment of Huntington disease and has spawned multiple TRiC-inspired reagents. A previous genome-wide RNAi screen in Caenorhabditis elegans identified six of the eight subunits of TRiC chaperonin (CCTs 1, 2, and 4–7) as suppressors of polyglutamine aggregation (21), whereas a co-overexpression assay in yeast identified the bovine TRiC subunits CCT1 and CCT4 as inhibitors of mHTT aggregation (17). In the same vein, the exogenously applied bovine recombinant purified CCT1 apical domain has been shown to penetrate cell membranes and decrease mHTT-Ex1 aggregation in vivo (22). Furthermore, recently, the purified human CCT5 subunit has been shown to form a homo-oligomeric complex and suppress mHTT aggregation in vitro (23). Interestingly, the CCT5 complex forms a double-ring barrel structure composed of 16 identical CCT5 subunits (23). This chaperonin-like homo-oligomeric complex hydrolyzes ATP and refolds both luciferase and human γ-crystallin (23). In short, the CCT5 complex looks and acts similarly to TRiC chaperonin. In addition, it has been reported recently that the CCT5 complex inhibits mHTT aggregation (24), leading us to question whether the CCT5 complex also utilizes the cap and contain structural mechanisms to inhibit mHTT aggregation.

Experimental Procedures

CCT5 Complex Purification—The human CCT5 construct was isolated as described previously (23), with a few modifications (24). Briefly, BL21(DE3)RIL Escherichia coli cells expressing human CCT5 with a His tag were lysed via French press. The lysate was centrifuged, and the supernatant was removed by pipetting, passed through a 0.45-μm filter, and loaded onto a cobalt-nitrilotriacetic acid column (Pierce). Fractions containing the CCT5 subunit were combined, and the His tag was removed using tobacco etch virus protease before the protein was again applied to the cobalt-nitrilotriacetic acid column, to which it no longer bound. Lastly, the fractions containing the CCT5 protein were combined and passed over a Superose 6 10/300 GL (GE Healthcare) size exclusion column.

CCT5 elutes as a homo-oligomeric, double-ring, barrel-shaped complex of ~1 MDa. Previous studies confirm that this TRiC-like complex has two rings with eight subunits per ring (23).

mHTTQ46-Ex1 Construct Purification—Huntingtin exon 1 with 46 glutamines fusion protein with a thioredoxin (TRX) tag at the N terminus and a His tag at the C terminus (25) was expressed as described previously (26). Purified mHTTQ46-Ex1-His (25 μM) was incubated alone or with the purified CCT5 complex (0.8 μM) at a ratio of two mHTTQ46-Ex1-His monomers to one CCT5 complex. Aggregation was initiated by addition of EkMax to cleave the TRX tag away from the mHTTQ46-Ex1 protein fragment (14 kDa). Samples were incubated at 30 °C, and aliquots of this mixture were taken at the 0, 6, and 24 h time points for filter trap and cryoEM analysis. These time points were chosen after optimization over a larger time range (0–48 h) with finer time intervals to both represent the full process of insoluble aggregate formation without the CCT5 complex present and also when the CCT5 complex was present to ensure visualization of the interaction between mHTT and the CCT5 complex.

Filter Retardation Analysis—Filter retardation was performed as described previously (24), originally on the basis of the work of Tam et al. (17). Briefly, aggregation of mHTTQ46-Ex1 was stopped at various time points by equal volume addition of 4% SDS and boiling for 10 min, followed by filtering through a 0.22-μm cellulose acetate membrane (GE Healthcare). The membrane was washed and blocked using 5% milk in TBS. MW8 antibody was used as a primary antibody, and an alkaline phosphatase-conjugated secondary antibody was used (Millipore), followed by membrane visualization using the alkaline phosphatase substrate kit (Bio-Rad). Quantification was performed using ImageJ (27).

Cryoelectron Microscopy and Tomography—Aggregation of mHTTQ46-Ex1 was stopped at various time points by cryofixation through plunge freezing (28). Quantifoil holey carbon (R 2/2 or R 2/1) copper grids were ethanol-cleaned and pretreated with 10-nm gold-BSA fiducials and plasma-cleaned or glow-discharged for 10 s before the sample was added and plunge-frozen.

Two-dimensional electron micrographs of mHTTQ46-Ex1 at various time points, with and without CCT5, were taken at various magnifications, depending on the size of aggregates, on a JEM2100 electron microscope at a dose of ~20 electrons/Å². To better display some of the images of very thick aggregates, we applied a tanh high-pass filter at 60 Å and then normalized to and subtracted from the original image to remove high-frequency noise.

