mRNA structure determines specificity of a polyQ-driven phase separation

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RNA promotes liquid-liquid phase separation (LLPS) to build membraneless compartments in cells. How distinct molecular compositions are established and maintained in these liquid compartments is unknown. Here, we report that secondary structure allows messenger RNAs (mRNAs) to self-associate and determines whether an mRNA is recruited to or excluded from liquid compartments. The polyQ-protein Whi3 induces conformational changes in RNA structure and generates distinct molecular fluctuations depending on the RNA sequence. These data support a model in which structure-based, RNA-RNA interactions promote assembly of distinct droplets and protein-driven, conformational dynamics of the RNA maintain this identity. Thus, the shape of RNA can promote the formation and coexistence of the diverse array of RNA-rich liquid compartments found in a single cell.

Formation of non–membrane-bound organelles through the condensation of macromolecules is a recently appreciated mechanism of intracellular organization. These liquid-like condensates form through liquid-liquid phase separation (LLPS) and are found are the cytoplasm and nucleus (1–2). A fundamental unsolved problem is how liquid droplets recruit distinct constituents and retain independent identities. RNA can drive LLPS and modulates the material properties of droplets (3–6), but it is unknown whether RNA controls the identity and maintenance of coexisting liquid compartments. Here, we show that mRNA secondary structure is required for droplet identity through directing interactions between mRNAs and RNA-binding proteins. Whi3, a polyQ-containing, RNA binding protein first identified in Saccharomyces cerevisiae (7), functions in morphogenesis, memory of mating, and stress responses, where it forms aggregates and associates with RNA-processing bodies (8–11). The homolog in the filamentous fungus Ashbya gossypii has an RNA recognition motif (RRM) and an expanded polyQ tract (fig. S1A), and both regions promote self-association. In vitro, Whi3 polyQ-dependent LLPS is driven by specific RNAs encoding regulators of either cell cycle (e.g., the cyclin CLN3) by specific RNAs encoding regulators of either cell cycle (e.g., the cyclin CLN3) or actin (e.g., the formin BNI1 and SPA2) (3). Distinct types of Whi3 droplets form in Ashbya cells: perinuclear CLN3 droplets and BNI1 droplets at sites of polarized growth at cell tips (12, 13) (Fig. 1A and movie S1). These two types of droplets have different Whi3 levels and Whi3 incorporation rates (Fig. 1, B and C), suggesting that they are structurally distinct. The distinct droplet properties may depend on extrinsic features of the local cytosolic microenvironment or arise due to different droplet constituents. CLN3 and BNI1 mRNAs minimally colocalized in the cytoplasm by single-molecule (sm) RNA fluorescence in situ hybridization (smFISH), although they were occasionally coexpressed by the same nucleus (Fig. 1, D and F). The lack of colocalization suggests there are intrinsic, compositional differences between droplets. In contrast, mRNA of the polarity regulator SPA2, significantly colocalized with BNI1 mRNAs, especially at growth sites (Fig. 1, E and F). Thus, mRNAs encoding functionally related proteins colocalize, whereas functionally unrelated mRNAs do not. How can distinct Whi3 mRNA droplets segregate to different droplets in a common cytoplasm?

To address this question, we employed a reconstruction system to investigate whether mRNA sequence was sufficient to generate droplet individuality (Fig. 2A). In vitro, as in cells, droplets composed of BNI1 mRNA displayed higher Whi3 to RNA molar ratios than droplets made with CLN3 mRNA (fig. S1B). Notably, when CLN3 mRNA was added to Whi3 droplets made with BNI1 mRNA, CLN3 preferentially assembled into new droplets, rather than incorporating into BNI1 droplets (Fig. 2, B and C, and fig. S1C). In contrast, BNI1 mRNA readily incorporated into preformed droplets (Fig. 2, B and C). Notably, SPA2 mRNA incorporated into BNI1 droplets (Fig. 2, B and C), and CLN3 did not incorporate into SPA2 droplets (fig. S1D). Thus, as in cells, cyclin and polarity mRNAs assemble into distinct and immiscible droplets in vitro, indicating that droplet identity is encoded by the mRNA.

mRNA sequences could influence droplet identity by favoring homotypic or specific heterotypic interactions between RNA molecules. To test for specific RNA-RNA interactions, we used a protein-free system to induce electrostatic-mediated phase transitions of the mRNA (14), where all mRNAs were capable of homotypic assembly into liquid or gel-like droplets (Fig. 2D). Strikingly, CLN3 mRNAs had minimal colocalization with BNI1 or SPA2 mRNAs, whereas BNI1 and SPA2 were significantly more colocalized (Fig. 2, E to G). Thus, sequence-encoded features of the mRNA can underpin the assembly of distinct, immiscible structures.

