Droplet and fibril formation of the functional amyloid Orb2

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Edited by Karin Musier-Forsyth

The functional amyloid Orb2 belongs to the cytoplasmic polyadenylation element binding (CPEB) protein family and plays an important role in long-term memory formation in Drosophila. The Orb2 domain structure combines RNA recognition motifs with low-complexity sequences similar to many RNA-binding proteins shown to form protein droplets via liquid–liquid phase separation (LLPS) in vivo and in vitro. This similarity suggests that Orb2 might also undergo LLPS. However, cellular Orb2 puncta have very little internal protein mobility, and Orb2 forms fibrils in Drosophila brains that are functionally active indicating that LLPS might not play a role for Orb2. In the present work, we reconcile these two views on Orb2 droplet formation. Using fluorescence microscopy, we show that soluble Orb2 can indeed phase separate into protein droplets. However, fluorescence recovery after photobleaching (FRAP) data shows that these droplets have either no or only an extremely short-lived liquid phase and appear maturated right after formation. Orb2 fragments that lack the C-terminal RNA-binding domain (RBD) form fibrils out of these droplets. Solid-state NMR shows that these fibrils have well-ordered static domains in addition to the Gln/His-rich fibril core. Further, we find that full-length Orb2B, which is by far the major component of Orb2 fibrils in vivo, does not transition into fibrils but remains in the droplet phase. Together, our data suggest that phase separation might play a role in initiating the formation of functional Orb2 fibrils.

In vivo, Orb2A is of relatively low abundance and only increases in concentration upon synaptic stimulation (1). However, its presence is essential for the aggregation of the common isoform Orb2B (3, 4). Orb2A has nine unique N-terminal residues that can form cross-β fibrils on their own (8) and whose deletion or mutation prevents Orb2 aggregation and LTM formation (4). The deletion of Orb2A’s C-terminus has no phenotype, whereas its deletion in Orb2B was lethal (3, 9). The N-terminus of Orb2B is Ser/Gly-rich and of unknown function. Hervás and coworkers recently determined the structure of the Orb2 fibril core, which is located in the Gln/His-domain, using cryo-EM (6).

The domain structure of Orb2 that combines low-complexity sequences with RNA-binding domains (RBDs) is reminiscent of a whole class of RNA-binding proteins, such as fused in sarcoma (FUS), that are able to undergo liquid–liquid phase separation (LLPS) (10–12). LLPS is often of functional relevance e.g., for RNA processing in the case of (stress) granules (13) or for neurotransmitter release and postsynaptic signal transmission in neurons (14, 15). Additionally, other Gln-rich proteins such as huntingtin exon-1 (HTTex1) or Whi3 have been reported to undergo LLPS (16, 17). When observed via light microscopy, LLPS manifests as droplet or puncta formation. These droplets, while relatively fluid at first, can mature over time, which results in the decreased ability to fuse with other droplets and low fluorescence recovery after photobleaching (FRAP) (18, 19). Structurally, this maturation is caused by reduced protein diffusion in the droplet phase, which becomes a rigid protein glass. These mature droplets can in cases such as FUS mutants or HTTex1 be the nucleation point of cross-β fibril formation (16, 20). Taken together, these findings suggest that Orb2 could also undergo phase separation, which could have a potential role for its function, or its aggregation into cross-β fibrils, or both. In the following, we answer the question of whether or not Orb2 can undergo phase separation by demonstrating that it indeed can. Further, we characterize droplet maturation and fibril formation of different Orb2 fragments to understand the relationship between Orb2 droplet and fibril formation.

