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## ***In Situ* Structure of Neuronal C9ORF72 Poly-GA Aggregates Reveals Proteasome Recruitment**

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### **Summary**

Protein aggregation and dysfunction of the ubiquitin-proteasome system are hallmarks of many neurodegenerative diseases. Here we address the elusive link between these phenomena employing cryo-electron tomography to dissect the molecular architecture of protein aggregates within intact neurons at high resolution. We focus on the poly-Gly-Ala (GA) aggregates resulting from aberrant translation of an expanded GGGGCC repeat in *C9orf72*, the most common genetic cause of amyotrophic lateral sclerosis and frontotemporal dementia. We find that poly-GA aggregates consist of densely packed twisted ribbons that recruit numerous 26S proteasome complexes, while other macromolecules are largely excluded. Proximity to poly-GA ribbons stabilizes a transient substrate-processing conformation of the 26S proteasome, suggesting stalled degradation. Thus,

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#### Author Contributions

Q. G. performed electron microscopy experiments, computationally analyzed the data and prepared figures. C. L., H. H. and M. S. H. performed cell culture and biochemistry experiments. A. M.-S. developed software procedures for data analysis. T. R. constructed structural models. F. B. contributed to the computational analysis of the data. M. P.-B. and F. F. contributed reagents. Q. G., M. S. H., F.-U. H., D. E., W. B. and R. F.-B. designed research. D. E. and R. F.-B. supervised experiments. Q. G., M. S. H., F.-U. H., D. E., W. B. and R. F.-B. wrote the manuscript.

#### Declaration of Interests

D. E. has a pending patent application on “Dipeptide-repeat proteins as therapeutic target in neurodegenerative diseases with hexanucleotide repeat expansion”.

poly-GA aggregates may compromise neuronal proteostasis by driving the accumulation and functional impairment of a large fraction of cellular proteasomes.

## Keywords

ALS; FTD; dipeptide-repeat proteins; UPS; cryo-electron microscopy; cryo-EM; cryo-ET; cryo-FIB

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## Introduction

The ubiquitin-proteasome system (UPS) is the main cellular pathway for targeted protein degradation (Collins and Goldberg, 2017; Hershko et al., 2000). UPS alterations have been implicated in many human diseases, including multiple neurodegenerative disorders (Dantuma and Bott, 2014; Hipp et al., 2014; Schmidt and Finley, 2014). In particular, frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) have been associated with mutations in UPS components (Deng et al., 2011; Johnson et al., 2010; Watts et al., 2004) and altered UPS function (Cheroni et al., 2009; Tashiro et al., 2012). However, the contribution of UPS dysfunction to neurodegeneration and its underlying mechanisms are not yet well understood.

UPS impairment has also been linked to *C9orf72* mutations, the most common genetic cause of ALS/FTD (Edbauer and Haass, 2016; Freibaum and Taylor, 2017; Gendron and Petrucelli, 2017; Lin et al., 2017). A massive expansion of a GGGGCC (G<sub>4</sub>C<sub>2</sub>) repeat in a non-coding region of the *C9orf72* gene to up to several thousand copies is found in 10–50% of familial ALS/FTD cases and in 5–7% of patients with sporadic disease (DeJesus-Hernandez et al., 2011; Majounie et al., 2012; Renton et al., 2011; van der Zee et al., 2013). Three non-mutually exclusive mechanisms have been suggested to mediate the toxicity of the G<sub>4</sub>C<sub>2</sub> repeat expansion: 1) loss of native function of the *C9orf72* protein due to reduced transcription of the mutant allele, 2) aberrant RNA interactions, and 3) production of toxic translation products and aggregates via repeat-associated non-ATG (RAN) translation (Zu et al., 2011).

Although the G<sub>4</sub>C<sub>2</sub> repeat is found in a non-coding region of the *C9orf72* gene, sense and anti-sense transcripts are unconventionally translated in all reading frames into five dipeptide-repeat proteins (Ash et al., 2013; Gendron et al., 2013; Mori et al., 2013a; Mori et al., 2013b; Zu et al., 2013): poly-GA, poly-GR, poly-GP, poly-PR and poly-PA. While all five proteins form TDP-43-negative, p62-positive inclusions in ALS/FTD patient brain, the vast majority of these aggregates contains poly-GA (Mackenzie et al., 2015; Mori et al., 2013b; Zhang et al., 2014).

Poly-GA expression leads to toxicity in heterologous cells, primary neuron cultures and mice (Jovicic et al., 2015; May et al., 2014; Schludi et al., 2017; Yamakawa et al., 2015; Zhang et al., 2016; Zhang et al., 2014). Similar to other toxic aggregating proteins (Olzscha et al., 2011; Park et al., 2013), poly-GA aggregates sequester critical cellular factors including Unc119 and multiple UPS components (May et al., 2014; Zhang et al., 2016). UPS impairment is critically involved in poly-GA-mediated toxicity (Yamakawa et al., 2015;

Zhang et al., 2016; Zhang et al., 2014), but our understanding of the underlying mechanisms remains incomplete. This is aggravated by the limited structural information currently available on poly-GA aggregates, especially within an unperturbed cellular context.

Here we address these challenges using state-of-the-art cryo-electron tomography (cryo-ET) technologies, which allow 3D imaging of the cell interior in close-to-native conditions and at molecular resolution (Beck and Baumeister, 2016). We reveal the structure and cellular interactions of poly-GA aggregates within intact neurons to an unprecedented level of detail. Interestingly, we find that poly-GA aggregates consist of densely packed twisted ribbons that recruit large numbers of 26S proteasome complexes. Structural analysis of these proteasome complexes by subtomogram averaging and classification into functional states provides mechanistic insights into proteasomal dysfunction in *C9orf72* ALS/FTD.

## Results

### Poly-GA Aggregates Contain Densely Packed Twisted Ribbons

To study neuronal poly-GA aggregates without interference from *C9orf72* loss-of-function and RNA-mediated toxicity, we transduced primary rat neuronal cultures with a GFP-tagged codon-modified synthetic construct expressing (GA)<sub>175</sub>-GFP and containing an ATG start codon (May et al., 2014). We have previously shown that lentiviral poly-GA expression results in inclusions of similar size and poly-GA intensity as in *C9orf72* patient tissue (May et al., 2014). Neurons were transduced at day *in vitro* (DIV) 5 and allowed to express the protein for another 5 days (DIV 5 + 5). The cultures were then vitrified and subsequently imaged by cryo-light microscopy to locate cellular poly-GA inclusions (Figure S1A). Correlative microscopy allowed the production of 100–200 nm-thick lamellas at the location of these aggregates using cryo-focused ion beam milling (Bauerlein et al., 2017; Rigort et al., 2012) (Figure S1B–D). Lastly, the samples were transferred to a cryo-transmission electron microscope for high resolution 3D imaging by cryo-ET (Figure S1E, F).

Poly-GA aggregate cross-sections were typically ~3 μm in diameter, and consisted of a dense network of elongated polymorphic ribbons (Figure 1A, B). Whereas the thickness of the ribbons was well defined (13 – 15 nm), their length (100 nm – 1 μm) and width (20 – 80 nm) varied considerably (Figure 1D top). Our measurements likely underestimate ribbon length, as parts of ribbons oriented perpendicular to the electron beam were not reliably detected due to missing information along this direction (Lucic et al., 2005). The ribbons were twisted along their axis with a variable helical pitch, and often bifurcated and/or associated laterally with neighboring ribbons (Figure 1D bottom). This polymorphism contrasts with the uniform fibrils forming polyQ-expanded huntingtin exon 1 aggregates in mammalian cells (Bauerlein et al., 2017). Poly-GA ribbons were also more densely packed than polyQ fibrils, which occupied a lower fraction of the inclusion volume (poly-GA, 10 %; polyQ, 4 %). Thus, different amyloids adopt different morphologies *in situ*.