Tilt series were collected using SerialEM (29) from ±55° with a step size of 5° on a JEM2200FS electron microscope with an in-column energy filter on a Gatan US4000 charge-coupled device camera (model 895) at ×25,000 magnification (4.52 Å/pixel) with a nominal defocus of 5 μm and a cumulative dose of ~70 electrons/Å². Tilt series were reconstructed using IMOD (30). Annotation of mHTTQ46-Ex1 fibrils and CCT5 complexes in a reconstructed three-dimensional tomogram was performed using Avizo (31) and a Wacom tablet. Subvol-
ume averaging of freestanding CCT5 complexes was performed using the single-particle tomography suite of programs in EMAN2 (32, 33).

Single-particle Tomography Processing Protocol—Single-particle tomography was performed on freestanding CCT5 subvolumes (n = 3201) incubated in the presence of mHTTQ46-Ex1 for various times (0, 8, and 12 h). These time points were chosen to ensure both a mostly unoccupied population and a mixed population of unoccupied and occupied CCT5 complexes. Ten different tomograms from various time points were used to box out subvolumes (723 pixels) in three dimensions using the IMOD and EMAN2 software packages. Mm-Cpn (EMDB-5140), a 16-mer, double-ring barrel structure of an archaeal chaperonin was phase-randomized and low-passed to 45 Å before use in the cross-correlation-based classification of experimental or control CCT5 subvolumes. This classification method created a continuum of subvolumes sorted on the basis of their similarity to the unoccupied Mm-Cpn barrel. The efficiency of sorting was assessed by mean density distribution analysis of the normalized, masked subvolumes (see “Results”). This indicated the necessity of a second sorting step to specifically identify occupied CCT5 complexes.

To create a model that would accurately sort on the basis of changes related to the presence of density inside the cavity of the CCT5 complex, we first removed the worst correlated 20% particles and then further processed the best correlated 20% (unoccupied) and the worst correlated 20% (occupied) from the Mm-Cpn barrel, sorting in the following way. A subset of subvolumes (n = 100) of each 20% group was aligned and averaged using an all versus all alignment methodology (34) with a restricted search space that allowed full rotation about the z axis, 180° flipping about the x axis, and up to 8 pixels of translational movement in x, y, and z. These parameters were chosen to reduce computational expense while still allowing the localization of any internal substrates. Additionally, the subvolumes were already coarsely aligned to the Mm-Cpn barrel from the original sorting, reducing the orientation space that needed to be searched. Both of these averages were then low-passed to 40 Å and D8-symmetrized to enhance contrast before being used as models for iterative refinement against either the top or bottom 20% datasets (n ~ 600), respectively, using the same alignment parameters as described above, with the addition of a restriction to generate the final averages using the best correlated half (n ~ 300). The final averages were low-passed to 40 Å.

These averages were used to create a difference map emphasizing the features of the occupied CCT5 complex (Fig. 1). The unoccupied final average was subtracted from the occupied final average. This model was low-passed to 40 Å, and then D8 symmetry was applied. The difference map was then used to recategorize the entire 3201-subvolume freestanding CCT5 complex dataset. As described above, the 20% groups

FIGURE 1. The methodology used to classify freestanding CCT5 complexes into unoccupied and occupied classes. A two-stage classification scheme was used to sort the entire population of CCT5 complexes into either unoccupied or occupied classes. The first stage of classification sorted specifically for unoccupied CCT5 complexes using Mm-Cpn as an unoccupied model, whereas the second stage of classification specifically sorted for occupied CCT5 complexes using a difference map generated during the first stage (yellow, positive density; purple, negative density). The gray boxes mark the final averages presented in Fig. 10, A and B.
were chosen and processed further using the same all versus all alignment strategy on a subset \( n = 100 \) of subvolumes, followed by iterative refinement using all of the particles against the model. The final occupied average was low-passed to 40 Å.

Control CCT5 Complex Processing—The CCT5 complex incubated without mHTTQ46-Ex1 acted as a control. Subvolumes were boxed out from two tomograms \( n = 1196 \). These subvolumes were processed exactly as described in the experimental protocol above, with 20% groups \( n = 240 \), using MmCpn as the initial classifier for unoccupied CCT5 complexes and the experimentally determined difference map as the secondary classifier for occupied CCT5 complexes. It is important to note that, in the case of the control, no or very few CCT5 complexes were expected to be occupied with native substrates and, when specifically sought for, led to a distorted, but still unoccupied, CCT5 structure.

Oligomeric Species Mass Calculations—After normalizing the final averages to a common scale, the internal cavity was separated for measurement by spherical masking. The appropriate cavity diameter (90 Å) was determined on the basis of the inclusion of as much density as possible in the occupied average and as little density as possible in the unoccupied average. Chimera (35) was used to measure the volume of the densities shown in both the masked unoccupied and occupied final averages at a more stringent (2×) threshold corresponding to the highest density found inside the cavity.