Results

To determine if Orb2 can phase separate in vitro, we studied full-length Orb2B and Orb2A and Orb2B fragments without the C-terminal RBD, which we refer to as ΔRBD constructs.
(Fig. 1A). We did not study full-length Orb2A because it does not require its RBD for proper function in vivo (3) and because we were not able to make it in large quantities. Sequence analysis using the database-based FuzPred algorithm (21) showed a high propensity for phase separation (pLLPS) for the N-termini of Orb2A and Orb2B until about the beginning of the RBD (Fig. 1B). Interestingly, the N-terminal residues unique to Orb2A showed a lower pLLPS than the corresponding residues in Orb2B. This propensity does not seem to be driven by charge density plots in Figure S1 indicate. To test if Orb2 can undergo phase separation, we started in conditions that favored a stable monomeric state, namely 100 mM KCl, 1 M urea, 10 mM HEPES, 0.05% v/v β-mercaptoethanol, pH 7.6, here referred to as HEPES–salt–urea buffer (HSU buffer). When left in HSU buffer, Orb2 monomers did not phase separate or form aggregates.

To introduce droplet formation, we exchanged each Orb2 construct from HSU buffer into H buffer (10 mM HEPES, 0.03% v/v NaNO3 and 0.05% v/v β-mercaptoethanol, pH 6.5). Immediately upon exchange, the solutions became visibly turbid (Fig. 2A). To further confirm that Orb2B, Orb2BARBD, and Orb2AΔRBD underwent droplet formation, we used differential interference contrast (DIC) and fluorescence microscopy of Oregon Green 488 labeled protein (Fig. 2B). Round protein droplets were visible for all constructs immediately after buffer exchange. We found these droplets to be predominantly in solution and did not observe any wetting of the glass slide in contrast to many other protein droplets. The average diameters of Orb2BARBD, Orb2AΔRBD, and Orb2B droplets were 1.3 ± 1.0 μm, 2.2 ± 1.1 μm, and 1.8 ± 1.4 μm, respectively (see Fig. S2). Morphological analysis (22) showed that the droplets had a high degree of roundness of 0.7–0.9. Besides H-buffer (i.e., low ionic strength), we found that both 10% PEG 8000 and polyA at a 1:10 RNA:protein m/m ratio were able to induce Orb2 droplet formation (see Fig. S3) when added to HSU buffer. Again, Orb2BARBD droplets are significantly smaller under these conditions.

Many protein droplets mature over time, undergoing a transition from a state in which proteins freely diffuse inside the droplet to a state with little to no protein diffusion (glass transition) (23). To characterize protein diffusion inside droplets, we measured FRAP. We bleached the center of Oregon Green 488 labeled Orb2 droplets using an argon laser and monitored the recovery by confocal microscopy for 5 min afterward. To our surprise, none of the Orb2 constructs showed any degree of recovery, even though FRAP experiments were performed within 1 min of droplet formation. These results indicate that Orb2 is static within droplets almost immediately after undergoing phase separation (Fig. 3). To further confirm these results, we did four consecutive 10 min FRAP experiments on the same cluster of Orb2B droplets. The first had still not recovered after the last experiment ended (Fig. S4).

In addition, we found several clusters of Orb2B droplets with overlap between individual droplet boundaries (Figs. 3 and S4). For many other proteins, this stage typically marks the beginning of a droplet fusion event. None of the droplets with overlapping boundaries completed the fusion process. These results support that Orb2B becomes static within droplets relatively quickly after phase separation. As a positive control, we used a N-terminally 6xHis and GFP-tagged FUS construct (His-GFP-FUS) for which we measured about 50% recovery in fluorescence after 22 s, similar to FRAP experiments on FUS droplet reported previously (20).

For some proteins, droplet maturation is followed by cross-β fibril formation (18). To test how Orb2 droplets evolve and if they eventually result in cross-β fibrils, we monitored Orb2 droplets over time using fluorescence microscopy, electron microscopy (EM), and Thioflavin T (ThT) fluorescence. Fluorescence microscopy and EM images of Orb2BARBD, Orb2ΔRBD, and Orb2B in H buffer are shown in Figure 4. In the case of Orb2ΔRBD, the initial droplets formed halos at 12 h in our fluorescence images, which coincided with unbundled fibrils radiating out from a dark center as seen by EM. Similarly, Orb2BARBD droplets turned into small fibrils and droplets at 12 h. At 48 h, we observed large objects in fluorescence microscopy, which coincided with thick, bundled fibrils in our EM images. In contrast, Orb2B droplets never progressed to form fibrils, but formed larger objects that were composed of merged droplets similar to those shown in Figure 3.

