Reentrant liquid condensate phase of proteins is stabilized by hydrophobic and non-ionic interactions

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Abstract

Many cellular proteins have the ability to demix spontaneously from solution to form liquid condensates. These phase-separated structures form membraneless compartments in living cells and have wide-ranging roles in health and disease. Elucidating the molecular driving forces underlying liquid–liquid phase separation (LLPS) of proteins has thus become a key objective for understanding biological function and malfunction. Here we show that proteins implicated in cellular phase separation, such as FUS, TDP-43, and Annexin A11, which form condensates at low salt concentrations via homotypic multivalent interactions, also have the ability to undergo LLPS at high salt concentrations by reentering into a phase-separated regime. Through a combination of experiments and simulations, we demonstrate that phase separation in the high-salt regime is mainly driven by hydrophobic and non-ionic interactions. As such, it is mechanistically distinct from the low-salt regime, where condensates are stabilized by a broad mix of electrostatic, hydrophobic, and non-ionic forces. Our work thus expands the molecular grammar of interactions governing LLPS of cellular proteins and provides a new view on hydrophobicity and non-ionic interactions as non-specific driving forces for the condensation process, with important implications for the aberrant function, druggability, and material properties of biomolecular condensates.

One Sentence Summary

Proteins implicated in cellular phase separation can undergo a salt-mediated reentrant liquid–liquid phase transition.

Introduction

Liquid–liquid phase separation (LLPS) has emerged as an important organizing principle in biology, where condensation of proteins and other biomolecules into liquid droplets has been shown to underlie the formation of membraneless compartments (*1*–*3*). Beyond compartmentalization, these biomolecular condensates have been implicated in diverse biological functions (*e.g.*, in chromatin reorganization (*4*), noise buffering (*5*), and sensing (*6*)), and their misregulation has been associated with the emergence of diverse pathologies, such as neurodegenerative diseases and cancer (*7*–*9*).

LLPS is a thermodynamic process in which interacting multivalent proteins, and in many cases oligonucleotides, find a minimal free energy state through demixing into a protein-depleted diluted phase and a protein-enriched condensed phase (*10*–*12*). LLPS becomes thermodynamically favorable at biomolecular concentrations and in solution conditions where the enthalpy gain from the dynamic formation of weak attractive intermolecular interactions (*13*), and the increase in entropy associated with the release of water molecules from the biomolecules' surfaces to the bulk (*14*), become sufficient to overcome the entropy loss due to the reduced number of available microstates upon demixing (*10*–*12*). This observation raises key questions about the nature of the molecular interactions that govern protein LLPS and of the factors that modulate them.

Previous experimental and theoretical studies have shed light on the molecular grammar underlying LLPS (*15*–*17*). Accordingly, the condensation process of proteins has been shown to be driven by the cooperation of both electrostatic and hydrophobic interactions, including charge–charge, cation–π, dipole–dipole, and π–π stacking interactions. The interplay of these factors underlies the phase separation behavior of proteins under conditions of ionic strength at or below those found physiologically.

Here we show that the proteins fused in sarcoma (FUS) (*18*–*22*), transactive response DNA-binding protein of 43 kDa (TDP-43) (*23*, *24*), and Annexin A11 (A11) (*25*), all of which are known to undergo LLPS via homotypic multivalent interactions at low salt concentrations, also have the ability to undergo LLPS at high salt concentration by reentering into a phase-separated regime from a well-mixed state at intermediate salt concentration. This transition is in contrast to the established RNA-mediated reentrant behavior of protein–RNA coacervates assembled through heterotypic multivalent interactions, which are stable only in the presence of intermediate RNA

concentrations, but exist as homogeneous solutions at both high and low RNA concentrations (*26–29*).

LLPS at high-salt conditions has been observed for a few polymer systems and proteins, but only at very high polymer/protein concentrations (*i.e.*, in the hundreds of micromolar to millimolar range), low temperatures and/or extremes of pH (*30–35*). Importantly, the reentrant protein LLPS we report here takes place at the usual low micromolar protein concentrations, temperature, and pH of physiological LLPS. This underscores the complexity of the dynamic processes that underlie condensate formation and dissolution (*1–3*), and the factors that control them, such as changes in scaffold concentration (*36*, *37*), fluctuations in the condensate environment (*38*, *39*), and many others (*28*).