However, similarly to GFP-tagged polyQ fibrils, GFP-labeled poly-GA ribbons were decorated by additional densities (Figure 1E). To investigate the nature of these densities, neurons were co-transduced with untagged poly-GA and tagRFP-p62, which co-localizes with poly-GA aggregates (May et al., 2014; Mori et al., 2013b; Yamakawa et al., 2015;

Zhang et al., 2014) and allows targeting untagged poly-GA by correlative microscopy. As for polyQ fibrils (Bauerlein et al., 2017), the decorating densities were absent from untagged poly-GA ribbons (Figure 1F), demonstrating that these additional densities require GFP for their formation and that the ribbons consisted indeed of poly-GA aggregates. Thus, poly-GA forms amyloid-like ribbons in neurons.

### 26S Proteasomes Are Recruited to Poly-GA Aggregates

Unlike polyQ fibrils (Bauerlein et al., 2017), poly-GA ribbons did not visibly interact with cellular endomembranes. However, both the aggregate interior and periphery were densely populated by macromolecular complexes (Figure 1A, C, E, F). Ribosomes were abundant around poly-GA aggregates but largely absent from their interior. In contrast, the space between poly-GA ribbons was densely populated with macromolecules that appeared as ~10 nm rings in tomographic cross-sections. Larger (~20 nm) cross-sectioned rings were also found at the aggregate periphery and occasionally in the interior. To investigate the identity of these macromolecules, we performed unbiased subtomogram averaging (Figure S2A). A small set of particles were hand-picked from the tomogram, aligned and averaged. The resulting average was used as a template to search the tomogram for additional occurrences of the same structure. These additional particles were then visually inspected, aligned, classified and averaged again to produce a higher resolution average. The iterative application of this procedure yielded an average structure unequivocally corresponding to the 26S proteasome (Chen et al., 2016a; Huang et al., 2016; Schweitzer et al., 2016; Wehmer et al., 2017) for the smaller ring-like structures, and to the TRiC/CCT chaperonin (Leitner et al., 2012; Zang et al., 2016) for the larger ones (Figure 2, Figure 3, Figure S3A–C, Movie S1). Other large UPS components such as p97/VCP did not appear abundant at poly-GA aggregates.

The abundance of TRiC/CCT complexes was not significantly different around poly-GA aggregates compared to the cell body of control neurons (untransduced or transduced with GFP only). However, the estimated concentration of proteasomes within the aggregate (~7  $\mu\text{M}$ ) was approximately 30-fold higher than in the cell body (Figure S4B) or the processes (Asano et al., 2015) of control cells. Given that poly-GA expression did not increase overall proteasome expression levels (Figure S4C), these data suggest that proteasomes are removed from other regions of the cell to accumulate at poly-GA aggregates. This is consistent with immunofluorescence staining (Figure S4A) and biochemical fractionation experiments showing reduced levels of Triton-soluble neuronal proteasomes (Figure S4C). Furthermore, our tomograms showed that 26S proteasomes accumulated almost exclusively within the aggregate interior (Figure 2, Figure S5A–D). Taken together, these results show that a substantial fraction of neuronal 26S proteasomes is sequestered into poly-GA aggregates.

To test the influence of the poly-GA expression level on proteasome recruitment, we analyzed aggregates formed in neurons at an earlier time point after transduction (DIV 5 + 3). Although these aggregates were smaller, they contained a similar concentration of proteasomes in their interior (Figure S5E). Importantly, analogous observations were made for poly-GA aggregates generated from a RAN-translated  $(\text{G}_4\text{C}_2)_{73}$  construct, which more closely mimics the *C9orf72* patient situation (Figure S4E, Figure S5F). Thus, poly-GA

aggregate morphology and proteasome recruitment were comparable in all the experimental conditions tested.

### Poly-GA Aggregation Alters Proteasome Structure

The 26S proteasome consists of a barrel-shaped 20S core particle (CP) that harbors the catalytic activity, bound to one or two 19S regulatory particles (RP). Regulatory particles are responsible for substrate recognition, unfolding and translocation into the CP for proteolysis. Recent single particle cryo-EM studies have revealed how RP conformational dynamics are coupled to the functional cycle of the 26S complex (Chen et al., 2016a; Unverdorben et al., 2014; Wehmer et al., 2017). Initial binding of substrates to the 26S proteasome presumably occurs in a low energy ground state (s1) (see also Lu et al., 2015). Bound substrates are committed for degradation (s2 state), and then translocated into the CP (s3-s4 states). In the s4 state the gate of the 20S CP is open, allowing the substrates to access the proteolytic chamber. We took advantage of the large number of proteasome complexes recruited to poly-GA aggregates to investigate their functional states *in situ* by subtomogram averaging and classification (Figure S2B).

We first sorted 26S proteasomes according to the number of regulatory particles (one or two) bound per CP. Previous structural (Asano et al., 2015) and biochemical (Tai et al., 2010) data indicated that in control neurons the large majority of 26S proteasomes contain only one RP (single-capped 26S). In striking contrast, 76% of poly-GA-associated 26S proteasomes were double-capped (Figure S3D, F). Therefore, the labile interaction between the proteasome core and regulatory particles (Kleijnen et al., 2007) is apparently stabilized within poly-GA aggregates.

We further classified 26S proteasomes according to RP conformation (Asano et al., 2015; Unverdorben et al., 2014). This yielded four well-defined classes, two of which (GS1, GS2) were consistent with the RP ground state conformation (s1), whereas the other two (SPS1, SPS2) corresponded to substrate processing states (s2-s4) (Figure 3). Interestingly, 37% of all 26S proteasomes belonged to substrate processing classes, almost twice the number than in control neurons (Asano et al., 2015) (Figure S3E, G). Thus, poly-GA aggregates recruit a large number of 26S proteasome regulatory particles, a substantial fraction of which adopts substrate processing conformations.

The relatively high resolution of the classes (11.8–15.4 Å, Figure 3, Figure S3H) enabled us to assign each class to a functional state. To this end we employed molecular dynamics flexible fitting (MDFF; Trabuco et al., 2009) initiated through the atomic models of the s1–s4 states of the yeast 26S proteasome (Figure 3C) (Wehmer et al., 2017). The s1 state was clearly the best fit for the GS1 and GS2 classes (Table S1). The yeast s1 structure fitted GS1 (49% of the total number of particles) without large discrepancies except for the position of the Rpn1 subunit, which in GS1 was similar to that observed in the human 26S proteasome (Chen et al., 2016a; Huang et al., 2016; Schweitzer et al., 2016). Also, in agreement with these studies, no prominent density was visible in our data for the Rpn13 subunit. The GS2 class (13.7% of particles) was overall similar to GS1, but the Rpn1 subunit pivoted 25° on its N-terminal region to shift its C-terminus towards the CP with respect to GS1 (Figure 4A–C). This is a novel conformation of Rpn1, a particularly dynamic subunit (Asano et al., 2015;

Huang et al., 2016; Schweitzer et al., 2016; Wehmer et al., 2017) that serves as binding hub for 26S regulatory cofactors containing ubiquitin-like (UBL) domains (Elsasser et al., 2002; Leggett et al., 2002) and was recently identified as an ubiquitin receptor (Shi et al., 2016).

