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Phase Separation Comes of Age: From Phenomenology to Single Molecules

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In this issue of *Molecular Cell*, Pitchiaya et al. (2019) use high-resolution single-molecule microscopy to dissect the localization of different types of RNAs with processing bodies (PBs) in cells, revealing novel insights about their dynamic recruitment to PBs.

Processing bodies (PBs) are distinct membraneless ribonucleoprotein (RNP) granules that assemble via liquid-liquid phase separation. Being enriched in translationally repressed messenger RNAs (mRNAs), microRNAs (miRNAs), and RNA processing enzymes, mammalian PBs are sites for RNA storage and translational regulation (for a review, see Protter and Parker, 2016). Despite intense research, we still lack a molecular-level description of how different types of RNAs localize to PBs and how the localizations and dynamics of these RNAs relate to their functions and regulations.

Here, Walter and colleagues (Pitchiaya et al., 2019) develop strategies to label different types of RNAs—mRNAs, miRNAs, and long non-coding RNAs (lncRNAs). Using single-molecule microscopy, they can now simultaneously visualize these different classes of RNAs and individual PBs in cells. Remarkably, they could identify and quantitate dynamic interactions between individual RNAs and the core or periphery of PBs (Figure 1). This supports prior observations that there can be substructures to such PBs (Cougot et al., 2012) and many other phase-separated structures (Feric et al., 2016), rather than a uniform compartment that one might have initially expected from a "droplet/blob/body"-type appearance in the cell.

Argonaute (AGO) proteins, key constituents of RNA-induced silencing complex (RISC), have been demonstrated to localize to PBs and recruit miRNAs (Liu et al., 2005; Pillai et al., 2005). Using their super-resolved imaging tools, Pitchiaya et al. (2019) found that the tumor-suppressive let-7 miRNAs demonstrated five distinct spatiotemporal localization patterns, involving either stable or transient interaction at the PB core or periphery. In contrast, a control DNA oligonucleotide lacking RNA silencing ability didn't localize to PBs, indicating a role for small double-stranded RNA oligonucleotides in miRNP-PB interactions. Interestingly enough, while mRNA-targeting and target-free miRNAs display similar PB localization and enrichment, their dynamics differed substantially; target-free miRNAs showed predominantly stable interactions with PBs. This result likely underscores a role for PBs in storing target-free miRNA for surveillance.

Pitchiaya et al. (2019) also interrogated whether the positioning of the miRNA response element (MRE) influences

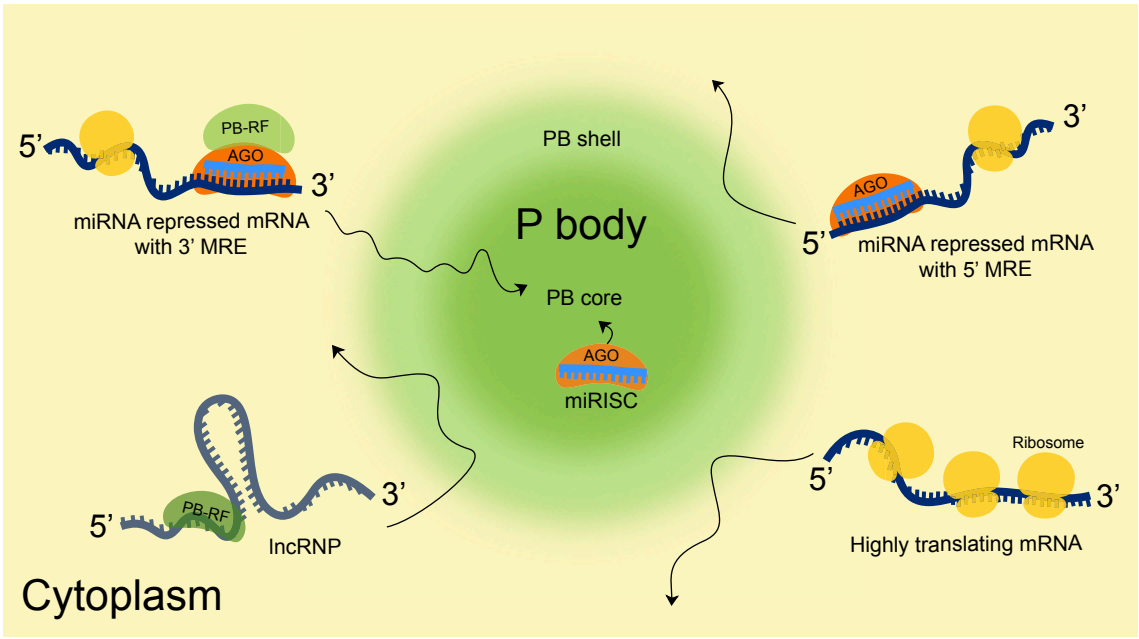

Figure 1. A Model for the Dynamic Recruitment of Functionally Distinct RNAs to PB
miRNA without mRNA target (center) stably binds to PB core. 3′-terminal positioning of miRNA target site (upper left) leads to more stable interactions with PB than 5′-terminal positioning (upper right). lncRNA with PB recruitment factor (PB-RF) (lower left) transiently yet specifically interacts with PB shell. Highly translating mRNA is excluded from PB (lower right).

mRNAs localization to PBs. They showed that 3′-terminal positioning of MREs promoted more stable PB interactions than 5′ MREs. The authors speculate that 5′ MREs involved translation suppression downstream of translation initiation sites, leading to polysome-bound nontranslating mRNAs, which thus impede PB interactions (Eulalio et al., 2007). By changing translational potential of mRNAs via tuning the number of MREs, Pitchiaya et al. also demonstrated that more translationally active mRNAs, i.e., mRNA with greater polysome association, colocalized to a lesser extent at PBs, with more transient and peripheral interactions.