We attributed the disappearance of Orb2BARBD and Orb2BARBD droplets over several hours to the maturation of these droplets into cross-β fibrils (Fig. 4). When examined using EM, Orb2BARBD fibrils were mostly unbundled,
occasionally radiating out from a common center, presumably a droplet. In contrast, Orb2AΔRBD fibrils were highly bundled. As can be seen from Figure S5, both fibril types did not show any fine structure or twist at higher magnification and had an average diameter of about 18 nm.

As an additional measure of droplet to fibril transition, we measured ThT fluorescence and OD_{600} kinetics for each Orb2 construct in both HSU and H buffer (Fig. 5). We did not observe any increase in ThT fluorescence in HSU buffer for any of the constructs. In H buffer, Orb2BΔRBD and Orb2AΔRBD showed an increase in fluorescence in a sigmoidal shaped curve typical for cross-β fibril formation. In contrast, Orb2B showed only a negligible increase in fluorescence in H-buffer compared with HSU buffer possibly due to light scattering from droplets. The OD_{600} curves of Orb2BΔRBD and Orb2AΔRBD decreased in the first 20 h and increased for Orb2AΔRBD after about 25–40 h. Only a slight increase for Orb2BΔRBD was observed after about 60 h. This initial decrease is compatible with the disappearance of Orb2BΔRBD and Orb2AΔRBD droplets. The later increase in scattering for Orb2AΔRBD correlates with the appearance of large, highly bundled fibrils as seen in our EM images that will scatter light much more than the unbundled Orb2BΔRBD fibrils seen by EM. The OD of Orb2B stayed

Figure 3. Droplets formed by Orb2BΔRBD, Orb2AΔRBD, and Orb2B show no protein diffusion via FRAP. Top, fluorescence microscopy images of Oregon Green 488 Orb2 constructs right after and 5 min after bleaching. Scale bars represent 5 μm. Bottom, average and standard deviation of the fluorescence intensity after bleaching (where 0 is the intensity after bleaching and 1 is the intensity of an unbleached region). None of the fragments showed measurable fluorescence recovery immediately after exchange into H buffer. GFP-labeled FUS, used as control, fully recovered after 3 min with 50% recovery after 22 s.
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Figure 4. Orb2ΔRBD droplets mature into fibrils, whereas Orb2B droplets do not. EM (upper row) and fluorescence microscopy (lower row) images of Orb2 constructs at 0, 12, and 48 h after exchange into H-buffer. Scale bars of EM and fluorescence microscopy images are 2 μm and 20 μm, respectively. Orb2ΔRBD and Orb2ΔΔRBD droplets disappear giving way to fibrils seen by EM that correlate with large fuzzy objects seen by fluorescence microscopy. In contrast, no fibrils were observed for Orb2B, whose droplets seemed to merge but not fuse over time.

Figure 5. ThT fluorescence and OD measurements confirm that Orb2ΔRBD and Orb2ΔΔRBD droplets disappear over time giving way to cross-β fibrils, whereas Orb2B droplets are stable. Top, ThT fluorescence kinetics for Orb2ΔRBD and Orb2ΔΔRBD were normalized to their maxima. Orb2B kinetics according to the maximum of Orb2ΔΔRBD because the fluorescence intensity did not increase significantly relative to background. Three biological replicates in H buffer are shown in colors, and an HSU buffer control is shown in gray. Bottom, OD600 kinetics as a measure of sample turbidity. Data were normalized to their first point. Where Orb2ΔRBD and Orb2ΔΔRBD show an initial decrease in turbidity, Orb2 turbidity was constant compatible with stable droplet formation.