SPS1 proteasomes (14.2% of particles) were most similar to the substrate commitment state s2 (Table S1). In contrast, the SPS2 class (23.1% of particles) was best fitted by the s4 model of actively translocating proteasomes (Table S1). This is remarkable because, *in vitro*, the s4 state was only recently detected in proteasomes incubated with non-hydrolysable nucleotide analogs (Wehmer et al., 2017), suggesting that it is normally a highly transient conformation. Thus, the interaction of poly-GA aggregates with the proteasome appears to stall its s4 conformation.

Both substrate processing classes showed prominent additional densities in the substrate binding region of the proteasome, in contact with the ATPase ring (Figure 3). In SPS1 the density was well defined (Figure 3), indicating a relatively stable interaction with the 26S complex that may in part correspond to bound UBL domain proteins (Aufderheide et al., 2015; Bashore et al., 2015). The density contacted Rpn1 (Figure 3, Figure 4D, E), consistent with the bound UBL domains of Rad23 or the ubiquitin C-terminal hydrolase 6 (Ubp6)/USP14 (Chen et al., 2016b; Shi et al., 2016), and extended to another binding site of Rad23 at the Rpn10 subunit (Hiyama et al., 1999; Mueller and Feigon, 2003; Walters et al., 2002) (Figure 4D, F). Interestingly, similar to our SPS1 class, Ubp6-bound proteasomes have been shown to mainly adopt the s2 conformation (Aufderheide et al., 2015). Because the Rpn1 and Rpn10 UBL binding sites also interact with ubiquitin, proteasome-bound ubiquitinated substrates may also contribute to the extra density. Consistent with this notion, the estimated molecular mass of the density (~ 70 kDa) was larger than Ubp6 (56 kDa) or Rad23 (40/44 kDa for Rad23A/B). For the SPS2 class, the additional density contacted the Rpn10 subunit but not Rpn1 (Figure 4G–I). The density was overall less well defined than for SPS1, indicating a more dynamic interaction, perhaps involving a more extensive participation of substrates (see below). Therefore, both substrates and cofactors may contribute to the additional densities found on substrate processing proteasomes.

### Direct Interactions with Poly-GA Aggregates Impair Proteasome Function

To address the physiological role of the different proteasome conformations observed, we investigated their cellular distribution by mapping the particles back into the tomograms (Figure 5). We found that proteasome conformation correlated with the distance to poly-GA ribbons (Figure 5B, C, E,  $p < 0.001$ , Chi-square test,  $N = 6080$  regulatory particles from 4 tomograms). For proteasomes directly touching (Figure 5D) or very close to ribbons the SPS2 class was overrepresented (36% vs 23% of the total proteasomes; Figure 5E), whereas the fraction of GS1 proteasomes was smaller than within the total (40% vs 49%; Figure 5E). The fraction of GS1 proteasomes increased with their distance to ribbons, whereas SPS2 proteasomes followed the opposite trend. For SPS2 proteasomes associated with poly-GA ribbons, the contact interface was consistent with the location of the additional density observed in this class (Figure 3, Figure 4G–I). Only small variations were found in the fractions of GS2 and SPS1 particles with respect to the distance to poly-GA ribbons (Figure



5E). These results indicate that association with poly-GA aggregates modifies the functional state of the 26S proteasome.

In agreement with this notion, functional measurements show that poly-GA expression impairs proteasome function (Figure S4D) (Yamakawa et al., 2015; Zhang et al., 2014). Interestingly, GA-rich sequences have been reported to slow or even stall proteasomal substrate processing in the context of the Epstein-Barr virus-encoded nuclear antigen 1 protein (Hoyt et al., 2006; Kraut, 2013; Levitskaya et al., 1997). Whereas *in vitro* the s4 state was only observed in the presence of non-hydrolysable nucleotide analogs (Wehmer et al., 2017), 23% of all proteasomes within neuronal poly-GA aggregates, and 36% of the proteasomes located in the immediate vicinity of poly-GA ribbons adopted the s4 conformation *in situ* (SPS2 class). Therefore, our results suggest that proteasomal degradation is slowed down by poly-GA-mediated stalling of the otherwise highly transient s4 state. This may play an important role in the proteostasis impairment observed in poly-GA models.

## Discussion

Previous studies using classical EM reported that cellular poly-GA inclusions consist of a network of filaments 15–17 nm in diameter (Zhang et al., 2016; Zhang et al., 2014). Our 3D imaging of unstained fully hydrated neurons shows that rather than filaments, poly-GA forms twisted ribbons similar to those observed *in vitro* for (GA)<sub>15</sub> (Chang et al., 2016). Whereas most amyloids are believed to be largely unbranched (Knowles et al., 2014), poly-GA ribbons bifurcated extensively. Together with their variable width, this suggests that *in situ* poly-GA ribbons are formed by different numbers of laterally stacked protofilaments. Furthermore, the similar morphology of (GA)<sub>15</sub> (Chang et al., 2016) and (GA)<sub>175</sub> (this study) ribbons is consistent with a molecular arrangement in which stacked GA-repeats give rise to the long axis of the protofilament.

Poly-GA aggregates recruited striking numbers of 26S proteasomes, whereas other macromolecules were excluded from the aggregate interior. This is remarkably different from our recent observations on polyQ inclusions, which interact with and may disrupt the membranes of the endoplasmic reticulum and other organelles, but do not harbor substantial numbers of 26S proteasomes or other large macromolecules (Bauerlein et al., 2017). This difference is surprising as proteasomes were also reported to colocalize with polyQ aggregates (Bennett et al., 2005; Waelter et al., 2001). Thus, different aggregating proteins may trigger UPS dysfunction and cellular toxicity by distinct mechanisms, and how proteasomes associate with other disease-related aggregates remains to be elucidated (Deriziotis et al., 2011; Myeku et al., 2016). Future work should also address the extent of proteasome recruitment by aggregates of poly-GA proteins expressed at endogenous levels, as well as by other *C9orf72* dipeptide-repeat proteins.

UPS impairment is known to play an important role in poly-GA induced toxicity (Yamakawa et al., 2015; Zhang et al., 2016; Zhang et al., 2014). The proteasome and other UPS components are major poly-GA interactors in neurons (May et al., 2014), and poly-GA expression leads to reduced proteasome activity (Yamakawa et al., 2015; Zhang et al., 2014

and our results). Our data provides mechanistic insights into these phenomena. First, poly-GA aggregates sequester a large fraction of cellular proteasomes, depleting them from other cellular functions critical for proteostasis maintenance, such as ER-associated degradation (Zhang et al., 2014). This may be particularly damaging to neurons given their extended morphology. Second, many of the poly-GA associated proteasomes may be functionally impaired. The fraction of double-capped proteasomes and proteasome RPs in substrate processing conformations was much higher within poly-GA aggregates than in control cells. Given that CP/RP interactions are stabilized during substrate degradation (Kleijnen et al., 2007), these data suggest that many poly-GA-associated proteasomes are processing substrates or stalled in the process. Consistently, our analysis shows that contact with poly-GA ribbons causes the proteasomes to adopt the so-called s4 conformation, an otherwise highly transient intermediate state of substrate translocation (Wehmer et al., 2017). This is in line with previous reports that GA-rich sequences slow proteasomal translocation or even stall it (Kraut, 2013; Levitskaya et al., 1997). Interestingly, despite the strong association between proteasomes and poly-GA aggregates observed here, inhibiting proteasomal degradation does not affect poly-GA levels (Yamakawa et al., 2015). Therefore, the recruitment of proteasomes to poly-GA aggregates may be unproductive and may not lead to poly-GA degradation. The mechanisms driving such recruitment, which may involve ubiquitination of poly-GA and/or of associated factors, require further investigation.

