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A call to order: Examining structured domains in biomolecular condensates

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ABSTRACT

Diverse cellular processes have been observed or predicted to occur in biomolecular condensates, which are comprised of proteins and nucleic acids that undergo liquid–liquid phase separation (LLPS). Protein-driven LLPS often involves weak, multivalent interactions between intrinsically disordered regions (IDRs). Due to their inherent lack of defined tertiary structures, NMR has been a powerful resource for studying the behavior and interactions of IDRs in condensates. While IDRs in proteins are necessary for phase separation, core proteins enriched in condensates often contain structured domains that are essential for their function and contribute to phase separation. How phase separation can affect the structure and conformational dynamics of structured domains is critical for understanding how biochemical reactions can be effectively regulated in cellular condensates. In this perspective, we discuss the consequences phase separation can have on structured domains and outline NMR observables we believe are useful for assessing protein structure and dynamics in condensates.

1. Condensates in biology

Cellular processes require the coordination of multiple steps in order to achieve a desired functional outcome. The evolution of multisubunit complexes that carry out sequential steps in a biochemical reaction has been essential for the spatial organization of these processes and is key to ensuring rapid, efficient responses to cell stimuli that maintain homeostasis. The relatively recent observation of liquid-like membraneless organelles (MLOs) in *C. elegans* ushered in a new paradigm for cellular organization in biology [1]. The formation of MLOs, or biomolecular condensates, results from the spontaneous partitioning of biomolecules into discrete compartments with a greater concentration relative to the surrounding solution [2,3]. Since their initial observation, numerous previously annotated cellular puncta have been shown to exhibit properties consistent with condensate formation. Together, the processes encapsulated within these puncta account for many essential aspects of cellular biochemistry. Among them includes the organization of heterochromatin and ribosome biogenesis in the nucleus, translational regulation and mRNA decay in the cytoplasm, as well as the organization of signaling cascades [4–9].

The molecular basis for condensate formation draws extensively from seminal studies in polymer chemistry [10,11]. Liquid-liquid phase separation (LLPS) and percolation are two predominant thermodynamic driving forces of condensate formation [12]. Briefly, phase separation of a single macromolecular system arises from unfavorable interactions between the macromolecule and surrounding solvent to cause concentrated sites enriched in homotypic intermolecular interactions (Fig. 1**A**, top). Percolation occurs when macromolecules with a high degree of multivalency lead to the formation of extended networks through oligomerization, protein–protein interactions or association with nucleic acids (Fig. 1**A**, bottom) [13]. Moreover, phase separation and percolation can be coupled to promote condensate formation. In this perspective we collectively refer to these processes as phase separation for simplicity.

Since a variety of cellular processes occur in condensates, the list of proteins observed and hypothesized to undergo phase separation *in vitro* and *in vivo* is also diverse and constantly growing. Thus, it has been difficult to devise a consensus sequence or structural feature of the types of interactions promoting phase separation, but a few notable patterns have emerged [14]. Namely, many proteins that are essential for or accelerate condensate formation are intrinsically disordered (IDPs) or contain structured domains flanked by intrinsically disordered regions (IDRs) (Fig. 1**B**). Atomistically, both electrostatic and hydrophobic interactions between amino acids have been shown to drive phase sepa-