Phase separation of Orb2ΔRBD and Orb2ΔΔRBD ultimately led to the formation of cross-β fibrils. Next we asked, how does the structure of these fibrils compare with the cryo-EM structure of Orb2 fibrils purified from Drosophila brain (6) and the structure of recombinant Orb2ΔA1,88 fibrils we investigated previously (8)? To answer this question, we expressed and purified U-13C -15N labeled Orb2ΔRBD and Orb2ΔΔRBD. We then prepared fibrils via phase separation for solid-state NMR measurements. One-dimensional 13C spectra of these fibrils are shown in Figure 6. Cross-polarization (CP) spectra detect static protein domains that in the case of cross-β fibrils often coincide with the fibril core. Refocused INEPT spectra, in contrast, are sensitive to dynamic protein regions that are often framing the static fibril core. As seen from the spectra, both Orb2ΔRBD and Orb2ΔΔRBD fibrils have static and dynamic domains. The aliphatic region of the CP spectra of these two fibril types overlaps relatively well indicating that both fibrils have similar structure. Both CP spectra also show His side chain resonances compatible with a His-rich fibril core. However, the small differences between the spectra indicate that their fibril core structures are not exactly the same. The more intense INEPT spectrum of Orb2ΔRBD indicates that these fibrils have larger dynamic domains.

To learn more about the amino acid residues found in the fibril core, we recorded CP-based, 2D 13C-13C DREAM (Fig. 7) spectra that highlight the most immobile residues in the fibrils, which often coincide with its core (8, 24, 25). These spectra allowed us to identify the amino acid residue composition of the core, which included Ala, Asn, Gln, His, Ile, Ser, Pro, Thr, and Val. To evaluate how the structure of these fibrils compared with those extracted from Drosophila by Hervás and coworkers, we predicted the NMR chemical shifts of Orb2B residues 178–204 of their cryo-EM structure (i.e., all but the first and last two residues from PDB access code 6VPS) using the program SHIFTX2 (26) and projected the expected cross-peaks onto our spectra. As can be seen from Figure 7, the predicted Glu Cα-Cβ, Cα-Cγ, and His Cα-Cβ peaks overlap well with the broad peak at about 55 and 32 ppm. In addition, the predicted cross-peak of L198, in the center of the Orb2 core, overlapped perfectly with the Ile Cα-Cβ and Cα-Cγ signals identified in our spectrum. Overall, these data suggest that the Orb2 fibril core determined by cryo-EM is also present in our fibrils. In addition, we identified many intense signals from residues that are not part of the Gln/His-rich core, namely Ala, Asn, Ile, Pro, Thr, and Val. The chemical shift of these resonances is generally compatible with an extended β-sheet conformation.

The 2D DREAM spectra confirm that the two fibrils give spectra that are very similar, indicating a similar core structure.
Nevertheless, we were able to identify few additional cross-peaks in the DREAM spectrum of Orb2AΔRBD that could not be seen in Orb2ΔARBD. These resonances are highlighted with arrows in Figure 7 and come from Thr, Val, and Ile residues. Interestingly, these resonances do not clearly point to an additional fibril core located at the N-terminal region unique to Orb2ΔRBD as could be expected, because Thr is not found in this N-terminus. We are currently working on the site-specific assignment of these samples to determine the location and structure of the additional static domains and their differences.

How do the Glu resonances in our Orb2 fibrils spectra compare with Glu resonances found in spectra of HTTex1 and Sup35p, two proteins that form fibrils with a Glu-rich core? Figure S6A shows the overlay of 2D $^{13}$C-$^{13}$C spectra of Orb2ΔARBD and HTTex1(Q25) fibrils, both of which were formed through phase separation. This overlay shows that the Glu peaks in Orb2ΔARBD do not correspond well to any of the two major Glu peaks (i.e., Glu A and Glu B) in the HTTex1 fibril spectra (27-32). Orb2ΔARBD’s $\gamma_C$ and $\beta_C$ chemical shift is compatible with an extended conformation and in between the shifts observed for Glu A and Glu B in HTTex1. Its side chain $\gamma_C$ shift is significantly higher than what has been observed for Glu A and GluB in HTTex1 and compatible with the minor Glu population in HTTex1 termed Glu C. This analysis suggests that the Glu conformations in the cross-$\beta$ cores of Orb2 and HTTex1 are different. We also compared the Glu resonance assigned in the Q-rich fibril core of Sup35p (33) with our spectra (Fig. S6B). Many of the Glu $\gamma_N-\beta_C$ and $\gamma_N-\gamma_C$ assignments fit well into the broad Glu resonance observed for Orb2ΔARBD suggesting that they have a similar structure.

