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RNA buffers the phase separation behavior of prion-like RNA binding proteins

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Prion-like RNA binding proteins (RBPs) such as TDP-43 or FUS are largely soluble in the nucleus, but form solid pathological aggregates when mislocalized to the cytoplasm. What keeps these proteins soluble in the nucleus and promotes aggregation in the cytoplasm is still unknown. We report here that RNA critically regulates the phase behavior of prion-like RBPs. Low RNA/protein ratios promote phase separation into liquid droplets, whereas high ratios prevent droplet formation in vitro. Reduction of nuclear RNA levels or genetic ablation of RNA binding causes excessive phase separation and the formation of cytotoxic solid-like assemblies in cells. We propose that the nucleus is a buffered system in which high RNA concentrations keep RBPs soluble. Changes in RNA levels or RNA binding abilities of RBPs cause aberrant phase transitions.

The intracellular environment is organized into membraneless compartments, which have been termed biomolecular condensates because they form by liquid-liquid phase separation (1, 2). These condensates often contain RNA binding proteins (RBPs) with distinctive domains, so-called prion-like domains, which are structurally disordered and contain polar amino acids (3) (Fig. 1A). Interactions between prion-like domains and additional interactions between RNAs and RNA binding domains drive the assembly of prion-like RBPs by phase separation (4, 5). However, several prion-like RBPs such as FUS, TDP43, and hnRNPA1 can also undergo an aberrant transition from a liquid-like state into solid aggregates that has been linked to neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) (4–6). One important aspect of these diseases is that aggregate formation is strongly associated with their subcellular location. Aggregates in patient neurons are usually found in the cytoplasm, whereas the nucleus is devoid of the aggregating protein (7–10), although there are some noteworthy exceptions (11). The importance of cytoplasmic localization is also highlighted by the fact that disease-causing mutations frequently affect the nuclear partitioning of prion-like RBPs (12, 13). Protein mislocalization to the cytoplasm causes loss-of-function and gain-of-function phenotypes that are thought to underlie disease (14–17). Importantly, genetic re-localization of FUS to the nucleus in

yeast strongly decreased its toxicity (18). This suggests that localization of FUS to the nuclear environment suppresses its pathological behavior. This raises two important questions: What prevents prion-like RBPs from forming solid-like aggregates in the nucleus? And why do these RBPs form aggregates in the cytoplasm?

To provide answers for these questions, we investigated the phase behavior of several prion-like RBPs (Fig. 1A). First, we determined the nuclear concentration of these proteins. The values ranged from 0.2 μ M for TAF-15 to 42.3 μ M for hnRNPA1 (Fig. 1, B to D, and supplementary methods). Next, we purified these proteins as GFP fusions and added them to a physiological buffer. At a concentration similar to the nuclear concentration (7.6 μ M), FUS phase separated into droplets (Fig. 1, E and F). This is in contrast to living cells, where only 1% of the nuclear FUS protein was contained in condensates (Fig. 1F), which are paraspeckles (19). The remaining 99% of nuclear FUS protein was diffusely localized. Similar observations were made for TDP43, EWSR1, TAF15, and hnRNPA1 (Fig. 1G, lower panel). This suggests that although the protein concentration is high enough for phase separation in the nucleus, there is an additional nuclear factor that prevents phase separation.

We hypothesized that nuclear RNA could regulate the

phase behavior of prion-like RBPs. To test this idea, we performed an *in vitro* phase separation assay with FUS in the presence of total RNA (Fig. 2A). In agreement with previous work (20–22), we found that low amounts of RNA promoted liquid droplet formation (Fig. 2B and fig. S1, A to D). RNA-containing droplets contained a higher FUS concentration than RNA-free droplets and they appeared slightly more viscous (fig. S2, A to C). Remarkably, however, upon further increasing the RNA/protein concentration ratio, the droplets became smaller and finally dissolved (Fig. 2, A and B, and fig. S3). Addition of RNase A resulted in droplet reappearance (Fig. 2D and figs. S4A, panels on the right, and S5), indicating that droplet solubilization depends on intact RNA. Similar results were obtained for EWSR1, TAF15, hnRNPA1, and TDP43 (Fig. 2C). Thus, we conclude that high RNA/protein ratios prevent phase separation, whereas low ratios promote phase separation.