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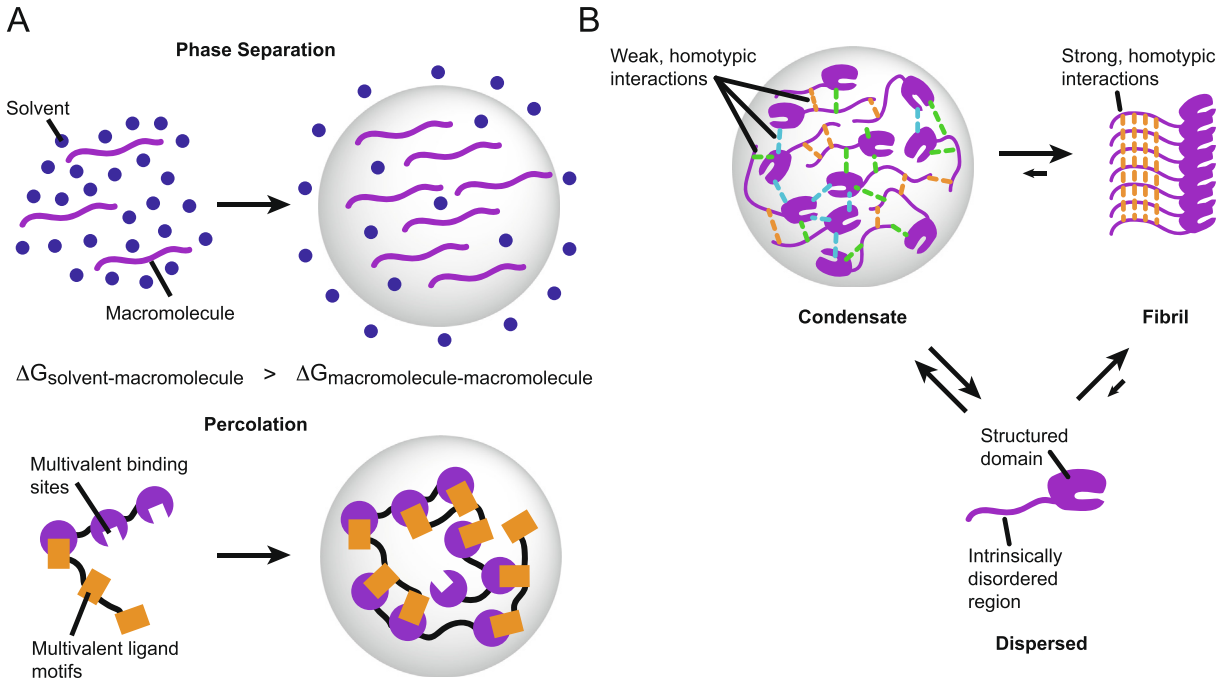

Fig. 1. Interactions control condensate formation and liquid-like properties. **A**, Condensate formation results from phase separation (top) and percolation (bottom). **B**, Intrinsically disordered regions often flank structured regions and promote liquid–liquid phase separation through weak, multivalent interactions. Disordered regions can also promote stable fibril formation through strong interactions that may follow maturation of condensates.

ration and alterations in these interactions can change the macroscopic behavior of droplets. For example, changing lysine:RNA interactions to arginine:RNA led to less dynamic condensates and decreasing the uniform distribution of aromatic residues caused hRNPA1 condensates to lose their liquid-like properties [15,16]. These and other observations underscore how the underlying molecular grammar driving phase separation can have important implications for tuning the microenvironment in condensates.

Protein and protein-RNA aggregates represent an extreme and often pathogenic form of MLOs. These stable aggregates result from the formation of amyloid-like fibrils that are seeded by interactions between glutamine and asparagine rich repeats within proteins [17,18]. Interestingly, several amyloidogenic proteins including FUS, Tau, hnRNPA1/2, and TDP-43 localize in various biomolecular condensates and undergo LLPS [19–22]. Moreover, mutations associated with neurodegenerative disease in TDP-43 inhibit its propensity to undergo phase separation while disease-associated mutations in tau enhance its phase separation and gelation [14,17]. The localization of FUS and TDP-43 to stress granules, for example, has been demonstrated to promote fibrilization and disease mutations associated with amyotrophic lateral sclerosis (ALS) prevented stress granule clearance and accelerated fibril formation [21,22]. Thus, the highly concentrated environment within condensates might represent a precarious precipice between homeostasis and pathogenesis whereby the liquid-like properties in condensates are an important checkpoint for clearing seeding intermediates to prevent nonreversible fibril formation.