Discussion

In the present study, we show that Orb2 can phase separate into droplets that quickly mature and transition into cross-$\beta$ fibrils in the absence of the C-terminal RBD. Because Orb2 has a domain structure similar to other RNA-binding proteins that phase separate, droplet formation of Orb2 was predicted to occur (10). However, Majumdar and coworkers showed that intracellular Orb2A puncta showed little mobility when assessed using FRAP assays suggesting that these were self-assembled oligomers rather than droplets (4). We reconcile these two views by showing that Orb2 phase separation does occur. In contrast to other proteins that undergo slow maturation from more liquid to more glass-like droplets with little to no protein mobility, the Orb2 droplets we describe either never go through a liquid phase or this state is so short-lived that we were not able to detect it. In this sense, Orb2 droplets are similar to those formed by the nuclear pore complex protein nucleoporin (34), the protein velo1 found in Balbiani bodies (35) or stress-induced A-bodies (36, 37). In contrast, HTTex1(Q25), which can form protein droplets that mature into fibrils with Glu-rich core similar to Orb2, forms initially liquid-like assemblies (16).

In our hands, phase separation is a necessary requirement to go from soluble Orb2ΔARBD into amyloid fibrils. We previously showed that a shorter fragment of Orb2A (i.e., Orb2A$_{1-88}$) forms cross-$\beta$ fibrils over time in HSU buffer (8). However, the fibril core of Orb2A$_{1-88}$ was not located in the Q/H-rich domain but rather at the very N-terminus unique to Orb2A. In contrast, Orb2ΔRBD fragments did not aggregate in HSU buffer and required phase separation for fibril formation.

Further, NMR spectra of Orb2ΔARBD fibrils are compatible with the presence of the Q/H-rich fibril core described in the recent cryo-EM structure of Orb2 fibrils (6). Our NMR spectra also show that there are additional static domains in Orb2ΔARBD and Orb2ΔARBD fibrils not explained by the Q/H-rich fibril core. These domains contain Ile, Pro, Val, and multiple Ala, Ser, and Thr residues. This amino acid composition does not allow us to locate these domains within the sequence. There is little evidence that they are located in the first eight residues of Orb2A because the prominent Phe peak that is characteristic for fibrils formed by these residues (8) was missing from our spectra. We are currently working on the assignment of these additional regions.

In addition to having slightly different fibril cores, Orb2ΔARBD and Orb2ΔARBD also form droplets that vary in size independent of conditions. Orb2ΔARBD fibrils are highly bundled, whereas Orb2ΔARBD fibrils show little tendency to bundle. Consequently, the different Orb2 N-termini must play a role in droplet size and fibril bundling.

How do the NMR spectra of Orb2ΔARBD fibrils compare with the NMR spectra of HTTex1 and Sup35p fibrils? This comparison is of interest because the Q/H-rich fibril core of Orb2 is reminiscent of the polyQ fibril core of HTTex1 and the Glu-rich core of Sup35p. Our comparison of the Glu cross-peaks of HTTex1 and Orb2ΔARBD fibril spectra indicates that the conformation of the Glu residues in both fibril cores is distinct, which would be in line with the fact that the Orb2 fibril core is an in-register parallel $\beta$-structure (6), whereas HTTex1 is not (38). This might also explain why the assignment of Glu resonances of Sup35p fibrils (33) fits our spectra better than HTTex1, since Sup35 fibrils were shown to form in-register parallel $\beta$-sheets (39). Ultimately, the best fit to the Glu resonances in our spectra was obtained by predicting chemical shifts from the cryo-EM structure of the Orb2 fibril core.