Strikingly, our data reveal that the molecular interactions stabilizing condensates in the high-salt reentrant regime are fundamentally distinct from those driving phase separation at low salt. At high salt concentrations, LLPS is mainly driven by hydrophobic and non-ionic interactions. This ability of salt to completely shift the molecular driving forces of protein LLPS is consistent with the wide body of work demonstrating the significance of salt in the modulation of protein stability (*40–43*), protein solubility (*44*, *45*), protein–protein interactions (*46*, *47*), and protein–nucleic acid interactions (*48–50*).

As such, this dominant role of hydrophobicity and non-ionic interactions expands the molecular grammar governing LLPS, and demonstrates that the driving forces for protein phase separation are not only dictated by the amino acid sequence but also by the condensate environment. Overall, these findings may have wide-ranging implications for the interactions, druggabilities, and material properties of biomolecular condensates, and thus provides a new lens for understanding biomolecular condensate behavior in health and disease.

Results and Discussion

We first discovered reentrant phase behavior for the protein FUS, when mapping out its phase diagram as a function of KCl concentration (Figure 1). FUS is phase separated in salt concentrations up to ~125 mM, in line with previous observations (*18–22*), then forms a well-mixed phase between 125 mM and 1.5 M, and reenters the phase separated regime above 1.5 M KCl. Hence, FUS exhibits two phase boundaries, at respective upper and lower transition concentrations of salt. Importantly, condensate formation at high KCl concentrations is fully reversible. Adjusting the KCl concentration back to the 500 mM to 1.5 M range yields a well-

mixed phase, which is also known to occur for condensates at low salt conditions upon increasing KCl concentration (*21*).

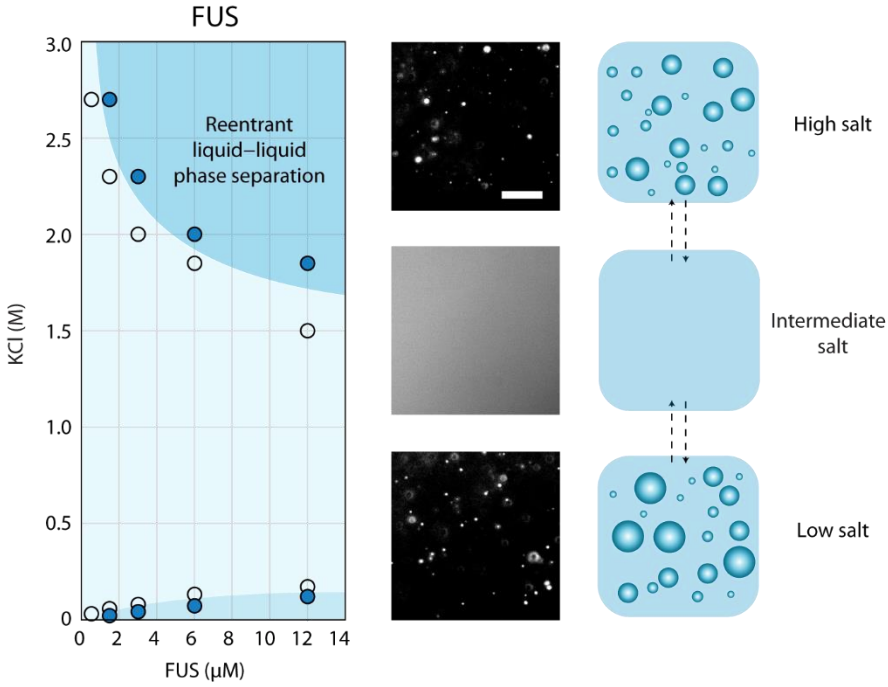

Figure 1. Reentrant phase separation of FUS at high salt. Phase diagram (left), representative images (center), and schematic (right) of FUS phase separation in the presence of increasing concentrations of KCl. In the phase diagram, markers filled with blue indicate concentrations where phase separation was observed in fluorescence images. Open markers indicate concentrations tested where phase separation did not occur. Darker blue regions are guides for the eyes indicating regions where phase separation of FUS occurs, and light blue is the region where no phase separation occurs. The reentrant phase separation regime is indicated. Fluorescent images of FUS (6 μM, EGFP labelled) were taken at 50 mM (low salt), 500 mM (intermediate salt), and 2.7 M KCl (high salt) in 50 mM Tris-HCl (pH 7.2). Scale bar is 20 μm.