Our data may also provide insights into the cellular mechanisms of proteasome regulation in the presence of protein aggregates. Poly-GA-associated proteasomes in substrate processing states showed additional densities that may correspond to bound ubiquitin and/or UBL domain-containing cofactors such as the deubiquitinating enzyme Ubp6/USP14 or the substrate shuttle factor Rad23 (Aufderheide et al., 2015; Bashore et al., 2015; Chen et al., 2016b; Shi et al., 2016). Although the binding of these factors to poly-GA-associated proteasomes remains to be conclusively demonstrated, several UBL domain proteins (Rad23, ubiquilin2 or Bag6) were highly enriched in the poly-GA interactome (May et al., 2014), and Rad23 is an important regulator of poly-GA induced toxicity (Zhang et al., 2016). UBL domain proteins strongly modulate proteasome activity (Finley et al., 2016) and have recently been implicated in neurodegeneration and aggregate clearance (Deng et al., 2011; Hjerpe et al., 2016). Our results are consistent with the notion that UBL domain cofactors regulate the interactions of the proteasome with protein aggregates.

## STAR Methods

### Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Wolfgang Baumeister (baumeist@biochem.mpg.de).

### Experimental Model and Subject Details

**Cell Culture**—HEK293 cells (female) stably expressing UbG76V-GFP (Dantuma et al., 2000; De Smet et al., 2017) were cultured in Dulbecco's modified Eagle's medium (DMEM; Biochrom) supplemented with 10 % (v/v) fetal bovine serum (Gibco), 2 mM L-glutamine



(Gibco), penicillin-streptomycin (Thermo Fisher) and non-essential amino acids (Gibco). Transfection was carried out using FuGENE 6 (Promega).

Rats (IGS background, Charles River; RRID:RGD\_734476) were housed in a pathogen-free facility with 12:12 h light/dark cycle and food/water available *ad libitum*. All animal experiments were performed in compliance with institutional policies approved by the government of Upper Bavaria following §11 Ab.1 TierSchG for the DZNE animal facility (Inst.-Nr. 04-26). Primary cortical neurons were prepared from embryonic day 19 animals of both sexes. Neocortex and hippocampus were dissected in ice-cold dissection media (HBBS, penicillin-streptomycin, 10 mM HEPES pH 7.3). The tissue was enzymatically dissociated at 37 °C (for cortices 20 min in 0.25 % trypsin, 0.7 mg/ml DNase I; for hippocampi 15 min in 0.15 % trypsin in dissection media) followed by gentle trituration.

For EM analysis, 250,000 cells/ml cortical neurons were plated on EM grids and cultured in Neurobasal medium containing 2 % B27 (Thermo Fisher), penicillin-streptomycin, 0.5 mM L-glutamine (Thermo Fisher). For biochemical analysis, 250,000 cells/ml cortical neurons were plated 771 on 12-well plates (Thermo Fisher) and cultured in Neurobasal medium. For immunofluorescence experiments, 85,000 cells/ml hippocampal neurons were plated on 12-well plates containing glass coverslips (VWR) coated with poly-D-lysine and cultured in Neurobasal medium supplemented with 12.5 µM glutamate.

## Method Details

**Lentivirus Packaging**—HEK293FT cells (female; from Thermo Fisher) of low passage number were plated in three 10 cm dishes (5,000,000 cells/dish) and cultured in DMEM (Thermo Fisher), penicillin-streptomycin, 1 % non-essential amino acids (Thermo Fisher) and 10 % fetal bovine serum (Sigma). A transfection mix was set up as follows: 18.6 µg transfer vector (FhSynW-(GA)<sub>175</sub>-GFP, FhSynW (GA)<sub>149</sub>, FU3a-tagRFP-p62 or FhSynW-GFP), 11 µg pSPAX2 and 6.4 µg pVSVg in 4.5 ml Opti-MEM were combined with 108 µl Lipofectamine 2000 in 4.5 ml Opti-MEM (Thermo Fisher) and incubated for 20 minutes. Cell media was replaced with 5 ml Opti-MEM and 3 ml of transfection mix were added per dish. The transfection media was replaced after 6 h by plating media supplemented with 13 mg/ml bovine serum albumin, and the supernatant was collected after additional 24 h. Lentiviral particles were harvested by ultracentrifugation using a Sw28 rotor (22,000 rpm, 2 h), resuspended in 150 µl Neurobasal media on a rocking platform overnight and stored in aliquots at –80 °C.

**Generation of pcDNA3.1 – STOP-GA(G<sub>4</sub>C<sub>2</sub>)<sub>73</sub>-GFP**—The method to generate the construct was adapted from Lee et al. (Lee et al., 2013). Complementary G<sub>4</sub>C<sub>2</sub> repeat oligonucleotides with phosphorylated ends (5'-PHOS-(G<sub>4</sub>C<sub>2</sub>)<sub>5</sub>-3' and 5'-PHOS-CC(G<sub>2</sub>C<sub>4</sub>)<sub>4</sub>GGCC-3') were energetically optimized to maximize heterodimer formation. After initial denaturation for 5 min at 95°C in T4 ligase buffer (New England Biolabs), the complementary oligonucleotides were allowed to anneal by slow cool down to room temperature during 2 h. Hybridized oligonucleotides were then self-ligated for 1 h to extend the G<sub>4</sub>C<sub>2</sub> DNA sequence. Adapter oligonucleotides containing NheI (5') and BamHI (3') restriction sites (5' adapter oligonucleotides containing NheI: 5'-

GCCGTC AAGGCCGCATCTAGTAGCTAGC-3' and 5'-PHOS-CCGCTAGCTACTAGATGCGGCCTTGACGGC-3'; 3' adapter oligonucleotides containing BamHI: 5'-PHOS-GGGATCCCTAGTACTGGGCCTCATGGGC-3' and 5'-GCCCATGAGGCCAGTACTAGGGATC-3') were separately hybridized and sequentially added in excess to the ligation reaction mix to stop G<sub>4</sub>C<sub>2</sub> elongation. Ligation products were separated in a 1.25% agarose gel, and bands running at the desired molecular size were excised. The DNA was then purified with the Zymoclean Gel DNA recovery kit (Zymo Research) according to the manufacturer's guidelines. The resulting blunt-end fragments were phosphorylated and cloned into a dephosphorylated SmaI-restricted pUC18 plasmid for amplification. Colonies were screened for G<sub>4</sub>C<sub>2</sub> repeat length, and a construct with 73 G<sub>4</sub>C<sub>2</sub> repeats was chosen for further experiments. The selected sequence was subcloned into the mammalian expression vector pcDNA3.1(+)/myc-His between NheI and BamHI in front of a GFP-encoding region. Finally, multiple stop codons were added by site-directed mutagenesis in every reading frame at the 5' of the sequence of interest, thereby removing all initiator codons between the T7 promoter and the sequence of interest. The sequence was verified by multiple sequencing reactions as well as restriction digest.

**Immunofluorescence and Cellular Fractionation**—Immunofluorescence stainings were performed on primary hippocampal neurons 5 days after infection with (GA)<sub>175</sub>-GFP or GFP lentivirus on day 5, or 3 days upon transfection with G<sub>4</sub>C<sub>2</sub> on day 3 using Lipofectamine 2000 (Thermo Fischer). Cells were fixed for 10 min at room temperature (4 % paraformaldehyde, 4 % sucrose in PBS). Anti-PSMC4 (Bethyl Laboratories, 1:250, RRID:AB\_2620201) or anti-GA (Mackenzie et al., 2013, 1:200) primary antibodies as well as secondary antibodies (Alexa 555, Alexa 647, Thermo Fisher, 1:400, RRID:AB\_2535850, RRID:AB\_141780, RRID:AB\_2535813) were diluted in staining buffer (0.1 % gelatin, 0.3 % Triton X-100, 450 mM NaCl, 16 mM sodium phosphate pH 7.4). After mounting the coverslips, images were taken using a LSM710 confocal microscope (Carl Zeiss, Jena) with a 63× oil immersion objective.