Figure 6. 1D $^{13}$C spectra of Orb2ΔARBD and Orb2ΔARBD fibrils indicate that both have similar structure with larger intrinsically disordered regions found in Orb2ΔARBD. Cross-polarization (CP, black) spectra detect static regions of the sample typically located in the fibril core. Histidine side chain resonances are highlighted in the aromatic region of the CP spectrum between 150 and 100 ppm. The refocused INEPT spectrum (INEPT, blue) detects highly dynamic, intrinsically disordered regions that are part of the fibril. Overlay of the two CP spectra indicates that both fibrils have similar structures.
suggesting that our fibrils are similar in structure to those found in vivo.

Where Orb2 fragments without the C-terminal RBD form cross-β fibrils after droplet formation, full-length Orb2B remains in a state of matured droplets, which merge but neither completely fuse nor transition into cross-β fibrils. This observation is compatible with previous in vivo work showing that Orb2B alone cannot form functional aggregates that activate the translation of target mRNA (4). Because Orb2BΔRBD forms fibrils via droplet formation, the C-terminal RBD likely plays an important role in preventing fibril formation of Orb2BA. Similar behavior has been observed for the polyQ-containing, RNA-binding protein Whi3, which can phase separate in the presence of RNA but forms more filamentous structures when its RRM is deleted (17).

The cross-β fibril of Orb2 has been shown to be a functional state important in LTM (6); however, the role of phase separation in LTM is unclear at this point. We speculate that phase separation might be the mechanism that induces fibril formation of Orb2 monomers in the cell. Orb2 aggregation and LTM in Drosophila depend on the increased half-live and thereby concentration of cellular Orb2A via phosphorylation (7). This increase in concentration could trigger phase separation. However, there may be other potential mechanisms that could induce Orb2 phase separation in the cell. Fibril formation via LLPS has been described for several proteins involved in neurodegenerative diseases (16, 20, 40). LLPS might be similarly important and a quite general mechanism for inducing fibril formation of functional amyloids.

In summary, our data establish droplet formation as a possible mechanism for inducing Orb2 fibril formation in vivo. This mechanism needs to be confirmed in vivo. Based on our ability to make monomeric Orb2 and induce its phase transition, our future work aims at building a functional Orb2 protein complex in vitro and describe the structural interactions of its components.
Experimental procedures

**Protein constructs**

The sequences for Orb2ΔRBD and Orb2ΔRBD were codon optimized for expression in *E. coli*, synthesized, and cloned into pET28b expression vectors with a C-terminal 6x histidine-tag (6xHis-tag) by Genscript USA Inc. Full-length Orb2B was cloned from a pDEST vector (provided by the lab of Dr Kausik Si) into a pET28b vector.

**Protein expressions and purification**

*Orb2*

The Orb2B was expressed in Rosetta 2 *E. Coli* (DE3), whereas Orb2ΔRBD and Orb2ΔRBD were expressed in T7 express or BL21 (DE3) (EMD Millipore). A single colony was used to inoculate 50 ml LB medium with the appropriate antibiotic (chloramphenicol 35 mg/ml and kanamycin 50 mg/ml) and grown overnight at 30 °C. The next day, 1–5 ml of this culture was expanded into 500 ml LB with the appropriate antibiotic and cells were grown at 37 °C to an OD600 of 0.6. Protein expression was subsequently induced with the addition of 1 mM IPTG and cells were grown for an additional 18–20 h at 18 °C. Cells were then harvested *via* centrifugation in a Sorvall SLC-6000 rotor (Thermo Fisher Scientific) at 4000 rpm for 20 min at 4 °C and were used immediately or stored at −80 °C for future use.