Results

The process of huntingtin aggregation, whereby monomers of mHTT exon 1 protein fragments assemble into higher-order structures, has been observed using many different techniques (36–38). Using a cleavable linker on a construct containing mHTT with 46 polyglutamines (mHTTQ46-Ex1) allowed us to follow the process of mHTT aggregation over time in the absence and presence of the CCT5 complex. The concentrations of mHTT and time points for these assays were chosen to balance the amount of insoluble aggregates both in the absence and presence of the CCT5 complex given the quick aggregation of mHTT and the strong inhibition of aggregation of the CCT5 complex.

![FIGURE 2. CCT5 suppresses the aggregation of mutant huntingtin over time.](image)

**A.** mutant huntingtin with or without the CCT5 complex was assayed at the 0, 6, or 24 h time points using a filter retardation assay with MW8 antibody and an alkaline phosphatase kit for visualization. **B.** this assay was repeated \( n = 3 \) and quantified (error bars show mean ± S.E.). mHTT aggregation was suppressed significantly by the CCT5 homo-oligomer complex at both the 6 and 24 h time points. *, \( p < 0.001 \).

![FIGURE 3. Representative cryoEM images describing the process of aggregation with and without the CCT5 complex.](image)

**A.** cryoelectron microscopy images of samples frozen at the 0, 6, and 24 h time points, both with and without the CCT5 complex starkly demonstrate the suppression of mHTT aggregation by the CCT5 complex. The inset at the 0-h time point with CCT5 shows a higher-magnification image with CCT5 complexes marked by arrows. The insets at the 24-h time point with and without the CCT5 complex are high pass-filtered, higher-magnification images of the edges of the large, electron-dense aggregate, showing tips of fibrils protruding from the main body of the aggregate. When the CCT5 complex is present (bottom row), these large aggregates are harder to find and generally smaller. **B.** at 6 h without the CCT5 complex, in addition to the prevalent small bundles of fibrillar aggregates shown in A, individual fibrils (left panel) and large bundles of fibrillar aggregates (right panel) can also be seen, with less frequency. Although we are able to synchronize the initiation of fibrillar growth, the aggregation process is not uniform, and many species of mHTT fibrils are found within the same time point. Scale bars = 300 nm.
The presence of substoichiometric levels of the synthetic CCT5 complex had a major impact on the amount of insoluble mHTTQ46-Ex1 aggregates, as seen in the filter retardation assay (Fig. 2). The inhibition of aggregation by CCT5 was significant \( p < 0.001 \) as early as 6 h post-aggregation initiation and continued to be so out to 24 h post-aggregation initiation, with \( \sim 9 \) - and 7-fold differences between CCT5 complex presence and absence at 6 and 24 h, respectively. The impact of CCT5 was confirmed through two-dimensional cryoEM (Fig. 3A). Although far fewer fibrils were observed in the presence of CCT5, it is important to note that fibrillar aggregates vary in size even within the same time point. The cryoEM images shown in Fig. 3A represent the size predominantly seen at each time point, whereas Fig. 3B shows examples of the various sizes present at just the 6-h time point in the absence of CCT5. Overall, aggregates were consistently larger and easier to find on the EM grid without CCT5 present, suggesting that more aggregates were present and consistent with the results from the filter retardation assay.

**FIGURE 4.** An example of the CCT5 complex interacting with mHTTQ46-Ex1 fibrils at their tips, potentially interfering with fibril growth through a steric capping mechanism. A, two-dimensional projection through a three-dimensional tomogram demonstrating a small aggregate of fibrils and many CCT5 complexes from the 24-h time point. The very dark, round objects are 10-nm gold particles. B, the same small aggregate of fibrils has been annotated in gold, CCT5 complexes in proximity to the fibrils in blue, and those freestanding (away from the fibrils) in red. C, a higher-magnification view of a single slice of the three-dimensional volume showing the densities as delineated into the three categories (fibrils and bound and freestanding CCT5). D–F, higher-magnification views of portions of the annotation demonstrating the interaction between the CCT5 complex and fibril tips.
Manual annotation of tomographic EM data showing a small bundle of mHTTQ46-Ex1 fibrils formed in the presence of CCT5 revealed two types of CCT5 complexes: those in close proximity (blue) to the mHTT fibril aggregate and those free-standing (red) away from the mHTT fibril aggregate (Fig. 4, supplemental Movie 1). The locations of the CCT5 complexes in close proximity to the fibrils indicate that they interact at the fibril tips. The heterogeneity of the system under study is a major hurdle for post-tomographic averaging of either the CCT5 complexes attached to fibril tips or of free-standing CCT5 complexes. Not only are there multiple proteins present (mHTT and CCT5 complexes), but the ways in which these two proteins interact with each other and, in the case of mHTT, with themselves add additional layers of complexity. Because of these challenges, no averaging of CCT5 complex attached to fibril tips was attempted. We also anticipated that the free-standing CCT5 might have different amounts of mHTT inside the cavity. Therefore, we undertook the more tractable task of deriving two average maps of the free-standing CCT5 corresponding to either unoccupied or highly occupied states. To this end, we developed a novel, two-step classification system to accurately sort the free-standing CCT5 complex population into unoccupied and occupied complexes (Fig. 1). Because we are limiting ourselves to a binary morphological question, “is the cavity occupied or unoccupied?”, the resolution necessary to provide the answer is relatively low compared with many other cryoelectron microscopy studies.