Biochemical experiments were performed on primary cortical neurons 10 days after infection with either (GA)<sub>175</sub>-GFP or GFP lentivirus on day 3. Cells were lysed (PBS with 1 % Triton-X-100, 15 mM MgCl<sub>2</sub>, 0.2 mg/ml DNase with protease inhibitor cocktail). Upon centrifugation (18,000 *xg*, 4 °C for 30 min) the soluble fraction was collected from the supernatant. The pellets were washed twice with lysis buffer yielding the insoluble fraction. After adding 4x Laemmli buffer (Bio-Rad), samples were denatured (95 °C for 10 min) and loaded on Tricine gradient gels (Thermo Fisher). The following antibodies were used: Anti-PSMC4 (Bethyl Laboratories, 1:1000), anti-GFP (NeuroMab, 1:5000, RRID:AB\_10671445) and anti-Calnexin (Enzo Life Sciences, 1:7000, RRID:AB\_10616095). The data in Figure S4C was normalized to the total proteasome levels in GFP-transduced control neurons.

**Flow Cytometry**—HEK293 cells stably expressing UbG76V-GFP were harvested 72 h after transient transfection with (GA)<sub>175</sub>-tagRFP or tagRFP as control and analyzed with a BD FACSAriaIII flow cytometer, as described previously (Hipp et al., 2012a; Hipp et al., 2012b). In brief, to ensure a sufficient number of cells with elevated levels of the transfected

proteins >100,000 events were analyzed per condition. To plot the level of the reporter protein versus the level of the transfected protein, a set of gates was established in the tagRFP channel. HEK293 cells transfected with tagRFP were used as single-color control to compensate the bleed-through between GFP and tag-RFP individually for each gate, and to correct for effects due to high expression of tagRFP. The compensated mean fluorescence of the 855 reporter protein UbG76V-GFP in each of these gates was plotted on the y-axis and the gate number (corresponding to the log of fluorescence intensity of the transfected protein) was plotted on the x-axis. The data shown in Figure S4D are from a single representative experiment out of three independent repeats. Cells expressing low levels of tagRFP (gates 1–9) and gates with < 1000 events were not included in the analysis. Raw flow cytometry data were analyzed using FlowJo software (version 9.9; Tree Star).

**Cryo-EM Sample Preparation**—Quantifoil grids (R2/1, Au 200 mesh grid, Quantifoil Micro Tools, Germany) were coated with an additional carbon layer (~20 nm thick) using a carbon evaporator (MED 020, BAL-TEC). Before use, the grids were glow discharged using a plasma cleaner (PDC-3XG, Harrick) for 20 seconds and neurons were seeded as described above. Neurons were vitrified 5 days after transduction with GFP or tagRFP-p62/untagged (GA)<sub>175</sub> on day 5, 3 or 5 days after transduction with (GA)<sub>175</sub>-GFP on day 5, and 3 days after transfection with 881 (G<sub>4</sub>C<sub>2</sub>)<sub>73</sub> on day 3. Untransduced and untransfected control neurons were vitrified on DIV 10.

For vitrification, the grids were blotted for 10 seconds from the back side using filter paper and immediately plunged into a liquid ethane/propane mixture (Tivol et al., 2008) using a manual plunge freezer. Grids were transferred to sealed boxes and stored in liquid nitrogen until usage.

**Cryo-Fluorescent Light Microscopy and Cryo-FIB Microscopy**—EM grids were mounted onto modified Autogrid (FEI, Hillsboro, OR, USA) sample carriers, allowing subsequent FIB milling with a shallow incident ion beam (Rigort et al., 2012), and then transferred to the cryo-stage of an FEI CorrSight microscope for cryo-light microscopy. Overview images of the grid and poly-GA-GFP/tagRFP-p62 signal were respectively acquired in transmitted light and the spinning disk confocal modes using a 20x lens (air, N.A. 0.8). Image acquisition was done with FEI MAPS software. The samples were then transferred into a FEI Scios dual beam cryo-FIB/scanning electron microscope (SEM) using a cryo-transfer system.

To improve the sample conductivity, a layer of organometallic platinum was deposited onto the grid using the *in situ* gas injection system with the following parameters: 10 mm working distance, 26°C pre-heating temperature, and 8 s gas injection time. MAPS software allowed correlation between cryo-light microscopy and SEM images via the 3-point alignment method. Thin lamellas were prepared in the regions of poly-GA-GFP/tagRFP-p62 fluorescence signal using the Ga<sup>2+</sup> ion beam at 30 kV under a 20° stage tilt angle. 0.5 nA beam current was used for rough milling, followed by sequentially lower currents during the thinning procedure. A current of 30 pA was used for the final polishing step to reach a final lamella thickness of 150–200 nm. SEM imaging was used to monitor the milling progress.

**Cryo-Electron Tomography and Reconstruction**—The specimens were examined at liquid nitrogen temperature in an FEI Titan Krios cryo-electron microscope operated at 300 kV and equipped with a field emission gun and a Gatan post column energy filter. Lamellas were loaded vertical to the tilt axis, and were precisely aligned by adjusting the  $\beta$  angle of the cryo-stage. Images were collected using a 4 k x 4 k K2 Summit (Gatan) direct detector camera operated in dose fractionation mode (0.4 s, 0.3 electrons/ $\text{\AA}^2$  per frame). Tilt series were recorded using SerialEM software (Mastronarde, 2005) at a nominal magnification of 42,000 X, resulting in a pixel size of 3.42  $\text{\AA}$  at the specimen level. Unidirectional tomographic tilt series were recorded from  $-50^\circ$  to  $+70^\circ$  with an increment of  $2^\circ$ . On average, 6 frames were collected for each image resulting in a total dose between 100  $e^-/\text{\AA}^2$  to 110  $e^-/\text{\AA}^2$  per tilt series. K2 frames were aligned using in house software (K2Align) based on previous work (Li et al., 2013). Tilt series were aligned using fiducial-less patch tracking, and the tomograms were reconstructed by weighted back projection using the IMOD software package (Kremer et al., 1996). For tomograms of lamellas superficially contaminated by ice crystals, a surface cleaning procedure was employed after alignment (Fernandez et al., 2016). The resulting tilt series were then aligned and reconstructed again to obtain the final reconstructions.

**Template Matching and Subtomogram Averaging**—To identify the macromolecules found in the tomograms, a *de novo* subtomogram averaging procedure without any external structural information was developed (Figure S2A). The MATLAB (Mathworks) TOM toolbox (Nickell et al., 2005) was used as general platform for image processing. Firstly, all the tomograms were binned twice (13.68  $\text{\AA}^3$  per voxel) for processing. In one tomogram, several identical small ring-like structures were hand-picked, aligned and averaged to obtain a first tube-like average structure. This was used as an initial template for template matching in all the binned tomograms using PyTom (Hrabe et al., 2012). The resulting subtomograms were cropped, CTF-corrected and classified using Relion (Bharat and Scheres, 2016). The resulting structure clearly showed a 20S proteasome core complex, but because of the missing wedge and the preferred orientation induced by the initial template, the 19S regulatory particles (RP) were not well resolved. This structure was used to perform a new round of template matching and classification, which clearly resolved proteasomes either single- or double-capped. The single-capped proteasomes were low-pass filtered to 40  $\text{\AA}$  and used as a reference for template matching again to produce the final dataset. In total, 10,367 proteasome subtomograms of 180<sup>3</sup> pixel volume were picked for further analysis from 9 tomograms containing large (GA)<sub>175</sub>-GFP aggregates (DIV 5 + 5). The same template matching procedure was applied to tomograms of control neurons, either untransduced (16 tomograms) or transduced with GFP only (17 tomograms).