2. Studying phase separation by NMR

Because proteins that undergo phase separation contain extensive disordered regions, exhibit dynamic properties and are formed through weak, transient interactions, they are refractory to traditional structural methods like X-ray crystallography and cryo-electron microscopy. This has made solution- and solid-state NMR particularly well-suited for gaining atomic information on systems undergoing LLPS (reviewed in [23–25]). *In vitro* droplets containing the IDR of DDX4 were observed to have a translational diffusion constant similar to an *E. coli* cell and similar results were obtained for FUS [26,27]. Despite such slow translational diffusion, 2D HSQC spectra could be recorded and interpreted due to significant flexibility of the IDR in the condensed phase, giving rise to relatively fast local rotational diffusion, and consequently, dephasing that is slow enough to allow resolution of multiple crosspeaks. At the other extreme, magic-angle spinning solid-state NMR (MAS-SSNMR) is a powerful approach to characterize the structure of fibrils and condensate maturation that are refractory to solution NMR methods [28–33]. Thus, the combination of solution and solid-state NMR experiments allows for interrogation of phase separation from initial seeding to aggregate formation.

Several studies have utilized solution-state NMR to elucidate the molecular interactions important for phase separation by IDPs and IDRs [23,24,26,34]. These studies have mainly combined analysis of proteins in dilute phase to make informed mutations that can be assayed biochemically to assess their effects on phase separation. Spectral crowding arising from the poor chemical shift dispersion of unstructured proteins can confound analysis. To overcome this problem, an array of ^{13}C-detected pulse schemes in conjunction with cryogenically cooled probes have been developed to allow assignment of IDPs [35–44]. The recent implementation of proton-detected haCONHA experiments have helped provide enhanced sensitivity to resolve structural changes that occur in proteins upon phase separation [45].

Reconstituted membraneless organelles can exist in liquid or hydrogel like states, much like lipid bilayers that can exist in fluid or gel phases [46]. Hybrid solution and solid-state NMR approaches in conjunction with MAS can be used to characterize, on a per residue basis, whether protein interactions in condensates are more liquid or gel-like. For example, both Methyl-TROSY and solid-state NMR studies of the enhancer of decapping protein Edc3 demonstrated its IDR remains largely flexible in condensates except for a small region that contacts the structured *C*-terminal

YjeF N domain, which had not been previously observed [47]. Furthermore, hybrid NMR studies of hydrogels were used to characterize the behavior of FG repeats in phase separation of the nuclear pore complex protein Nup98 and to identify specific serine residues that contribute to the liquid-to-gel transition of human HP1α [48,49]. Finally, the study of squid beak proteins revealed that histidine deprotonation is an early step in their phase separation followed by tyrosine-tyrosine interactions and hydrophobic interactions [50]. The above studies highlight the power of NMR for determining how interactions change during LLPS, gelation and fibril formation, which can inform on biological function and disease.

3. Consequences of phase separation on molecular structure

The demixing of biomolecules from solution has several consequences for controlling both enzymes and scaffolding proteins [3,51]. The simplest view is that there is a division of labor between the IDR and structured enzymatic core domains, with the former promoting LLPS and the latter carrying out a specific function such as scaffolding or an enzymatic reaction. Thus, the simplest direct consequence of phase separation is an increase of protein concentration in the condensed phase, which can accelerate enzyme catalyzed reactions [52–54]. However, there are several examples whereby condensate formation leads to inhibition or activation of functional processes beyond simple concentration effects, suggesting the unique chemical environment of the condensed phase can bias structured domains to achieve a switch-like response in output [55–59]. In this case, we imagine the IDRs are poised to respond to these changes and alter the physical interactions to influence structured domain conformation. For example, the scaffolding of proteins within condensates can increase enzymatic rates by enhancing the association of reactants and enzyme (decreased K_M) or preventing nonproductive interactions compared to the unscaffolded proteins [2,60]. Alternatively, the condensed phase may promote nonproductive interactions that inhibit function by masking allosteric sites or substrate binding, for example. Finally, the unique solvation properties within condensates could affect reaction rates by biasing interactions between cofactors and enzymes or altering the free energy of the transition state [51].

Given that multi-step processes can be enriched into a single condensate, there is the potential for numerous types of interactions to occur. Altering these interactions can have consequences for favoring the types of reactions that take place in condensates. For example, the nucleolus has been shown to organize into a condensate with distinct molecular composition that thermodynamically promotes the release of matured ribosomal RNA because of decreased multivalent interactions [61]. In addition, arginine-phosphate interactions were important for phase separation and mRNA partitioning to influence whether FMRP/CAPRIN1 condensates promote translation or deadenylation (repression) [62]. We also recently showed that mRNA decapping activity in condensates is regulated in a composition-dependent manner because a structured domain of the activator Edc3 interacts with motifs in the IDR of the decapping enzyme Dcp2 to change the molecular network promoting phase separation [56]. Thus, favoring certain interactions between structured and disordered regions in condensates is a robust mechanism to control cellular biochemistry.