We next tested whether different types of RNAs differ with respect to their ability to dissolve FUS droplets. Individually, rRNA, tRNA or a non-coding RNA that is known to bind to FUS (Neat1) were all able to solubilize FUS droplets, suggesting a general effect, but smaller RNAs were more potent than larger ones (fig. S4, A to D). Secondary structure was important for enriching FUS in droplets, consistent with previous work (20), but both secondary structure (fig. S4, A to E) and binding affinity (fig. S6) only slightly affected droplet solubilization. We next asked if the cellular RNA concentration is high enough to suppress phase separation of FUS. We estimated that the nuclear RNA concentration is about 10.6-fold higher than required for droplet dissolution *in vitro* (fig. S7 and supplementary methods). However, about 1% of nuclear FUS formed condensates (Fig. 1E), called paraspeckles, by binding to the non-coding RNA Neat1 (19). To test whether Neat1 could nucleate FUS droplets in the presence of a high background concentration of RNA, we added Neat1 RNA to a FUS sample that had been solubilized with tRNA. Remarkably, this led to a reappearance of FUS droplets (Fig. 2E and fig. S4F). We attribute this to the ability of Neat1 to form large RNA assemblies (fig. S4C), which subsequently recruit FUS. This suggests that highly structured RNAs such as Neat1 could act as scaffolds that promote the nucleation of condensates in the high RNA concentration environment of the nucleus. A similar scenario may apply for stress granules in the cytoplasm, which contain high amounts of structured polyA-mRNA (fig. S8).

To test experimentally whether the high nuclear RNA concentration keeps FUS soluble, we microinjected RNase A into the nucleus of HeLa cells. Immediately after RNase A injection, FUS-GFP condensed into many liquid-like droplets (Fig. 3A, fig. S9, and movie S1), and this effect was not due to a general loss of nuclear integrity (figs. S10 and S11). As an al-

ternative approach to decrease RNA/protein ratio, we injected purified FUS-GFP into the nucleus, which led to an immediate increase in the number and size of nuclear FUS assemblies (fig. S12). RNase A microinjection into the nucleus also triggered rapid phase separation of hnRNPA1, EWSR1, TDP43 and TAF15 (figs. S13 and S14). To investigate whether FUS forms complexes with RNA in living cells, we employed fluorescence correlation spectroscopy (FCS). We identified two populations of FUS, one slow moving and one fast moving (see supplementary methods for details). We estimate that the amount of slow FUS in the nucleus is 10-fold greater than in the cytoplasm (Fig. 3, B to D; fig. S15, A to E; and supplementary methods). The fraction of slow FUS in the nucleus was decreased by mutating RNA binding domains in FUS (FUS-mutRRM/ZnF, FUS-mutRGG2) and was further decreased by removing all RNA binding domains (FUS-PLD) (Fig. 3, E and F, and figs. S15, F to I; S16; and S17). This indicates that a large fraction of nuclear FUS is complexed with RNA. To further investigate the solubilizing role of RNA, we performed genetic experiments with transfected FUS-GFP encoding plasmids. We observed that the number of nuclear FUS assemblies was directly proportional to the nuclear FUS concentration (Fig. 3G). We further found that FUS variants with a weaker capacity to bind RNA generally formed a higher number of assemblies (Fig. 3, H to J, and figs. S16 and S17). Thus, reduced RNA binding directly affects the solubility and decreases the saturation concentration at which FUS phase separates.

We showed previously that FUS *in vitro* initially forms liquid-like assemblies, but these mature into more solid-like gels and fibrils over time (5). These solid-like states are reminiscent of pathological aggregates in ALS (8, 9). Thus, we next tested whether addition of RNA prevents the formation of fibrils *in vitro*. Remarkably, addition of RNA kept the droplets in a soluble state, and fibers were not seen (Fig. 4A). We next investigated whether RNA also changes the material properties of FUS assemblies *in vivo*. We set up an *in vivo* aging assay, in which we microinjected RNase A into HeLa cells and then followed the dynamics of the liquid-like drops. After about 30 min, the FUS drops no longer fused (Fig. 4, B and C, and movie S2), but stuck together in large clusters, similar to phenotypes seen previously *in vitro* (Fig. 4D and movie S3). A change in the material properties was also evident from photobleaching experiments (Fig. 4, E and F, and fig. S18, A and B). Similar results were obtained for TDP43, but the transition was much faster (fig. S19 and movie S4). We next used a genetic approach to test how RNA binding affects the dynamics of FUS *in vivo*. Complete abrogation of RNA binding resulted in a dramatic decrease of mobile FUS (Fig. 4, G, H, and J, and fig. S18C) and the formation of sticky droplet clusters (Fig. 4K). Lastly, we employed a chemical approach using the dye F22 to reduce RNA binding (23). In F22-treated cells,