The subtomograms were then 3D classified using Relion (Figure S2B). They were firstly divided into single-capped and double-capped proteasomes. To further rule out reference bias, a single-capped proteasome, a double-capped proteasome and a mirrored single-capped proteasome were used as references for classification. The results were similar, indicating negligible reference bias during classification. To further analyze the conformational status of the 19S regulatory particles, all subtomograms were cut *in silico* between the  $\beta$ -rings of the 20S, resulting in two independent particles for the double-capped proteasomes (Asano et

al., 2015). All cut-out half proteasomes were merged into a new dataset for another round of classification. Ground state and substrate processing state structures were distinguished by the relative orientation of the Rpn5 and Rpn6 subunits (Asano et al., 2015; Unverdorben et al., 2014). Ground state and substrate processing state structures were further classified by applying a soft sphere mask in the RP region only. Identical subclasses were merged resulting in two ground state and two substrate processing state classes, which were further refined to achieve the final structures. A similar analysis could not be applied to control neurons (GFP-transduced or untransduced) due to the low numbers of proteasomes found in these samples. Therefore, the results from untransduced neurons from a previous study (Asano et al., 2015) were used as reference. Visualization of the subtomogram averages was performed in UCSF Chimera (Pettersen et al., 2004). Resolution was determined using the 0.143 criterion according to the gold standard Fourier Shell Correlation (Scheres and Chen, 2012).

To identify the larger ring-like structures found in the tomograms, a similar template matching procedure was employed. A spherical structure was generated after processing, which showed a clear eight-fold symmetry by rotational correlation coefficient analysis (Figure S3B). Furthermore, the size of the average fitted well with the crystal structure of TRiC/CCT (PDB 4V94) (Leitner et al., 2012), indicating that these larger rings correspond to the TRiC/CCT chaperonin. A total of 1,366 TRiC subtomograms were used for averaging. For ribosome template matching, a human 80S ribosome structure (EMDB 2938) (Khatter et al., 2015) was filtered to 40 Å and used as a template.

**Atomic Model Fitting**—The *S. cerevisiae* proteasome models of the states s1 (PDB 5MP9, 5MPD), s2 (PDB 5MPA, 5MPE), s3 (PDB 5MPB), and s4 (PDB 5MPC) (Wehmer et al., 2017) were used as initial models. The core particles of the models were fitted into each of the groups obtained by classification of the poly-GA proteasome dataset using rigid body docking within UCSF Chimera. For each class, MDFF was performed to refine the s1–4models according to the density maps. Then, the RMSD between the best fitting atomic models and the initial s1–4state models was calculated (Table S1). This showed that the GS1 class reflected the s1 state with the Rpn1 positioning of the human s1 state (PDB 5L5K) (Schweitzer et al., 2016), GS2 reflected the s1 state with a rotated Rpn1 position, SPS1 reflected the s2 state and SPS2 reflected the s4 state. To obtain the final atomic rat models, the best fitting yeast structures for each class were used as templates for comparative modeling and real-space structure refinement (Goh et al., 2016). MDFF simulations were prepared using QwikMD (Ribeiro et al., 2016), analyzed with VMD (Humphrey et al., 1996), and carried out with NAMD (Phillips et al., 2005).

**Segmentation of Poly-GA Aggregates and Distance Measurement**—Poly-GA ribbons are locally planar and were consequently segmented using a filter based on tensor voting, which distinguishes planar-like from line- and blob-like structures (Martinez-Sanchez et al., 2014). The filter outputs a scalar map, where voxel intensity value is proportional to the local similarity with a plane. The final segmentation was generated by thresholding the filter output combined with a manually generated mask to discard other locally planar structures like membranes and correct minor artifacts. Due to the missing

information along the electron beam direction (Lucic et al., 2005), poly-GA ribbons were not clearly visible when oriented within the xy plane. 984 Segmentations were visualized using Amira (Thermo Fischer, RRID:SCR\_014305).

For Figure 5, proteasome regulatory particles and poly-GA ribbons were represented by an isosurface, properly placed and oriented in their original tomogram. For regulatory particles, surfaces were obtained by applying the marching cubes algorithm, implemented in the VTK Open Source library ([www.vtk.org](http://www.vtk.org); RRID:SCR\_015013; Schroeder et al., 2006), on the corresponding subtomogram average. Isosurface threshold was set manually for every case with the criterion of choosing the minimum value avoiding noisy features. For poly-GA ribbons, isosurfaces were generated from the output of the planar filter using the marching cubes algorithm. The final ribbon surfaces were obtained by masking the isosurface with the corresponding ribbon segmentation from the tomogram. To calculate the fraction of the volume of poly-GA inclusions occupied by poly-GA ribbons, total ribbon volume was measured from the segmentations and divided by the total tomogram volume. For this calculation, tomograms (N = 4, (GA)<sub>175</sub>-GFP transduced neurons at DIV 5 + 5) were selected in which the large majority of the volume contained poly-GA aggregates.

985 RP to poly-GA ribbon distance was defined as the shortest Euclidean distance between the center of the RP to any surface point of any ribbon in the same tomogram. A custom Python software package was developed to measure RP-to-ribbon distance for every RP in all tomograms. The results were grouped by particle class to facilitate statistical analysis. The shortest distance among surfaces was computed with the help of a VTK library (Schroeder et al., 2006).

### Quantification and Statistical Analysis

To measure proteasome concentration, for each tomogram (N = 9, (GA)<sub>175</sub>-GFP transduced neurons, DIV 5 + 5; N = 17, GFP transduced neurons; N = 16, untransduced neurons) the number of proteasomes found by template matching and subtomogram averaging was divided by the total tomogram volume. Because other cellular structures (such as poly-GA aggregates, other macromolecules or cellular organelles) were also present in the tomogram volume, this calculation underestimates the cytosolic concentration of proteasomes. For poly-GA aggregates, only tomograms with more than half volume occupied by aggregates were considered for the concentration calculation. In Figure S4B, the top and bottom boundaries of the boxes indicate  $\pm 2x$  standard error, and whiskers extend to the maximum and minimum values. Statistical analysis was performed by the non-parametric Mann-Whitney test, as not all data was normally distributed according to the Shapiro-Wilk test.

For the analysis of proteasome conformation as a function of the distance to poly-GA ribbons (Figure 5B, C, E), 6080 regulatory particles from 4 tomograms of (GA)<sub>175</sub>-GFP transduced neurons (DIV 5 + 5) were analyzed. The data were divided in a 4x4 table (4 RP states x 4 distance bins) resulting in 9 degrees of freedom. Statistical analysis was performed by Chi-square test.

The proteasome levels in the total, soluble and insoluble fractions of (GA)<sub>175</sub>-GFP transduced neurons were quantified from western blots by measuring the grey levels using



Fiji (Schindelin et al., 2012). Error bars in Figure S4C indicate standard error. All data was normally distributed according to the Shapiro-Wilk test (95 % confidence level). Statistical analysis was performed by two-sided paired t-test (N = 6 replicates from 4 independent experiments). All graphs were plotted using OriginPro (OriginLab).