Phase separation is regulated by molecular conformation and we envision the unique environment in condensates can perturb molecular conformation. G3BP1, an essential component of stress granule formation, must undergo a conformational change from an autoinhibited to open state that reveals an IDR to interact with RNA and promote phase separation [63,64]. The heterochromatin protein HP1α changes conformation because of phosphorylation of its *N*-terminal disordered region or ligand binding to cause phase separation and the fission yeast protein Swi6 promotes heterochromatin phase separation by distorting the nucleosome core [4,65]. As these examples illustrate, molecular conformation represents an additional mechanism to control condensate formation, composition, and function.

4. Considerations for sample preparation

Using NMR to studying macromolecules that undergo phase separation leads to several challenges and considerations that need to be assessed to ensure monitoring of the appropriate observable. For instance, studying interactions in the dilute phase provides valuable insights into the initial events in phase separation but are an indirect readout of the interactions in condensates [66,67]. Direct observation of the condensed phase is difficult because it usually requires large quantities to create a homogenous monophasic sample across the entire NMR coil to avoid the effects of settling and higher viscosity that reduce signal intensity [23]. In addition, condensate-glass interactions can alter the magnetic susceptibility of the sample to cause additional crosspeaks that confound analysis [68]. This latter challenge was overcome by the suspension of condensates in an agarose hydrogel that mimics the cytoplasm and maintains a more physiologic surface area-to-volume ratio [68]. Gaining a complete understanding of the regulatory mechanisms imparted by phase separation will likely require a combination of the sample types described here and presents an opportunity for the advancement of spectroscopic hardware and molecular labeling schemes.

5. Approaches for studying structured domains in phase-separated systems

So far, most NMR analyses of condensates have focused on the IDRs and these studies have been crucial for illuminating the interactions important for phase separation [23]. While IDRs are replete in the proteome, they are often found adjacent to structured domains that are necessary for proper protein function [69]. In addition, IDRs can regulate the function of structured domains through direct and allosteric mechanisms [70]. In the context of biomolecular condensates, structured domains can promote phase separation and alter the material properties of condensates *in vitro* and *in vivo* [71,72]. Thus, examining the behavior of structured domains in condensates is important to understanding how biochemical processes are regulated in condensates. However, how phase separation may influence structured domains of proteins has remained poorly studied, likely due to the challenges in expressing and purifying these proteins as well as the additional spectral complexity arising from additional crosspeaks and slower rates of molecular tumbling.

It is our view that studying the impact of IDRs on the dynamics of structured domains in solution is a good prelude for understanding how biological condensates can achieve additional control of function. This necessitates a combination of liquid, solid-state NMR and pulsed EPR approaches such as DEER [68,73]. A major goal in the field will be to answer the fundamental question of how conformational landscapes can be impacted by phase separation to impart additional regulation.

Systems with globular domains and IDRs will have a great degree of spectral complexity. One workaround is to introduce spectroscopically active labels at specific positions throughout the protein sequence, a now common practice for NMR studies of large proteins and complexes [74–77]. The labeling of terminal methyl groups in isoleucine, leucine, valine, methionine, and alanine (ILVMA) residues has greatly enhanced the sensitivity of

NMR signals for the study of large protein assemblies, and we have outlined some of its applications for studying phase separation above (Fig. 2) [74,78].