In addition to FUS, we find salt-induced reentrant phase behavior in the pathological G156E mutant of FUS (*18*, *19*), TDP-43 (*23*, *24*), and A11 (*25*) (Figure 2). Like FUS, all proteins phase-separate via homotypic multivalent interactions at low salt and are implicated in neurodegenerative disorders. Notably, in all cases, high-salt condensates have similar size distributions and shapes as their low-salt counterparts. An analysis of droplet shape revealed that both low- and high-salt condensates exhibit a high degree of circularity (>95%) (see Supplementary Materials, Figure S1), substantiating their liquid-like character in both salt regimes.

The observed reentrant phase behavior provides an opportunity to shed light on the molecular processes that lead to condensate formation in the high-salt regime, and to probe whether these phenomena are the same at high and low salt concentrations. Condensation at and below physiological salt concentrations, for example of FUS (*18*, *19*), is mainly driven by an interplay of both electrostatics (*21*, *51*) and hydrophobic interactions (*52*, *53*), and can be modulated by RNA (*20*) and ATP (*54*, *55*) both of which favor and disrupt phase separation depending on their concentration. This broad mix of forces that drive and modulate phase separation at physiological salt concentrations is supported by the widespread use of charge-modifying post-translational modifications in cells to control the strength of protein–protein (*56*) and protein–DNA interactions (*57*), and the ability of uncharged proteins to fold and self-assemble into large complexes (*58*).

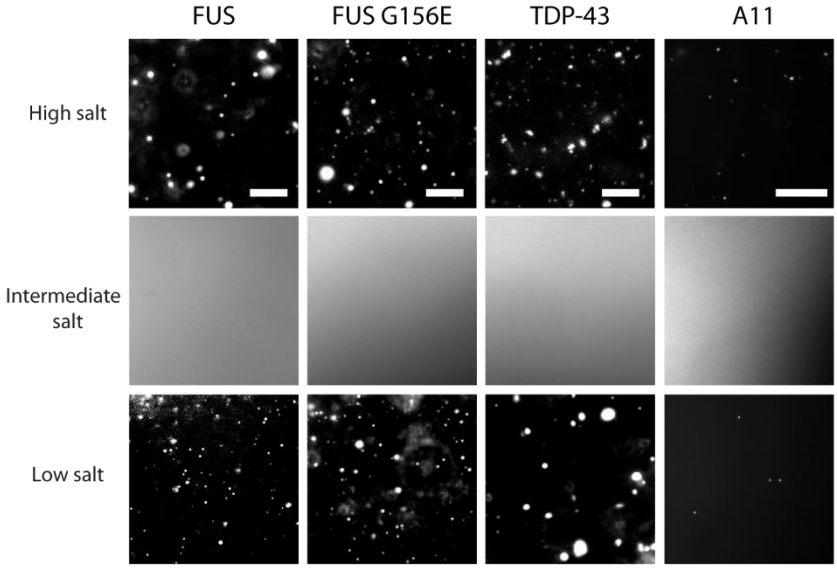

Figure 2. Salt-mediated reentrant phase separation of FUS, FUS G156E, TDP-43, and A11. Representative images of FUS, FUS G156E, TDP-43 at 50 mM (low salt), 500 mM (intermediate salt), and 2.7 M KCl (high salt) in 50 mM Tris-HCl (pH 7.2); and of A11 at 22.5 mM (low salt), 225 mM (intermediate salt), and 500 mM NaCl (high salt) in 20 mM HEPES (pH 7.0). For fluorescence imaging, both FUS variants and TDP-43 were tagged with EGFP and studied at a protein concentration of 6 μM. A11 was labelled with AlexaFluor647 and studied at a protein concentration of 15 μM. Scale bars are 20 μm in all images.

From these observations it follows that the molecular processes that lead to demixing and condensate formation in the high-salt regime are likely connected to electrostatic protein–protein

interactions becoming negligible and the hydrophobic effect being heightened. Indeed, the significant drop in protein solubility upon the addition of salt, which can result in precipitation for many proteins (*i.e.*, the 'salting out' effect), has been attributed to hydrophobic effects (*40*, *59*). Thus, we reasoned that enhanced hydrophobic interactions and weakened ionic interactions might be the key drivers of protein reentrant phase separation at high-salt concentration.