### Data and Software Availability

The Python scripts used to calculate the distance between proteasomes and poly-GA ribbons are available at <https://github.com/anmartinez/poly-GA.git>

The tomogram analyzed in Figure 2 has been deposited at the Electron Microscopy Data Base (EMDB) with accession number EMD-4191. The structures of the proteasome and TRiC/CCT obtained by in situ subtomogram averaging have been deposited at EMDB with the following accession numbers: EMD-3916 (GS1 proteasome), EMD-3913 (GS2 proteasome), EMD-3914 (SPS1 proteasome), EMD-3915 (SPS2 proteasome), EMD-3917 (TRiC/CCT). The fitted atomic models of the different proteasome states have been deposited at the Protein Data Base with the following accession numbers: 6EPF (GS1), 6EPC (GS2), 6EPD (SPS1), 6EPE (SPS2).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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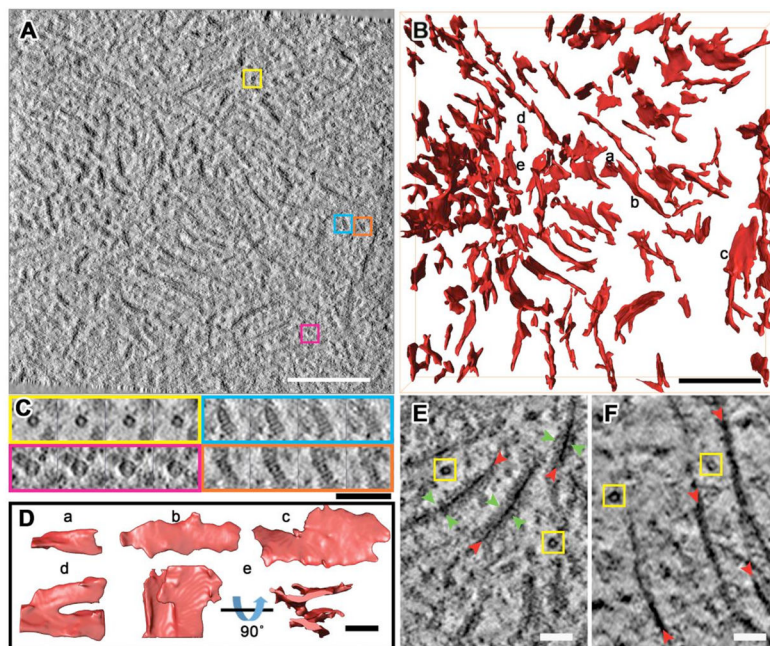
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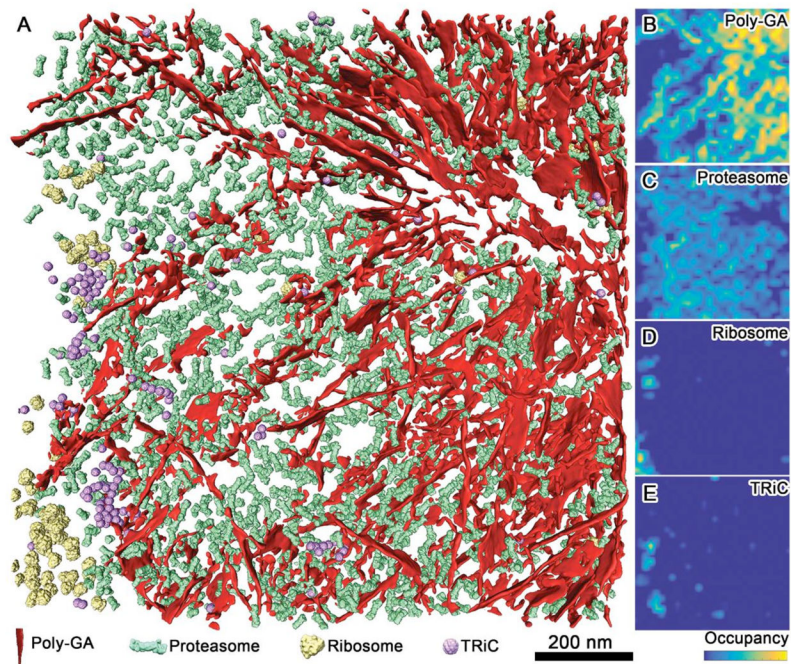
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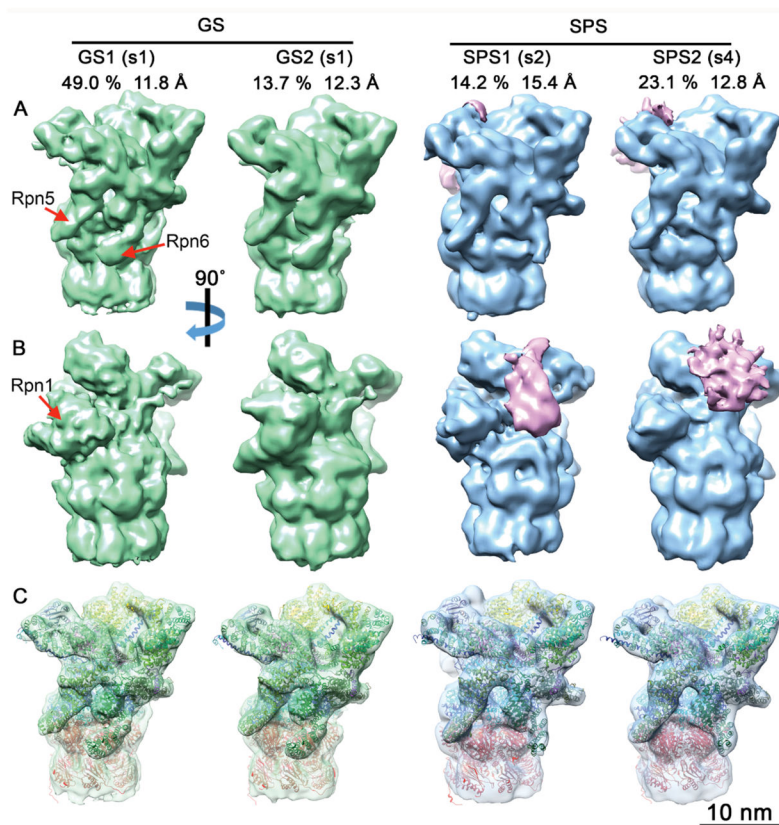
**Figure 1. *In Situ* Neuronal Poly-GA Aggregates Form Twisted Ribbons**

(A) Tomographic slice of an aggregate within a (GA)<sub>175</sub>-GFP-transduced neuron (DIV 5 + 5). Colored boxes show macromolecules magnified in (C). (B) 3D rendering of the aggregate shown in (A). Selected poly-GA ribbons (red) magnified in (D) are indicated. (C) Series of higher magnification tomographic slices of representative protein complexes detected in the tomogram shown in (A). Yellow and magenta boxes show the typical smaller (yellow) and larger (magenta) ring-like structures found in the aggregate region. Blue and orange boxes show side views of single-capped (blue) and double-capped (orange) 26S proteasomes. (D) Selected ribbons from (B) rotated and magnified for visualization. Note the variable width of the ribbons (a–c). Some ribbons show bifurcations (d–e). (E, F) Higher magnification tomographic slices of aggregates within neurons transduced with (GA)<sub>175</sub>-GFP, DIV 5 + 5 (E) or untagged (GA)<sub>175</sub>, DIV 5 + 5 (F). Yellow boxes mark similar small ring-like structures as in (A). Note that (GA)<sub>175</sub>-GFP ribbons (red arrowheads) are decorated by additional densities (green arrowheads), which are missing from untagged (GA)<sub>175</sub> ribbons. Tomographic slices are 5 nm thick. Scale bars: 200 nm (A, B), 50 nm (C–F). See also Figure S1.