In addition, the conjugation of NMR reporter groups such as ^{13}C-methyl-methanethiosulfonate at reactive amino acids (i.e. cysteine) following purification allows for expression of proteins of interest in insect and mammalian cultures, which may be required to obtain NMR quantities of proteins that are recalcitrant to *E. coli* expression (Fig. 2) [79]. Furthermore, the incorporation of fluorine into biomolecules at aromatic residues and through chemical conjugation has been applied to the study of biomolecular interactions and dynamics [80–82]. In particular, the development of aromatic ^{19}F-^{13}C TROSY pulse sequences has extended its application to larger biomolecules, including RNA (Fig. 2) [83]. Given its favorable spectroscopic properties, sensitivity to chemical environment, and lack of natural incorporation into biomolecules, fluorine may be a powerful tool for studying molecules within condensates using solution or SSNMR.

Another means for minimizing spectral overlap is through the segmental labeling of proteins wherein only one region of interest is NMR active. Segmental labeling has the advantage of maintaining the protein in a near-native state. Two protein-based methods for ligation have been the most extensively used for NMR studies: intein- and sortase-mediated ligation [84,85]. In the former, two components of interest (exteins) are ligated through the formation of an intermediate thioester that is resolved by a *N*-terminal cysteine on the second extein (Fig. 3**A**). The *N*-terminal cysteine is required for ligation and either needs to be synthesized or exposed upon proteolytic cleavage during purification. Sortase-mediated ligation makes use of the protein encoded by the *S. aureus* SortaseA gene, a member of the transpeptidase family of enzymes that anchor proteins to the cell wall of gram-positive bacteria (Fig. 3**B**) [86]. A recognition sequence LPXTG (X = any amino acid) is appended to the C-terminus of one fragment and ligated to an *N*-terminal glycine residue of the second protein fragment. Thus, the mutational scar LPXTG$_n$ (n = number of glycine residues) is present in the final ligated protein. We recently used SortaseA ligation to study how the disordered region in the decapping enzyme Dcp2 influences the conformation of the structured catalytic domains (Fig. 3**C,D**) [56]. In addition, the labeling and ligation methods outlined here can be used in combination to produce differential labeling schemes within a single polypeptide that would allow for greater coverage of the total protein structure[87]. Moreover, these strategies can also be combined to label multiple proteins that together undergo phase separation.

Conformational landscapes of structured core domains may be remodelled by interactions with IDRs[88,89]. Paramagnetic relaxation enhancement (PRE) NMR experiments can also be employed to observe transient, lowly-populated conformations that may be present in solution[87]. With known structures of core domains, the ability to back-calculate PREs allows one to ask if and how the IDR would influence conformation of dynamic core domains [90]. This approach is quite complementary to pulsed EPR approaches such as DEER that are carried out on frozen solutions[73,91]. PRE NMR has been extensively used to characterize the intra- and intermolecular interactions important for controlling phase separation of FUS, TDP-43, and hnRNPA2 in the dilute phase and how post-translational modifications influence these interactions[92,93]. In addition, NMR and pulsed EPR studies employing DEER to generate distance distribution functions from nitroxide spin pairs incorporated into FUS demonstrated it undergoes compaction upon phase separation[68]. Cross-linking mass spectrometry (XL-MS) corroborated these results and demonstrated more extensive interactions between and within FUS molecules in condensates[94]. The combination of these methods and the development of new ones would greatly enhance our ability to understand how structured regions are influenced by phase separation.