The first stage in the classification of the free-standing CCT5 complex was on the basis of the correlation of each subvolume with an unoccupied 16-mer barrel-like template, the homo-oligomeric archaeal Mm-Cpn chaperonin (Fig. 1). This classification step created a continuum of correlation scores corresponding to subvolumes sorted according to their similarity to the unoccupied Mm-Cpn barrel. The top members of the sorted list are those that are best correlated with the unoccupied Mm-Cpn barrel, and the bottom members of the sorted list are the worst correlated with unoccupied Mm-Cpn barrel. The main difference between the top and bottom of the list is the absence or presence of density inside the barrel cavity (Fig. 5). Efficiency of sorting was assessed by breaking the sorted list into quintiles and calculating the mean density distribution of each particle within the top or bottom quintile after either the first classification stage using Mm-Cpn as a model (Fig. 1) or the second classification stage using the difference map as a model (Fig. 1). Both model-based classifications created sorted cross-correlation-based continuums of subvolumes that are most like (first quintile) or least like (fifth quintile) the respective model. Boxes are 72 pixels (33 nm) in width.

FIGURE 5. Projections of CCT5 complex averages by quintile for each classification step. Two-dimensional projections of the average of each quintile of particles (n ~600) after sorting with either the Mm-Cpn empty barrel model or the difference map model (Fig. 1). Both model-based classifications created sorted cross-correlation-based continuums of subvolumes that are most like (first quintile) or least like (fifth quintile) the respective model. Boxes are 72 pixels (33 nm) in width.

FIGURE 6. Assessment of sorting efficiency. A and B, mean density distribution analysis of each particle within either the top or bottom percentage of the whole dataset after either the first classification stage using Mm-Cpn as a model (A) or the second classification stage using the difference map as a model (B). Groups expected to be occupied are labeled in green, whereas groups expected to be unoccupied are labeled in purple. Even when considering only a small fraction of the total dataset (10%), it is clear that the first classification step does not adequately sort the CCT5 complexes into both unoccupied and occupied classes. However, after the second stage of classification, both the 10% and 20% groups show good separation. To maximize the number of subvolumes included in the analysis while still maintaining a good sort, 20% was used as the cutoff for particle inclusion at both stages of sorting.
into various group sizes: top and bottom 10%, 20%, 30%, 40%, and 50%. Mean density distribution analysis of the normalized, masked subvolumes belonging to each group was performed (Fig. 6A). The mean density distribution for a population of occupied CCT5 complexes would be expected to be higher in density than that of unoccupied CCT5 complexes. This expectation is fulfilled overall because the top of the sorted list is less dense than the bottom of the list on average. However, the bottom group is clearly poorly sorted at each percentage, with many subvolumes falling in line with the top, unoccupied CCT5 complex peak. This indicated the necessity of a subsequent sorting step to specifically identify occupied CCT5 complexes.

To better sort for the occupied CCT5 complexes, the 20% populations that were best and worst correlated to Mm-Cpn were independently processed using an all versus all alignment strategy to create an initial model for each that was then iteratively refined to create a final average (See Fig. 1 and “Experimental Procedures” for details). These two final averages were then used to create a difference map that emphasized the features of the occupied CCT5 complexes and was used in a second classification step to re-sort all of the subvolumes on the basis of correlation to this new model. This again generated a continuum of correlation scores (Fig. 5).

Our second stage of sorting was to use the difference map as a template to classify the entire dataset. In this sorting, the best correlation scores were associated with the positive difference densities (the occupied particles), and the worst scores were associated with the negative difference densities. The efficiency of this second sorting step was again assessed using mean density distribution analysis for particle subsets of various sizes (Fig. 6B). After this sorting, the occupied CCT5 complex population is better defined regardless of the size of the subset taken, with much less overlap between the unoccupied and occupied groups (Fig. 6B). The 20% group size was chosen for further processing to maximize particle numbers while minimizing overlap between groups. Again, an all versus all alignment strategy was used to create an initial model for each group, followed by iterative refinement of each respective dataset to create two more final averages (Fig. 1).