We next investigated which features of the mRNA sequence generate specificity. An mRNA with scrambled CLN3 coding sequence (cln3 scr) with intact Whi3 binding sites formed Whi3 droplets (fig. S1E) but no longer showed specificity (Fig. 3, A and C). Because the length, nucleotide composition, and Whi3 binding sites were identical, we hypothesized that the secondary structure could promote specificity. CLN3 mRNA heated to 95°C to disrupt secondary structure also readily incorporated into Whi3-BNI1 droplets (Fig. 3, A and C). Melted CLN3 mRNA that was slowly refolded (CLN3 refold) showed significantly less recruitment than melted but more than native CLN3 (Fig. 3, A and C). Mixing between melted CLN3 and melted BNI1 occurred in the presence of Whi3 and in RNA-only reactions, suggesting that mixing is initiated by RNA-RNA interactions (fig. S2). Thus, specificity information in CLN3 mRNA can be eliminated by disrupting secondary structure.

To identify what features of CLN3 mRNA secondary structure promote specificity, we performed selective 2′-hydroxyl acylation analyzed by primer extension and mutational profiling (SHAPE-MaP), which identifies highly flexible regions in RNA (15), to determine secondary structure changes on native, refolded, and scrambled CLN3 mRNA (Fig. 3D and fig. S3, A and B). The first 400 nucleotides in the CLN3 sequence exhibited especially low SHAPE reactivity (fig. S3C, purple shaded regions), suggesting many paired nucleotides and a highly folded structure. Refolded CLN3 had a significant increase in SHAPE reactivity compared with native CLN3 (fig. S3A) (P < 0.001, Wilcoxon rank sum test), indicating a transition to a more unstructured state (Fig. 3, D and E). Melting and refolding thus allows the RNA to sample different conformations from those formed during transcription. As expected, cln3 scr showed a different SHAPE profile with dramatically altered secondary structure (Fig. 3, D and E, and fig. S3B).

We hypothesized that secondary structure influences mRNA sorting, because stem loops may selectively display or mask sequences capable of hybridizing with other RNAs. CLN3 contains five complementary regions to BNI1 (fig. S4A),...
most of which had low SHAPE reactivity and therefore were more structured (fig. S4B), suggesting that these regions are inaccessible for hybridizing with BNI1. We hypothesize that these regions became available to pair with BNI1 when CLN3 is melted, causing the structure-dependent loss of droplet specificity. To test this hypothesis, oligonucleotides (i.e., oligos) complementary to these regions were added to melted CLN3 and significantly decreased the coassembly with BNI1, restoring the formation of distinct CLN3 droplets (Fig. 3, B and C). Additionally, cln3sm, a mutant perturbing structure and exposing complementarity, colocalized with BNI1 transcripts in vitro and at polarity sites in cells (Fig. 3F and fig. S5) (>60% tips colocalized). Thus, secondary structure can regulate RNA sorting into distinct droplets through altering the capacity to form intermolecular interactions.

We next asked whether exposed complementarity explains coassembly of BNI1 and SPA2 into the same droplets. Indeed, SHAPE-MaP analysis of BNI1 and SPA2 showed complementary regions between these colocalizing mRNAs having significantly higher SHAPE reactivity and less structure compared with the CLN3/BNI1 regions (figs. S4 and S6) (P < 0.002, t test). Addition of complementary oligos to these regions disrupted colocalization in the presence of Whi3 and in RNA-only reactions (fig. S7, A and B). We predicted that CLN3 may self-assemble and indeed cln3 codon, a CLN3 mutant whose codons have been randomized but Whi3 binding sites remain intact, does...
not colocalize with endogenous CLN3 mRNA in cells, further supporting RNA-RNA interactions in coassembly of related RNAs (fig. S7C). These data suggest that RNA-RNA interactions based on intermolecular hybridization direct RNAs into the same or different droplets.