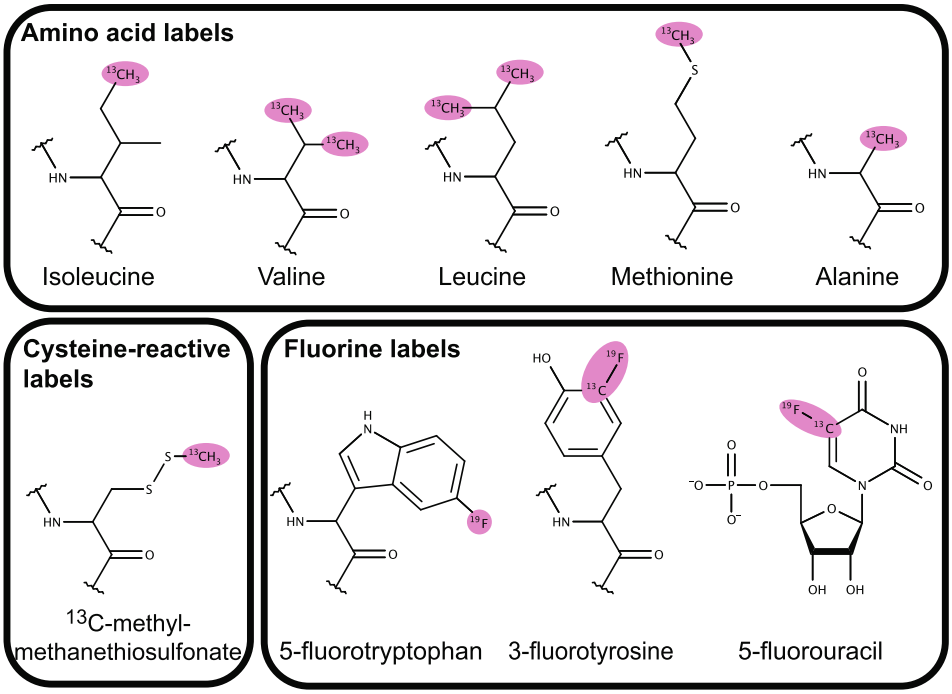

Fig. 2. Amino and nucleic acid derivatives used in biomolecular NMR. These labels have advantageous NMR relaxation properties useful for studying large macromolecular assemblies such as condensates. Observable signals are highlighted in pink.