Interestingly enough, RNA interaction dynamics at PBs seems to be more nuanced than being simply governed by translational potential as seen from lncRNA-PB interaction dynamics. lncRNAs are a group of long regulatory transcripts without coding potential. Pitchiaya et al. (2019) demonstrated that lncRNA-PB interactions are distinct from those involving miRNAs and mRNAs. The highly conserved testis-associated oncogenic lncRNA, THOR, and androgen receptor-regulated lncRNA, ARlnc1, which bear binding motifs for PB binding proteins, showed a similar fractional extent of PB localization as mRNAs bearing 3′ MREs. However, unlike mRNAs, lncRNAs predominantly interacted transiently and at the periphery of PBs. lncRNAs lacking recognition motifs for PB-associated proteins showed minimal PB interaction, suggesting a role for specific PB protein-lncRNA interactions in PB recruitment of lncRNAs. Capturing such transient yet specific lncRNA-PB interaction is a strength of the employed single-molecule techniques and not easily achieved by other approaches.

Noteworthy also is the hypothesis made by Pitchiaya et al. (2019) that the most efficient RNA degradation happens at submicroscopic PB assemblies. This is supported by simulations and indirect experimental evidence where hyperosmotic stress-induced PB coalescence downregulated mRNA repression. If indeed smaller PB assemblies provide access to distinct functionality, then the search for a mechanism (e.g., spatial constraint by the cytoskeleton) that works against the thermodynamic process that favor emergence of large droplets (Oswald ripening, coalescence; for a review, see Hyman et al., 2014) has just begun.

Reinforcing the findings by Pitchiaya et al., two recent studies (Moon et al., 2019; Wilbertz et al., 2019) using similar single-molecule imaging strategies also showed a biphasic nature of mRNA interactions with RNP condensates, including stress granules (SGs) and PBs. SG and PBs are frequently mentioned in one context, and yet they are also distinct in function and composition. These two studies also found stable and transient interactions depending on RNA and condensate types and demonstrated exchange of mRNAs between PBs and SGs as well as the correlation between mRNA size and PB interaction dynamics. This is well in line with the phase-separated nature of SGs and PBs, which allows constant exchange of materials with the environment and also between the different droplet-like structures. The dynamic, multicomponent, and heterogeneous nature of such phase-separated assemblies is the very same reason that studying those bodies is considered challenging, and why we are still far from a complete molecular characterization of

PBs or other complex phase-separated structures in the cell. The work by Pitchiaya et al. (2019), Moon et al. (2019), and Wilbertz et al. (2019) shows the way forward.

Future work should address the molecular mechanism behind PB size distribution and its link to RNA processing and degradation kinetics and provide a precise characterization of the different client proteins involved in regulating spatiotemporal dynamics of the interaction between different RNA classes and PBs. The ability to study PBs and SGs at the single-molecule level will allow a better disentanglement between the function and composition and their underlying mechanisms in the cell and sets the direction for high-resolution molecular investigation of phase-separation-driven membraneless organelles. While phase behavior for a liquid state is relatively easy to validate for large droplets, higher-resolution experimental studies will be also needed to define "phase-separation phenomena" when investigating sub-resolution objects or nano-assemblies, especially in a complex cellular milieu. This will become more important the more we start thinking about molecular mechanisms and the role of phase separation in biology at ultra-resolution.

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Getting in LINE with Replication

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Sultana et al. (2019) and Flasch et al. (2019) determined integration patterns of human LINE-1 (long interspersed element-1) retrotransposons highlighting their interaction with DNA replication guided by their 5′-TTTT/AA-3′ integration motif and nucleotide biases in the genome.

Host genomes tolerate transposable elements, which, despite being mutagenic, can in fact be beneficial depending on their location in the genome. In line with their host spectrum and co-evolution with their hosts, different transposon families have evolved different strategies as to where to insert into the host genome. Moloney murine leukemia virus (MLV) and human immunodeficiency virus (HIV) prefer open chromatin and insert close to or into expressed genes. Being viruses that quickly move on, they suffer less penalty when harmful to their host than endogenous transposons that only survive along with their host. Ty1 and Ty3 LTR retrotransposons in *S. cerevisiae* specifically seek RNA-polymerase-III-transcribed loci, such as tDNA clusters, to integrate in an extremely gene-dense genome. Other transposons, like the DNA transposon *Sleeping Beauty*, exhibit a seemingly random insertion profile ideal for gene therapeutic applications. LINE-1 (L1) retrotransposons are the most active retrotransposons in humans and occupy ~17% of our genomes but also co-mobilize other extremely abundant repeats such as short interspersed elements (SINEs). Despite their enormous impact on mammalian genomes, until recently, L1 integration preference had only been determined for relatively few *de novo* insertions. The Cristofari and Moran laboratories have now undertaken systematic, large-scale, sequencing-based detection of tens of thousands of *de novo* L1 insertion sites, revealing