Does Whi3 protein influence the identity of droplets? The majority of Whi3 binding sites are exposed on stem loops in CLN3, BNI1, and SPA2 (Fig. 3E and figs. S8 and S9). Notably, refolding or scrambling the CLN3 sequence rearranges the presentation of Whi3 binding sites (Fig. 3E). Therefore, RNA secondary structure may influence Whi3 binding and contribute to droplet composition and immiscibility in addition to RNA complexing. SHAPE-MaP analysis of CLN3 mRNA in the presence of Whi3 supports that Whi3 binding sites are occupied by protein (Fig. 4A).

**Fig. 2. Polarity and cyclin complexes segregate in vitro.** (A) Experimental schematic of in vitro droplet recruitment assay. (B) CLN3 mRNA (pink) is not efficiently recruited, but BNI1 or SPA2 mRNA (pink) are recruited into preformed Whi3-BNI1 droplets (green) based on fluorescence microscopy. Scale bar, 10 µm. (C) Recruitment coefficients of mRNA from (B). Boxes indicate interquartile range, line is median, whiskers contain points within three times the interquartile range, and outliers are indicated with dots. NS, not significant, \( P > 0.05; ** P < 0.01; *** P < 0.001 \) (t test). \( n \geq 500 \) droplets for \( N \geq 3 \) biological replicates. (D) Cartoon schematic and representative images showing in vitro RNA-only droplet assay where CLN3, BNI1, and SPA2 mRNAs assemble into liquid or gel-like droplets. Scale bar, 5 µm. (E) Fluorescence microscopy images showing that BNI1 RNA (green) colocalizes with SPA2 RNA (pink) in droplets. (F) Fluorescence microscopy images showing that CLN3 RNA (pink) does not colocalize with SPA2 (green) and BNI1 (green) droplets. Scale bar, 5 µm. (G) Quantification of colocalization between BNI1 and SPA2, SPA2 and CLN3, or BNI1 and CLN3 RNAs. NS, not significant, ***\( P < 0.001 \) (Wilcoxon rank sum test). \( n \geq 200 \) droplets for \( N \geq 3 \) biological replicates.
Fig. 3. RNA secondary structure determines specificity and identity of Whi3-CLN3 droplets. (A) Fluorescence microscopy images showing the recruitment of scrambled (cln3 scr), melted (CLN3 ml), and refolded CLN3 (CLN3 refold) mRNA (pink) into preformed Whi3-BNI1 droplets (green). (B) Fluorescence microscopy images showing the loss of recruitment of CLN3 ml when mixed with oligonucleotides targeting complementary sequences of CLN3 to BNI1. Scale bar, 10 µm. (C) Quantification of (A) and (B). *P < 0.05; **P < 0.01; ***P < 0.001 (t test). n ≥ 500 droplets for N ≥ 3 biological replicates. (D) Base pairing probability from SHAPE-MaP of CLN3, cln3 scr, and CLN3 refold show differences in the secondary structure in CLN3. Arcs connect base pairs and are colored by probability. (E) Secondary structure models from SHAPE-MaP for the first 400 nucleotides of CLN3, CLN3 refold, and cln3 scr. Whi3 binding sites are in orange. (F) CLN3 structure mutant (cln3 sm) mRNA is significantly recruited to Whi3-BNI1 droplets in vitro and in vivo. **P < 0.01 (t test). Green arrows denote sites of colocalization between BNI1 mRNA (green) and cln3 sm mRNA (pink) by smFISH. Scale bar, 10 µm for in vitro, 2 µm for in vivo. n ≥ 500 droplets for N ≥ 3 biological replicates.
Fig. 4. Whi3 binding alters RNA behavior. (A) Differences in SHAPE reactivities (ΔSHAPE) were calculated by subtracting CLN3 SHAPE reactivities from CLN3 + Whi3 reactivities. Positive ΔSHAPE values indicate protection from modification in the presence of Whi3, and negative ΔSHAPE reports enhanced reactivity in the absence of Whi3 protein. (B) Base-pairing probability compared between CLN3 and CLN3 with Whi3 shows rearrangements in CLN3 structure in the presence of Whi3. Arcs connect base-pairing sites and are colored by probability. (C) Schematic of smFRET experiment. (D) FRET histograms before (gray) and after (green) 0.5 or 5 µM Whi3 addition. Purple shaded regions denote high and mid FRET states for CLN3 and BNI1, respectively. (E) Averaged cy3 (green), cy5 (red) intensities, and representative FRET traces (blue) obtained from smFRET experiments of CLN3 and BNI1 in the presence of 5 µM Whi3. Dwell-time analysis reveals slower FRET fluctuations for CLN3 than BNI1 in the presence of Whi3. (F) Proposed model in which RNA-RNA interactions derived from mRNA structure promote the selective uptake of distinct RNAs and protein constituents into droplets leading to distinct dynamics (orange zigzags) of different droplet complexes.
and fig. S10A) and revealed that protein binding causes structural rearrangements (Fig. 4B). We therefore hypothesize that Whi3 binding may have important contributions to structural rearrangements of target RNAs relevant to droplet identity.