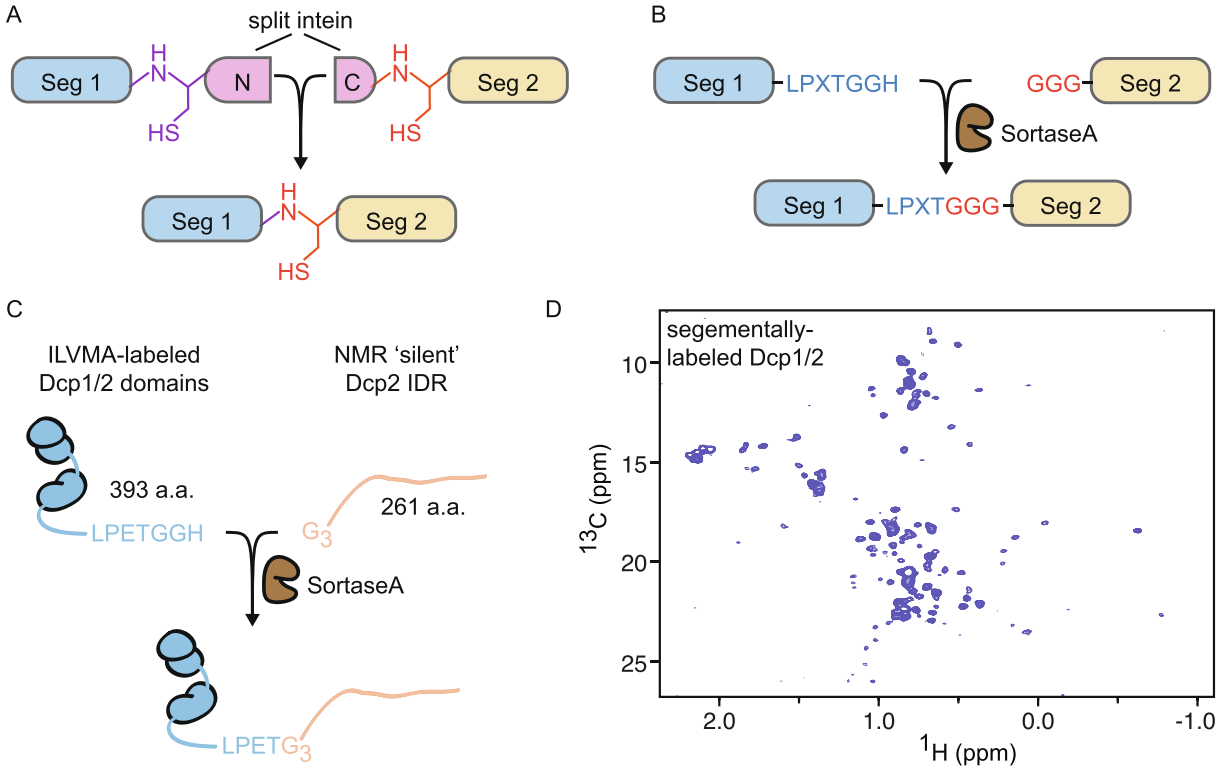

Fig. 3. Protein conjugation strategies for segmental labeling of proteins. **A**, Split intein can be fused to two polypeptide fragments (Seg1 and Seg2) that are subsequently ligated to form a single chain. **B**, SortaseA enzyme ligates a protein fragment (Seg2) with *N*-terminal glycine residues to its C-terminal recognition sequence in a second fragment (Seg1). **C**, SortaseA ligation applied to *S. pombe* decapping complex Dcp1/2 **D**, $^{1}H–^{13}C$ HSQC ILVMA spectrum of structured domains in segmentally-labeled Dcp1/2.

6. Consequences of phase separation on structured domains

6.1. What interactions emerge in condensates?

Protein IDRs are often crucial for condensate formation through multivalent interactions. In addition, short linear motifs (SLiMs) embedded within IDRs can promote phase separation and regulate protein function. In dilute solution, interactions between a regulatory SLiM and structured domain may be transient but their partitioning into condensates creates an environment that stabilizes the interaction and exerts emergent regulatory control (Fig. 4**A**). We have observed that the *C*-terminal IDR of the decapping protein Dcp2 mediates its phase separation with the essential cofactor Dcp1, which leads to enhanced repression of activity[56]. Our study did not characterize the interactions between the inhibitory IDR of Dcp2 and structured core domains of Dcp1/Dcp2 in condensates directly, but we observed by solution state NMR that the Dcp2 *C*-terminal IDR stabilizes an inactive conformation of the Dcp1/Dcp2 complex. We hypothesize that the high local concentrations and unique solvation properties present in condensates may stabilize interactions between the *C*-terminal IDR and the structured core domains of the Dcp1/Dcp2 complex. In addition, as we describe in the next section, phase separation may allow proteins to access lowly-populated states through alterations in their energetic landscape.

6.2. How are conformational landscapes of multidomain proteins affected by LLPS?

Proteins can explore a wide conformational landscape with motions spanning from picoseconds to hours or longer and these conformational fluctuations are often important for proper function[95]. The unique properties of biomolecular condensates, including increased viscosity, high molar concentrations, and altered dielectric constants have the potential to reshape a protein's conformational landscape (Fig. 4**B**). A recent cross-linking mass spectrometry (XL-MS) study demonstrated that a RRM domain in FUS undergoes a partial denaturing upon LLPS that is not readily observed in dilute solution[94]. In addition, the heterochromatin protein HP1 ortholog from *S. pombe*, Swi6, causes distortions in buried regions of the nucleosome and disrupting the distortions abrogates phase separation *in vitro* and leads to improper heterochromatic centers *in vivo*[65]. Thus, it seems probable previously 'invisible' states can be accessed and stabilized in the condensed phase. It is also intriguing to consider that structured oligomeric states not readily detectable in dilute solution become accessible in condensates to increase cooperativity and avidity in reactions. In sum, these 'new' states could impart additional regulation, enhance function, or represent unstable intermediates on pathway to amyloid formation. However, the population and structural features of these states has not been studied, but we believe NMR is well-suited to rigorously address these questions.

7. Concluding remarks

The work of many research groups has advanced our knowledge of how intrinsic disorder contributes to protein function and, more recently, its fundamental role in liquid–liquid phase separation. The increasing suite of biophysical and computational methods is a promising integrated framework to address the questions presented here regarding emergent interactions and altered conformational landscapes in biomolecular condensates. The ability to apply and expand existing NMR approaches to study the interplay between disordered and structured regions in condensates presents an exciting opportunity to uncover new modes of regulation.