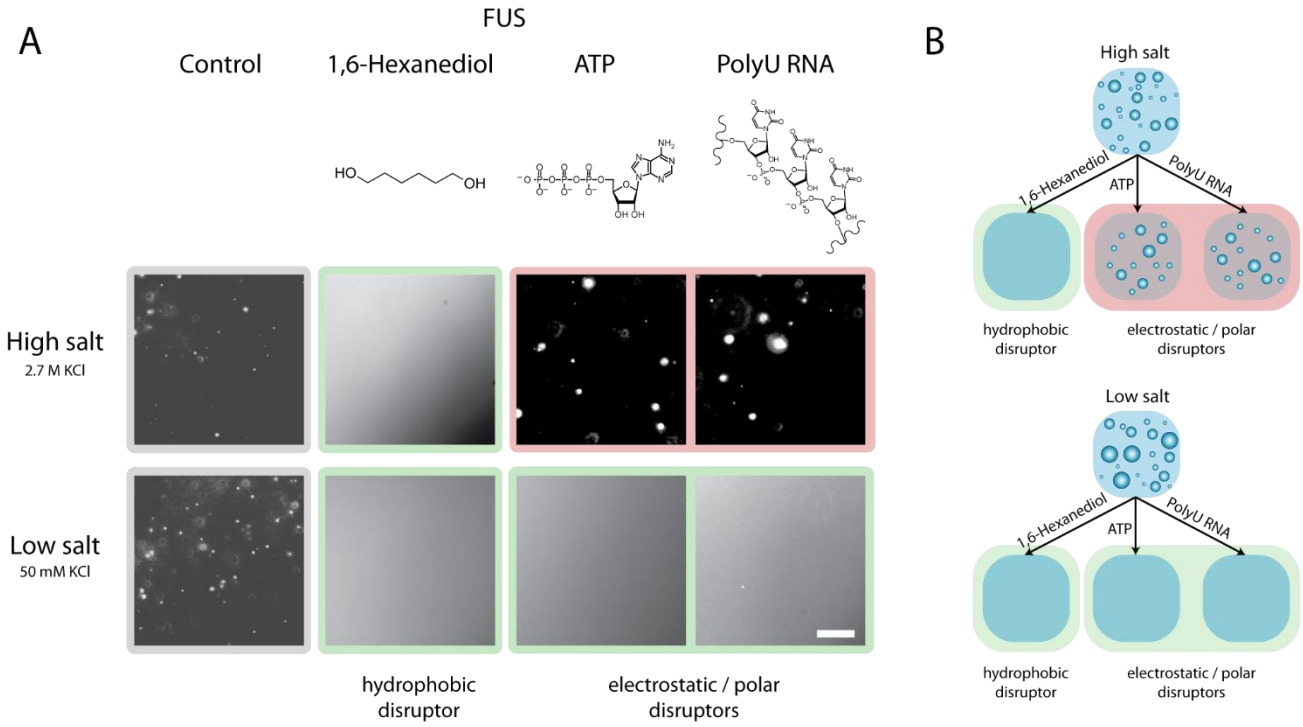

Figure 3. Dissolution assay of FUS condensates in the high- and low-salt regime using hydrophobic and electrostatic/polar disruptors. (A) Representative images of FUS condensates upon addition of 1,6-hexanediol, ATP, and PolyU RNA are shown. Total protein concentration was 4.5 µM and final additive concentrations were 10% 1,6-hexanediol, 1.25 mg/mL PolyU RNA, 12.5 mM ATP in 50 mM Tris-HCl (pH 7.2) at 50 mM (low salt) and 2.7 M KCl (high salt). Conditions at which the disrupters dissolved the condensates are highlighted in green and those where condensates remained intact are highlighted in red. Scale bar is 20 µm. **(B)** Schematic representation of the ability for electrostatic/polar disruptor molecules ATP and PolyU RNA to dissolve condensates in the low-salt regime but not in the high-salt regime, and for the hydrophobic disruptor 1,6-hexanediol to dissolve condensates in both regimes.