the fraction of RNA-bound FUS was strongly diminished (fig. S20 and supplementary methods) and this caused a strong reduction in the mobile fraction of FUS (Fig. 4, I and J, and fig. S18, E and F). Together, these findings show that RNA keeps condensates formed by prion-like RBPs in a dynamic state and prevents the formation of solid assemblies that could cause disease.

To investigate how reduced RNA binding affects cell viability, we transiently transfected HeLa cells with wild type and mutant FUS and monitored cell survival. Expressing a nuclear variant with reduced RNA binding (FUS-mutRRM/ZnF) affected the rate of cell death only slightly (Fig. 4, L and M, and fig. S16), presumably because the high nuclear RNA concentration compensates for the genetic defect. However, targeting the very same variant to the cytoplasm by removing the NLS (FUS-mutRRM/ZnFΔNLS) led to a strong increase in cell death, which was likely caused by the high propensity of this variant to form solid aggregates (figs. S21 to S23). Importantly, this increase was not observed for a cytosolic variant of FUS with normal RNA binding (FUSΔNLS). Thus, we conclude that excessive phase separation in the cytoplasm due to low RNA levels induces a pathological state that leads to cell death.

One of the key questions in protein misfolding diseases caused by prion-like RBPs is why these proteins aggregate in the cytoplasm, rather than the nucleus. In this paper, we show that this is due in part to different RNA concentrations in the cytoplasm and the nucleus. More specifically, the higher RNA concentration in the nucleus suppresses phase separation of prion-like RBPs, while the lower concentration in the cytoplasm stimulates phase separation. Therefore, by keeping the proteins in the nucleus, the cell ensures that they are in a soluble and non-toxic state, shuttling them out of the nucleus only upon stress. After removal of stress, the proteins shuttle back into the nucleus where they are again kept in a soluble and well-mixed state. The consequence is that any insult that prolongs the stress will tend to increase the propensity for aggregation because they prolong the time these proteins spend in the cytoplasm (fig. S24).

Our data also has important implications for the control of phase separation in cells. We find that paraspeckles are likely induced by locally concentrating Neat1 RNA, which has a strong affinity for FUS. Similar phenomena have been seen for nucleoli, who depend on local production of ribosomal RNA (24). Therefore, at least in the nucleus, local production of RNAs with high affinity for specific RBPs could provide the specificity to induce phase separation in a system buffered by non-specifically interacting RNA. Thus, the phase behavior of FUS in the nucleus is likely controlled by many different types of specific and nonspecific RNAs. This situation does not apply to the cytoplasm. Here, the RNA concentration is

only slightly higher than the concentration required to suppress phase separation in vitro, and there is no buffering of phase separation by RNA. This results in a much higher propensity of FUS to phase separate. However, it also brings with it an increase in the tendency of FUS to form cytotoxic solid-like aggregates. Indeed, high amounts of RNA have been shown to suppress the toxicity of prion-like RBPs (25–28). Moreover, there are many cases of familial ALS in which mutated prion-like RBPs mislocalize to the cytoplasm and form cytotoxic aggregates. For example, mutations in FUS have been shown to increase its cytoplasmic concentration, thus causing the formation of aberrant solid-like aggregates (8, 9, 29–31). We predict that local changes in RNA levels or RNA binding abilities of proteins are frequent causes of age-related protein misfolding diseases.