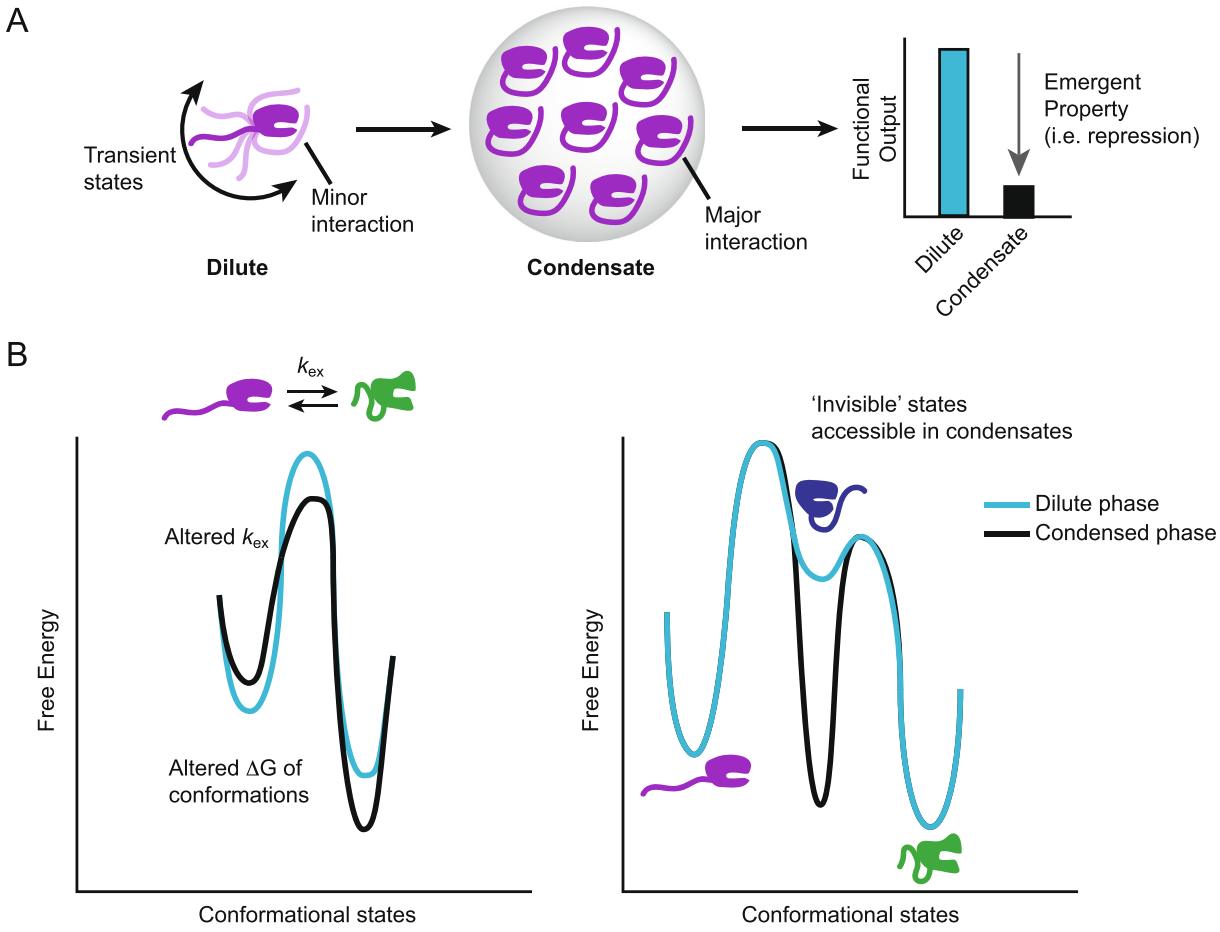

Fig. 4. Potential effects of phase separation on structured domains. **A**, In dispersed solution, an IDR samples many conformational states, but one minor interaction may become stabilized in condensates and cause an emergent functional outcome for activity (i.e. repression, activation, etc.) **B**, The condensate environment can alter the conformational landscape of proteins in multiple ways, including changes to the rate of conformational exchange or the free energy of a given state.

These contributions will further our understanding of why phase separation is a common feature of diverse cellular processes.

CRediT authorship contribution statement

Ryan W. Tibble: Conceptualization, Writing – original draft, Writing – review & editing. **John D. Gross:** Conceptualization, Writing – original draft, Writing – review & editing, Funding acquisition, Supervision.

Data availability

No data was used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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