Two-stage classification of the same dataset creates a situation where a single subvolume might fall into both the unoccupied (from the first classification stage) and occupied (from the second classification stage) final averages (Fig. 1, shaded boxes). We would expect a low overlap between these two groups because of the two different sorting strategies used to create them, and we find only 1% overlap, corresponding to just 7 subvolumes (Fig. 7). These results, in conjunction with the mean density distribution analysis (Fig. 6), indicate that our two-stage classification method has accurately sorted the population into essentially pure groups of occupied or unoccupied CCT5 complexes.

An additional worry with cryoelectron tomography data is the missing wedge artifact that arises from the limited range of data collection. The effect of the missing wedge is an elongation of the reconstructed tomograms along the z axis, which appears as a literal wedge of missing information in Fourier space. This artifact can be effectively overcome by subvolume averaging because it combines information from many different subvolumes, corresponding to particles in random orientations throughout the tomogram. Neither the unoccupied nor occupied CCT5 complex final averages show characteristics of the missing wedge artifact in their Fourier space representations (Fig. 8).

Of the four final averages that have been created and validated using our two-stage sorting strategy, the one that best represents the unoccupied CCT5 complex is the one that was created by specifically sorting for unoccupied complexes, i.e. the final average that was produced with the subvolumes that are best correlated to Mm-Cpn (Fig. 9A). Likewise, the final average that best represents the occupied CCT5 complex is the one that was created using the subvolumes that are best correlated to the difference map because it emphasizes the features of the occupied CCT5 complex (Fig. 9B). These averages have no symmetry imposed and are free from model bias because of the all versus all alignment strategy used to create initial models from the raw subvolumes.

As a control, CCT5 complex without mHTTQ46-Ex1 was imaged and processed as above. The same unoccupied barrel-like template (Mm-Cpn chaperonin) and difference map were used as classifiers to search the control dataset for both unoccupied and occupied CCT5 complex populations. In both cases, the final control CCT5 complex averages were unoccupied (Fig. 9, C and D), indicating that the density found under the experimental conditions for which mHTT was present is due to mHTT rather than potential bias in processing.

The unoccupied and occupied CCT5 complex final averages show clear morphological differences, including the presence of density in both chambers of the CCT5 complex in the occupied final average (Fig. 9, A and B). This is in striking contrast to the control dataset that shows unoccupied cavities even when the processing protocol is sorting specifically for density inside the cavity, leading to a distorted CCT5 complex (Fig. 9, C and D). On the basis of our results from processing both the experimental and control datasets, we conclude that the density inside the occupied CCT5 complex is due to the encapsulation of a heterogeneous mixture of mHTTQ46-Ex1 monomeric or oligomeric species. The TRX tag and EkMax present in the reaction are not likely to account for the density found inside some of the freestanding CCT5 complexes. EkMax is present at a very low concentration in the final reaction (1:500 dilution from 1 unit), while the TRX tag is small in size (~12 kDa) and does not oligomerize (39). Additionally, because of improvements in the CCT5 complex purification protocol (24) and the

![FIGURE 7. Subvolume overlap by classification](image-url)
lack of density in the control CCT5 complex population, even when searched specifically for occupied CCT5 complexes (Fig. 9D), it is unlikely that the density found in the experimentally occupied CCT5 complex is due to native proteins present during purification.

When the contrast is enhanced in the two-dimensional projection of the occupied CCT5 complex average, it becomes clear that the averaged density inside the cavity takes a specific shape (Fig. 10A). Applying a more stringent threshold to the three-dimensional average reveals that the density in the cavity takes the shape of a bridge across the equatorial region of the complex (Fig. 10B). At this threshold, the bridge density measures ~40 Å in width and spans the full-length of the internal cavity (~100 Å). The volume-to-mass conversion factor (1.21 Å³/Da (40)) was then used to calculate the mass of the density included in the masked unoccupied (4.9 kDa) and occupied (131 kDa) cavities at this threshold. To account for the mass because of the CCT5 complex included in the mask, the occupied mass measurement was subtracted from the occupied mass measurement, giving a total of 126 kDa of mass in the occupied CCT5 complex cavity. This mass corresponds to nine monomers of the mHTTQ46-Ex1 construct used here. We interpret this to mean that the internal density is a heterogeneous mixture of monomers and oligomers, with an average size corresponding to a 9-mer oligomeric species.

Discussion
This study follows in the footsteps of previous experiments describing the structural role of TRIC chaperonin inhibition of mHTT aggregation (16). However, in this study, we present a more rigorous data classification scheme (Fig. 1) to demon-
strate the encapsulation of the mHTT oligomer inside the CCT5 complex cavity. Here a similar but different construct for mHTT is used to provide additional structural details of the process of fibril formation and aggregation. The construct used in this study contains 46 rather than 51 polyglutamines, putting it squarely in the range of polyglutamine repeats found in HD patients (41). In addition, the 46Q construct used here has N17 and C38 flanking regions with a C-terminal His tag, whereas the previous construct was composed of N17 and C36 flanking regions with no C-terminal tag (16, 25). Both of these constructs represent the newly discovered aberrant splice variants (7, 8), which have been described previously as highly toxic (42). Confirming the previous fibril formation and aggregation observations with a new mHTT construct further solidifies these findings. Moreover, the synthetic chaperonin-like CCT5 complex, which has been shown recently to also inhibit mHTT aggregation (24), was structurally characterized to provide information on its mechanism of interaction with mHTT. As shown previously, it is possible to overexpress the CCT5 subunit in bacteria, producing the CCT5 complex (23, 24). It is intriguing to raise the question of whether CCT5, as a homooligomer complex, is native to mammalian cells, which awaits future investigation.