Cell pellets ranging from 2.5 to 3 g were resuspended in HSU buffer (1 M urea, 100 mM KCl, 10 mM HEPES, 0.1% v/v Tween-20 and 0.05% v/v β-mercaptoethanol, pH 7.6) containing 1 mg/ml lysozyme and 2 µl/ml of 10X DNase I. Cells were then sonicated using a Q125 ultrasonic homogenizer (Qsonica), and the cell lysate was centrifuged at 20,000 rpm for 20 min at 4 °C using a Sorvall SS-34 rotor (Thermo Fisher Scientific). The supernatant was collected and filtered through a 0.22 µm vacuum filtration system (Corning Inc). The 6xHis-tagged protein samples were loaded onto pre-equilibrated Ni2+ affinity resin (HisTrap HP 5 ml, GE Healthcare). The column was sequentially washed with HSU buffer containing 0.5% triton-X, 500 mM NaCl, 20 mM imidazole, and 1 M NaCl (each was wash five column volumes). The protein was then eluted with 500 mM imidazole and 1 M NaCl in HSU buffer. Protein elution was traced using 280 nm UV absorption. Fractions containing the protein of interest were pooled together and loaded onto a pre-equilibrated size-exclusion column (HiPrep 16/60 Sephacryl S-300, GE Healthcare). The protein was then eluted using HSU buffer, and the protein fractions were used immediately or frozen in liquid nitrogen and stored at −80 °C.

*HTTex1*

HTTex1(ΔRBD) was expressed and purified as described earlier (28, 41). Reversed-phase purified and lyophilized HTTex1(ΔRBD) was dissolved in 0.5% trifluoroacetic acid in methanol (TFA-MeOH) and dried in a borosilicate glass tube using nitrogen. The dried protein film was reconstituted in 20 mM phosphate buffer, pH 7.4, containing 150 mM NaCl and 10% dextran such that the final protein concentration was 300 μM. The solution was incubated at room temperature for 14 h. Under these conditions, HTTex1(Q25) forms fibrils via phase separation. These fibrils were harvested by centrifugation at 40,000 rpm for 30 min in a Beckman Coulter TLA-100.3 rotor. The fibrils were washed with 20 mM phosphate buffer, pH 7.4, containing 20 mM NaCl before they were packed into a solid-state NMR rotor.

**Fluorescence and DIC wide-field microscopy**

Protein samples that were used for microscopy either eluted from SEC in a HSU buffer that contained 5 mM TCEP or exchanged into a HSU buffer with 5 mM TCEP using a desalting column. The protein samples were then labeled with either 5 mM thiol-reactive or 5 mM amine-reactive fluorescent dyes over night at 4 °C with light shaking. Orb2B and Orb2ΔRBD were labeled with thiol-reactive Oregon Green 488 through maleimide chemistry (Thermo Fisher). Due to the lack of cystine residues in the Orb2ΔRABD protein construct, it was labeled with lysine-reactive succinimidyl ester Oregon Green 488 (Thermo Fisher). The excitation and emission maxima of these fluorescent bioconjugates are 496 nm and 524 nm, respectively. To induce droplet formation, protein was either exchanged into H buffer (10 mM HEPES, 0.03% v/v NaN3 and 0.05% v/v β-mercaptoethanol, pH 6.5), or into HSU buffer, at pH 6.5 followed by the addition of 10% v/v PEG 8000 or 1:10 m/m PolyA:protein.

Fluorescence images were taken by exiting at 488 nm and imaging at 505 nm using a Zeiss AxioImager wide-field light microscope with white light laser. DIC microscopy images were also recorded on Zeiss AxioImager microscope seconds after the corresponding fluorescence images were taken, by switching from a Zeiss Filter set 38 HE (optimized for emission at 488 nm) to a default Zeiss DIC filter. Images of protein droplets were captured at 0, 12, and 48 h post exchanged into the H buffer. Images were analyzed using Fiji image analysis software (42, 43) including the BioVoxxel plugin. Microscopy was repeated on three biological replicates.

**Fluorescence recovery after photobleaching (FRAP)**

Oregon Green labeled protein samples were prepared analogous to those described above. Protein samples were then exchanged into H buffer with 5 mM TCEP using a PD-10 G-25 media desalting column and FRAP measurements were done using Leica-SP8X confocal laser scanning microscope with a 63× oil immersion objective. Droplets were bleached with an average diameter of 5 nm, to avoid whole-droplet bleaching artifacts in droplets of smaller diameters (44). Bleached regions of interest (ROI) of 0.5 µm were created with a pinhole Argon laser with a scanning speed ranging from 50 to 150 ms using the Leica software. FRAP data were analyzed and plotted with in-house python scripts using the pandas, numpy, scipy, and matplotlib libraries. Recovery curves ±SD were generated by normalizing and averaging across three biological replicates.
Thioflavin T fluorescence assay