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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S24

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Movies S1 to S4

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Fig. 1. Prion-like RBPs phase separate at their physiological concentrations. (A) Domain structure. PLD: prion-like domain; RRM: RNA recognition motif; RGG: arginine- and glycine-rich region; ZF: zinc finger; NLS: nuclear localization sequence. (B) Representative images of immunostained HeLa cells. Dashed line indicates nuclear boundary. (C) Quantification of the nuclear enrichment of RBPs. Error bars represent SD. Scale bar, 5 μm . (D) Calculated cellular and nuclear concentrations of RBPs in HeLa cells. (E) Left: Live HeLa cell nucleus expressing GFP-tagged FUS from a BAC. Arrows point to paraspeckles. Right: FUS-GFP phase-separated in vitro at 7.5 μM . Scale bar, 2 μm . (F) Quantification of the fraction of FUS present in the condensed or the soluble state in vivo and in vitro. Error bars represent SD. (G) Top panel: HeLa cell nuclei expressing GFP-tagged RBPs from BACs. White arrows indicate condensates. Lower panel: purified RBPs phase separate at their respective nuclear concentrations. Scale bar, 2 μm .

Fig. 2. RNA regulates the phase behavior of prion-like RBPs. (A) Representative images of purified FUS-GFP (5 μ M) in vitro in the presence of total RNA. (B) Quantification of the fraction of condensed FUS-GFP. Error bars represent SD. (C) In vitro phase separation assay with EWSR1, TAF15, hnRNPA1 or TDP43 in the presence of total RNA. (D) Addition of RNase A to a sample of FUS-GFP (5 μ M) solubilized with 300 ng/ μ l of total RNA. Scale bar, 2 μ m. (E) Left: FUS-GFP (5 μ M) solubilized with 800 ng/ μ l tRNA in vitro. Right: Addition of 100 ng/ μ l Neat1 RNA triggers FUS phase separation in the presence of tRNA (800 ng/ μ l).

Fig. 3. RNA keeps prion-like RBPs in a soluble state in the nucleus. (A) Montage of a HeLa cell expressing FUS-GFP after microinjection with RNase A. Scale bar, 2 μ m. (B) HeLa cells expressing FUS-GFP. White lines indicate cell outlines, orange lines indicate nuclear outlines and boxes indicate regions of FCS measurements. Scale bar, 5 μ m. (C) Autocorrelation curves obtained from FCS of FUS-GFP. (D) Quantification of the amount of slow FUS (see methods). Error bars represent SD. (E) Autocorrelation curves obtained from FCS of FUS-GFP variants in the nucleus. (F) Quantification of slow FUS in the nucleus, obtained from two component fits of the curves in E (see methods). Error bars represent SD. (G) HeLa cells showing variable FUS-GFP expression. (H) HeLa cells expressing different FUS-GFP variants with mutations in RNA binding domains (fig. S16). (I) Number of nuclear FUS-GFP assemblies per cell as a function of mean protein intensity (AI) ($n > 30$). (J) Number of cells with more than 100 nuclear assemblies, $n > 100$ cells. Error bars represent SD. * $P < 0.05$ and ** $P < 0.01$ as compared to Wt.

Fig. 4. RNA regulates aberrant liquid-to-solid phase transitions of prion-like RBPs. (A) In vitro phase separated FUS(G156E)-GFP in the absence or presence of total RNA after 24 hours. Scale bar, 2 μ m. (B) FUS-GFP expressing HeLa cell nucleus after RNase A microinjection. (C) Montage of FUS-GFP droplets formed after RNase A microinjection. The droplets fuse in the first 5 min (blue box), but not after 30 min, resulting in “sticky droplets” (red box). (D) Montage of FUS-GFP droplets formed in vitro (7 μ M). Fusion of freshly formed droplets is compared to 3-hour old droplets. (E) Fluorescence recovery after photobleaching of nuclear FUS-GFP assemblies less than 5 min (blue box) or more than 30 min (red box) after RNase A microinjection. (F) FRAP of nuclear FUS-GFP assemblies in HeLa cells after RNA degradation as shown in B and E ($n > 10$). (G to I) FRAP of nuclear assemblies in HeLa cells expressing full-length FUS (G, I) or FUS-PLD (H). The cell in (I) was also treated with F22. (J) Mobile fraction of photobleached assemblies in G-I ($n > 15$). Error bars represent SD. (K) 3D rendering of FUS-PLD nuclear assemblies. The inset shows aberrant “sticky droplets”. Scale bar, 1 μ m. (L) Time series to track the life time of FUS-GFP HeLa cells. H2B-mCherry was used to detect cell death. (M) Quantification of the fraction of cells undergoing cell death.