Here we report that the CCT5 complex acts on both forms of mHTT species observed in Huntington disease (6, 10, 11), namely fibrils and oligomers. Specifically, the CCT5 complex interacts via two complementary mechanisms with mHTT. It caps mHTT fibril tips and encapsulates oligomers, suggesting physical blockage of mHTT fibril growth and sequestration of mHTT oligomers as mechanisms of aggregation inhibition. Interestingly, the CCT5 complex both suppressing mHTT aggregation and capping mHTT fibril tips implies that one mechanism of fibril growth is through the addition of mHTT monomers or oligomers at the tips of fibrils. Capping fibril tips and encapsulating oligomers are the same mechanisms by which TRiC was shown to interact with mHTT (16), indicating a shared mode of action: cap and contain. These behaviors contrast with previously proposed models for the action of chaperones in suppressing aggregating misfolded proteins, which tend to focus on the binding of monomeric forms of the misfolded protein (43, 44). Although it is possible that some of the density within the occupied cavity average is due to the presence of monomeric or smaller oligomeric forms of mHTT, the highest density may represent the form of a large oligomer spanning the internal chamber of the CCT5 complex, corresponding to a 9-mer species of mHTT. In addition to the size, the shape of the density inside the cavity of the CCT5 complex and the multiple interaction sites between this density and the CCT5 complex indicate that the predominant species of mHTT encapsulated by the CCT5 complex is oligomeric. Although the resolution and sample size of this study are not high enough to distinguish between the multiple heterogenous forms of mHTT oligomer that are encapsulated by the CCT5 complex, the presence of the high density indicates that it is representative of a large portion of the oligomeric population found within the CCT5 complex cavity. Additionally, this species of oligomer is larger than expected on the basis of previous research of productive encapsulation and folding of substrates (45). However, it is worth noting that, in this case, both the chaperonin and substrate are atypical and, therefore, not appropriate as models for normal chaperonin function to assist protein folding. In fact, our results suggest that the CCT5 complex is acting to suppress aggregation through sequestration of mHTT rather than productive refolding. This interaction represents a simplified system of substrate recognition on the basis of a single subunit of TRiC chaperonin and provides an intriguing possibility of exploring either the full-length or a small domain of CCT5 as a reagent to suppress mHTT aggregation.

Cellular aggregates of mHTT protein have been found in inclusion bodies and large tangles of amyloidogenic fibrils (9, 43, 44, 46, 47). It is unclear whether these aggregations are a part of the progression of the disease itself or a cellular mechanism to suppress the progression of the disease. This is due, in part, to the uncertainty surrounding the question of what the toxic species is. Multiple groups have reported soluble oligomers of mHTT as the toxic species (6, 10, 11). Additionally, fibrillar inclusion bodies also contain other important cellular proteins, leading to dysregulation of cellular transcription (9, 46, 47). In both cases, targeting the oligomers or the fibrils would potentially ameliorate the disease burden of the cell. As seen in Fig. 2, CCT5 does not arrest aggregation but does significantly decrease the rate of aggregation and the size of aggregates. Because most patients develop HD late in life, this slowing may translate to a delay in the age of onset sufficient to allow the disease to bypass a large number of potential patients. The results presented here and reported previously for apiCCT1 (22) highlight the idea that one single subunit or a specific peptide component of TRiC is sufficient for the suppression of huntingtin aggregation. Additionally, a subunit of CCT5 or a specific peptide derived from CCT5 are appropriate sizes for packaging into an adeno-associated virus vector capable of treating the brain. Coupled with the above findings, this may suggest the option of exploring the use of CCT5 or a fragment of CCT5 as a reagent to prevent mHTT aggregation, which may contribute to the development of HD pathology.
Structural Basis of mHTT Aggregation Inhibition by CCT5

Author Contributions—W. C. J. A. K., R. L., M. C. D., and O. A. S. designed the study. O. A. S. and J. M. I. purified proteins and, with M. C. D., performed and analyzed the experiments shown in Figs. 2 and 3. M. C. D. performed manual annotation of the data shown in Fig. 4. M. C. D., in consultation with M. F. S. and J. G.-M., designed the processing protocol described in Fig. 1, producing the results shown in Figs. 5–10. M. C. D. and O. A. S. wrote the paper, and all authors reviewed, edited, and approved the final version of the manuscript.