Protein samples were exchanged into an H buffer using a PD-10 G-25 media desalting column (GE Healthcare) to induce droplet formation. In total, 200 μl of 10–20 μM protein was mixed with ThT at a final concentration of 50 μM and added to a clear flat-bottom 96-well plate (Greiner Bio-One). The solution was then excited at 442 nm, and the emission at 482 nm was measured every 10 min for 80–160 h using an Eppendorf AF2200 plate reader at 25 °C. Three independent measurements were recorded for each protein and normalized and plotted using in-house python scripts (available upon request).

Electron microscopy

Electron microscopy specimens were prepared by submerging a Formvar carbon-coated copper grid (Electron Microscopy Sciences) in 10 μl of protein solution and incubating it for 5 min. These grids were then stained with 10 μl of a 1% uranyl acetate solution for 2 min at room temperature. The grids were then tapped on two additional 10 μl uranyl acetate drops and finally, rinsed in deionized water. Filter paper was used to remove the excess of stain and water before imaging. The negatively stained samples were examined for droplets or fibrils with a JEOL JEM-1400 TEM electron microscope. Images were acquired using a Gatan Orius digital camera at magnification of 5000–10,000×. Fibril images were analyzed using ImageJ including the FibrilJ plugin (45).

Solid-state NMR spectroscopy

NMR spectra were recorded on an Agilent DD2 600 MHz solid-state NMR spectrometer using a T3 1.6 mm probe. All hard 1H and 13C radio frequency (rf) pulses had amplitudes of 200 kHz and 100 kHz, respectively. 1H-13C CPs were done with 60 kHz rf-amplitude on 13C and a 1H rf-amplitude that was larger by the MAS frequency. A 10% amplitude ramp was used to remove the excess of stain and water before imaging. One-dimensional CP, DE, and refocused INEPT spectra were recorded at 30 kHz MAS using a spectral width of 200 kHz in both dimensions and a mixing time of 4.5 ms. For spectra were recorded at 50 kHz MAS with a spectral width of 50 kHz and a mixing time of 50 ms. For Orb2B 500 indirect, complex increments were recorded with 64 acquisitions each.

All spectra were processed using Lorentz to Gauss transform window functions. Adamantane spectra were used to reference the chemical shifts externally to DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) (49). The spectra were analyzed using CARA (26) and plotted using in-house python scripts (available upon request) based on the numpy, matplotlib, and nmrglue packages (50, 51).

Data availability

Spectroscopy and microscopy raw data are available from the corresponding author upon request.

Supporting information—This article contains supporting information.

Author contributions—K. A., A. S. F., C. H., S. G., S. A. C., A. R., and A. B. S. investigation; K. A., A. S. F., C. H., and S. G. methodology; K. A., C. H., and A. B. S. writing-original draft; A. S. F. and A. B. S. conceptualization; A. S. F. and A. B. S. data curation; A. S. F. and A. B. S. formal analysis; S. A. C. and A. B. S. supervision; S. A. C. and A. B. S. project administration; A. R. resources; A. B. S. funding acquisition; A. B. S. visualization; A. B. S. writing-review and editing.

Funding and additional information—A.B.S. would like to acknowledge funding from the National Institutes of Health (R01NS084345, R01GM110521). A.S.F. would like to acknowledge funding from the National Institutes of Health (F31GM120858). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: CP, cross-polarization; CPEB, cytoplasmic polyadenylation element binding; DIC, differential interference contrast; EM, electron microscopy; FRAP, fluorescence recovery after photobleaching; FUS, fused in sarcoma; HSU, HEPES–salt–urea; HTT, huntingtin exon-1; LPPS, liquid–liquid phase separation; LTM, long-term memory; RBD, RNA-binding domain; rf, radio frequency; ROI, regions of interest; RRM, RNA recognition motif; ThT, Thioflavin T.

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