**Figure 2. Mapping Macromolecules within Poly-GA Aggregates Shows a Substantial Recruitment of 26S Proteasomes**

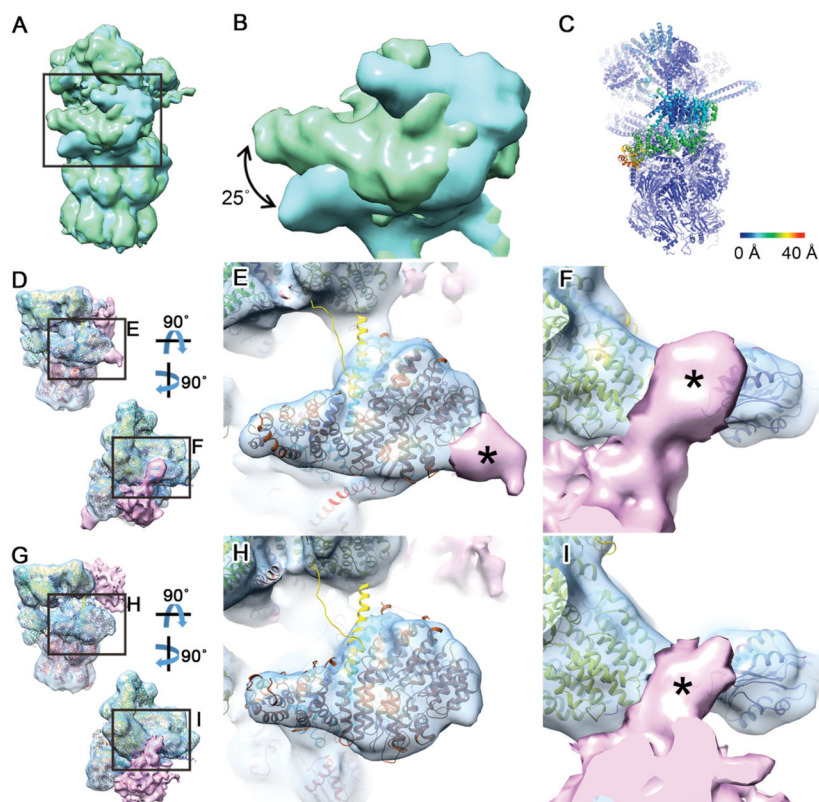
(A) 3D rendering of an aggregate within a neuron transduced with  $(GA)_{175}$ -GFP (DIV 5 + 5) showing different macromolecules found either within or at the periphery of the aggregate. Poly-GA ribbons (red), 26S proteasomes (green), ribosomes (yellow), TRiC/CCT chaperonins (purple). The macromolecules are mapped in their original locations and orientations, computationally determined by template matching and subtomogram averaging. (B–E) Maximum intensity projection heat maps of the molecular species shown in (A). Note that the proteasomes are mostly found in between poly-GA ribbons, whereas ribosomes occur almost exclusively outside of the aggregate. TRiC/CCT molecules mostly populate the aggregate periphery, but some can also be found between poly-GA ribbons. See also Figure S2A, Figure S3, Figure S4, Figure S5 and Movie S1.



### Figure 3. Subtomogram Classification of 26S Proteasomes Reveals Enrichment of Substrate Processing Conformations

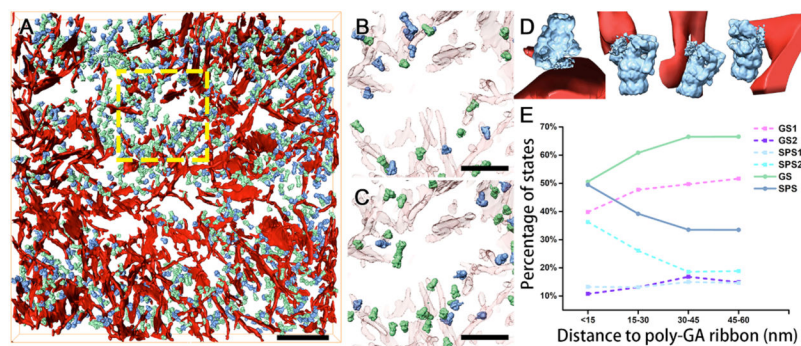
To analyze the functional state of proteasome regulatory particles single- and double-capped proteasomes were cut by the half of the CP. The resulting half proteasomes were classified according to RP conformation into ground or substrate processing states (Asano et al., 2015), yielding two ground states (GS1, GS2) and two substrate processing classes (SPS1, SPS2). (A, B) The four density maps are displayed in solid surface representation in two different views. The positions of the Rpn1, Rpn5 and Rpn6 subunits are indicated. Prominent densities in the substrate binding region of SPS1 and SPS2 are colored in pink. For each class, the percentage of the total number of classified particles and the global resolution are indicated. (C) Same view as (A), with semi-transparent maps superimposed with the atomic models generated by MDFF. The classes respectively represent the s1 state with different Rpn1 positions (GS1, GS2), the s2 state (SPS1) and the s4 state (SPS2). Atomic models are colored by subunits: Rpn1 (brown), Rpn2 (yellow), Rpn9/5/6/7/3/12 (different shades of green), Rpn8/Rpn11 (light/dark magenta), Rpn10 and Rpn13 621 (purple), AAA-ATPase hexamer (blue), and CP (red). See also Figure S2B, Figure S3 and Table S1.





**Figure 4. Detailed Structural Differences between 26S Proteasome Conformations Highlight its *In Situ* Structural Dynamics and Interactions**

(A) Superimposition of GS1 (green) and GS2 (cyan) density maps aligned by their CP. Both classes are consistent with the s1 state and differ only in the position of Rpn1. (B) Magnified view of the region boxed in (A) showing a 25° rotation of the Rpn1 subunit in the GS2 map. (C) Atomic model of the GS2 class colored according to the root mean square deviation (RMSD) from the GS1 model. Note that the only substantial differences are found in the Rpn1 region. (D) Two views of the SPS1 map (consistent with the s2 state) shown in surface representation superimposed with its atomic model. A prominent density in the substrate binding region is colored in pink. (E, F) Magnified view of the regions boxed in (D). The atomic models of Rpn1 (E), Rpn2 and Rpn10 (F) are shown in brown, yellow and purple respectively. Parts of the additional density denoted by asterisks may correspond to proteasome-bound ubiquitin or UBL domain proteins. (G) Two views of the SPS2 map (consistent with the s4 state) shown in surface representation superimposed with its atomic model. A prominent density in the substrate binding region is colored in pink. (H, I) Magnified view of the regions boxed in (G). Note that the density in the Rpn2/10 region is similar in the SPS1 and SPS2 class averages (I), whereas no additional density was found on the Rpn1 region of the SPS2 map (H).



**Figure 5. Spatial Mapping of Proteasomes within Poly-GA Aggregates Reveals Poly-GA Influence on Proteasome Conformation**

(A) 3D rendering of an aggregate within a neuron transduced with (GA)<sub>175</sub>-GFP (DIV 5 + 5). Poly-GA ribbons (red), proteasomes in ground state (green), proteasomes in substrate processing states (blue). Proteasome location and orientation were determined by template matching and subtomogram averaging. (B, C) Magnification of the region boxed in (A) showing only proteasomes less than 15 nm away from poly-GA ribbons (B) or 30 – 45 nm away (C). Poly-GA ribbons are shown as a transparent red surface. Note that substrate processing proteasomes are more abundant close to poly-GA ribbons. (D) Examples of SPS2 proteasomes directly touching poly-GA ribbons in the tomogram shown in (A). The additional density in the substrate binding region overlaps with the poly-GA ribbons. (E) Plot of proteasome conformation vs. distance to poly-GA ribbons. The influence of the distance to poly-GA ribbons in proteasome conformation was statistically significant ( $p < 0.001$ , Chi-square test,  $N = 6080$  regulatory particles from 4 tomograms). Scale bars: 200 nm (A), 100 nm (B, C). See also Figure S4.