References
1. Bates, G. P. (2005) History of genetic disease: the molecular genetics of Huntington disease: a history. Nat. Rev. Genet. 6, 766–773
2. Walker, F. O. (2007) Huntington’s disease. Lancet 369, 218–228
3. Wetzel, R. (2012) Physical chemistry of polyglutamine: intriguing tales of a monotonous sequence. J. Mol. Biol. 421, 466–490
4. Davies, S. W., Turmaine, M., Cozens, B. A., DiFiligia, M., Sharp, A. H., Ross, C. A., Scherzinger, E., Wanke, E. M., Mangiarini, L., and Bates, G. P. (1997) Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. Cell 90, 537–548
5. DiFiligia, M., Sapp, E., Chase, K. O., Davies, S. W., Bates, G. P., Vonsattel, J. P., and Aronin, N. (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 277, 1990–1993
6. Hatters, D. M. (2012) Putting huntingtin “aggregation” in view with windows into the cellular milieu. Curr. Top. Med. Chem. 12, 2611–2622
7. Gipson, T. A., Neuerer, A., Wexler, N. S., Bates, G. P., and Housman, D. (2013) Aberrantly spliced HTT, a new player in Huntington’s disease pathology. RNA Biol. 10, 1647–1652
8. Sathasivam, K., Neuerer, A., Gipson, T. A., Landles, C., Benjamin, A. C., Bondulich, M. K., Smith, D. L., Faull, R. L., Roos, R. A., Howland, D., Deltoff, P. J., Housman, D. E., and Bates, G. P. (2013) Aberrant splicing of HTT generates the pathogenic exon 1 protein in Huntington disease. Proc. Natl. Acad. Sci. U.S.A. 110, 2366–2370
9. Clabough, E. B. (2013) Huntington’s disease: the past, present, and future search for disease modifiers. Yale J. Biol. Med. 86, 217–233
10. Margulis, B. A., Vigont, V., Lazarev, V. F., Kanzacheeva, E. V., and Guzhova, I. V. (2013) Pharmacological protein targets in polyglutamine diseases: Mutant polypeptides and their interactors. FEBS Lett. 587, 1997–2007
11. Van der Putten, H., and Lotz, G. P. (2013) Opportunities and challenges for molecular chaperone modulation to treat protein-conformational brain diseases. Neurother. 10, 416–428
12. Behrends, C., Langer, C. A., Boteva, R., Böttcher, U. M., Tempst, P., and Hartl, F. U. (1992) Function in protein folding of TRiC, a cytosolic ring complex containing TCP-1 and structurally related subunits. EMBO J. 11, 4767–4778
13. Thulasiraman, V., Yang, C. F., and Frydman, J. (1999) In vivo newly translated polypeptides are sequestered in a protected folding environment. EMBO J. 18, 85–95
14. Braig, K., Otwinowski, Z., Hegde, R., Boisvert, D. C., Joachimiak, A., Horwich, A. L., and Sigler, P. B. (1994) The crystal structure of the bacterial chaperonin GroEL at 2.8 A. Nature 371, 578–586
15. Yang, W., Dunlap, J. R., Andrews, R. B., and Wetzel, R. (2002) Aggregated polyglutamine peptides delivered to nuclei are toxic to mammalian cells. Hum. Mol. Genet. 11, 2905–2917
16. Sontag, E. M., Joachimiak, L. A., Tan, Z., Tomlinson, A., Housman, D. E., Glabe, C. G., Potkin, S. G., Frydman, J., and Thompson, L. M. (2013) Exogenous delivery of chaperonin subunit fragment ApoCCT5 modulates mutant huntingtin cellular phenotypes. Proc. Natl. Acad. Sci. U.S.A. 110, 3077–3082
17. Sergeyeva, O. A., Chen, B., Haase-Pettingell, C., Ludkte, S. J., Chiu, W., and King, J. A. (2013) Human CCT4 and CCT5 chaperonin subunits expressed in Escherichia coli form biologically active homo-oligomers. J. Biol. Chem. 288, 17734–17744
18. Sergeyeva, O. A., Tran, M. T., Haase-Pettingell, C., and King, I. A. (2014) Biochemical characterization of mutants in chaperonin proteins CCT4 and CCT5 Associated with hereditary sensory neuropathy. J. Biol. Chem. 289, 27470–27480
19. Bennett, M. J., Huey-Tubman, K. E., Herr, A. B., West, A. P., Jr., Ross, S. A., and Bjorkman, P. J. (2002) A linear lattice model for polyglutamine in CAG-expansion diseases. Proc. Natl. Acad. Sci. U.S.A. 99, 11634–11639