To examine the consequence of Whi3 binding to RNA, we used smFRET (fluorescence resonance energy transfer) (Fig. 4C) to measure the conformational dynamics of CLN3 mRNAs with and without Whi3 (16). In the absence of protein, CLN3 RNA showed high FRET values indicative of a compacted state, whereas BNI1 RNA showed lower FRET values, indicating a less compact state (Fig. 4D, purple shaded regions). Upon addition of Whi3, CLN3 FRET values decreased, indicating that more extended RNA conformations were induced, dependent on the ability of Whi3 to bind mRNA (fig. S10, B and C). In contrast, bound to Whi3, BNI1 RNA showed a more substantial broadening of FRET values (Fig. 4D), indicating that Whi3-BNI1 complexes are more dynamic. Dwell-time analysis revealed that Whi3-induced dynamics are three times faster for BNI1 than CLN3 (Fig. 4E). Different mRNAs thus react differentially in their intramolecular fluctuations to the presence of Whi3, providing an additional mode of RNA droplet regulation.

These FRET studies suggest that Whi3 binding alters the conformational dynamics of target RNAs. We speculate that these differential dynamics help maintain droplet identities established by RNA-RNA interactions. Once RNA-RNA interactions are formed, Whi3 binding may reduce the ability of the RNA to resample many alternate RNA structures to maintain the identity. Additionally, the slower fluctuations of CLN3 bound to Whi3 may be one source of exclusion from the more rapidly fluctuating BNI1-Whi3 complexes in those droplets. Such dynamics may drive the droplet material properties reported previously (3) and serve as barriers to homogenization.

We show that mRNA structure defines the ability of an RNA to engage in homo- or heteromeric interactions and thus drives specificity in the composition of liquid droplet compartments (Fig. 4F). This mechanism is likely relevant for the sorting of specific RNAs to other RNA granules, such as stress and P granules, and P bodies (17, 18). Future work will address the timing and location of how mRNA secondary structure influences selective uptake of cellular constituents into droplets. Protein binding to different RNAs can lead to varied dynamics of complexes, further distinguishing the physical properties of different droplets and promoting immiscibility of coexisting droplets. Given the large number of distinct, RNA-based condensates in the cell, these mechanisms are likely broadly relevant to explain how droplets achieve and maintain individuality.

REFERENCES AND NOTES


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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S10

References (19–22)

Movie S1

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RNA and membraneless organelles
Membraneless compartments can form in cells through liquidliquid phase separation (see the Perspective by Polymenidou). But what prevents these cellular condensates from randomly fusing together? Using the RNA-binding protein (RBP) Whi3, Langdon et al. demonstrated that the secondary structure of different RNA components determines the distinct biophysical and biological properties of the two types of condensates that Whi3 forms. Several RBPs, such as FUS and TDP43, contain prion-like domains and are linked to neurodegenerative diseases. These RBPs are usually soluble in the nucleus but can form pathological aggregates in the cytoplasm. Maharana et al. showed that local RNA concentrations determine distinct phase separation behaviors in different subcellular locations. The higher RNA concentrations in the nucleus act as a buffer to prevent phase separation of RBPs; when mislocalized to the cytoplasm, lower RNA concentrations trigger aggregation.
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