20. Fodale, V., Kegulian, N. C., Verani, M., Caroli, A., Azzollini, L., Petricca, L., Daldin, M., Boggio, R., Padova, A., Kuhn, R., Pacifici, R., Macdonald, D., Schoenfeld, R. C., Park, H., Isas, J. M., Langen, R., Weiss, A., and Caricasole, A. (2014) Polyglutamine- and temperature-dependent conformational rigidity in mutant huntingtin revealed by immunoassays and circular dichroism spectroscopy. PloS ONE 9, e112262
21. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to Image: 25 years of image analysis. Nat. Methods 9, 671–675
22. Lepault, J., Boyo, F. P., and Dubochet, J. (1983) Electron microscopy of frozen biological suspensions. J. Microsc. 129, 89–102
23. Mastronarde, D. N. (2005) Automated electron microscope tomography using robust prediction of specimen movements. J. Struct. Biol. 152, 36–51
24. Kremer, J. R., Mastronarde, D. N., and McIntosh, J. R. (1996) Computer visualization of three-dimensional image data using IMOD. J. Struct. Biol. 116, 71–76
25. Pruggnaller, S., Mayr, M., and Frangakis, A. S. (2008) A visualization and segmentation toolbox for electron microscopy. J. Struct. Biol. 164, 161–165
26. Tang, G., Peng, L., Baldwin, P. R., Mann, D. S., Jiang, W., Rees, I., and Ludkte, S. J. (2007) EMAN2: An extensible image processing suite for electron microscopy. J. Struct. Biol. 157, 38–46
27. Galaz-Montoya, J. G., Flanagan, J., Schmid, M. F., and Ludkte, S. J. (2013) Single particle tomography in EMAN2. J. Struct. Biol. 190, 279–290
28. Schmid, M. F., and Booth, C. R. (2008) Methods for aligning and for averaging 3D volumes with missing data. J. Struct. Biol. 161, 243–248
29. Goddard, T. D., Huang, C. C., and Ferrin, T. E. (2007) Visualizing density maps with UCSF Chimera. J. Struct. Biol. 157, 281–287
30. Bhardwaj, V., Panicker, M. M., and Udgaonkar, J. B. (2014) Fluorescence anisotropy uncovers changes in protein packing with inclusion growth in a cellular model of polyglutamine aggregation. Biochemistry 53, 3621–3636
31. Caron, N. S., Hung, C. L., Atwal, R. S., and Truant, R. (2014) Live cell imaging and biophotonic methods reveal two types of mutant huntingtin inclusions. Hum. Mol. Genet. 23, 2324–2338
32. Legleiter, J., Mitchell, E., Lotz, G. P., Sapp, E., Ng, C., DiFiligia, M., Thompson, L. M., and Muchowski, P. J. (2010) Mutant huntingtin fragments form oligomers in a polyglutamine length-dependent manner in vitro and in vivo. J. Biol. Chem. 285, 14777–14790
39. Gronenborn, A. M., Clore, G. M., Louis, J. M., and Wingfield, P. T. (1999) Is human thioredoxin monomeric or dimeric? Protein Sci. 8, 426–429
40. Harpaz, Y., Gerstein, M., and Chothia, C. (1994) Volume changes on protein folding. Struct. 2, 641–649
41. Myers, R. H. (2004) Huntington’s disease genetics. NeuroRX 1, 255–262
42. Barbaro, B. A., Lukacsovich, T., Agrawal, N., Burke, J., Bornemann, D. J., Purcell, J. M., Worthge, S. A., Caricasole, A., Weiss, A., Song, W., Morozova, O. A., Colby, D. W., and Marsh, J. L. (2015) Comparative study of naturally occurring huntingtin fragments in Drosophila points to exon 1 as the most pathogenic species in Huntington’s disease. Hum. Mol. Genet. 24, 913–925
43. Hendrick, J. P., and Hartl, F. U. (1995) The role of molecular chaperones in protein folding. FASEB J. 9, 1559–1569
44. Young, J. C., Agashe, V. R., Siegers, K., and Hartl, F. U. (2004) Pathways of chaperone-mediated protein folding in the cytosol. Nat. Rev. Mol. Cell Biol. 5, 781–791
45. Rüßmann, F., Stemp, M. J., Mönkemeyer, L., Ettchells, S. A., Bracher, A., and Hartl, F. U. (2012) Folding of large multidomain proteins by partial encapsulation in the chaperonin TRiC/CCT. Proc. Natl. Acad. Sci. 109, 21208–21215
46. Arrasate, M., and Finkbeiner, S. (2012) Protein aggregates in Huntington’s disease. Exp. Neurol. 238, 1–11
47. Arrasate, M., Mitra, S., Schweitzer, E. S., Segal, M. R., and Finkbeiner, S. (2004) Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. Nature 431, 805–810