To test this hypothesis, we probed the ability of pre-formed FUS condensates to dissociate when exposed to a range of additional components acting as phase separation disruptors, as shown in Figure 3A. We selected a representative set of compounds with the ability to modulate both electrostatic interactions, such as poly-uridine (PolyU) RNA and ATP, both highly negatively charged molecules previously described to disrupt phase separation (*20*, *54*, *55*), as well as 1,6-

hexanediol, an aliphatic alcohol known to disrupt weak protein–protein hydrophobic interactions and selectively dissolve liquid condensates but not solid ones (*60*). At low salt concentrations, PolyU RNA, ATP, and 1,6-hexanediol were all able to dissolve FUS condensates, confirming that both hydrophobic and electrostatic interactions contribute to the stability of FUS condensates in the low-salt regime. At high-salt conditions, 1,6-hexanediol was the only disruptor that could dissolve FUS condensates, while addition of PolyU RNA and ATP did not show any effects. These observations, summarized in Figure 3B, suggest that reentrant high-salt phase separation of proteins is indeed primarily a hydrophobically driven process where electrostatics are screened out.

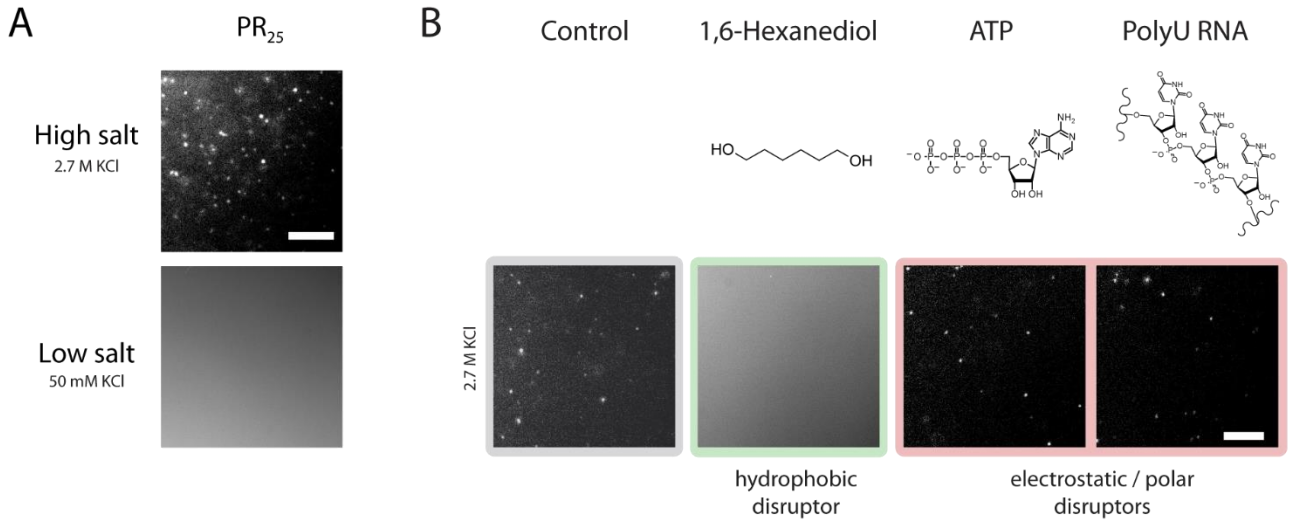

Figure 4. Phase separation and disruptor-mediated dissolution behavior of the PR_{25} peptide at high and low salt concentrations. (A) Representative images of PR_{25} at 50 mM (low salt) and 2.7 M KCl (high salt) in 50 mM Tris-HCl (pH 7.2). Unlabeled peptide was mixed with a small amount of the same peptide labeled with AlexaFluor546; total peptide concentration was 72 μM. **(B)** Dissolution assay of PR_{25} condensates in the high-salt regime using hydrophobic (1,6-hexanediol) and electrostatic/polar disruptors (ATP and PolyU RNA). Final peptide concentration was 54 μM PR_{25} and final additive concentrations were 10% 1,6-hexanediol, 1.25 mg/mL PolyU RNA, 12.5 mM ATP in 2.7 M KCl, 50 mM Tris-HCl (pH 7.2). Conditions at which the disruptors dissolved the condensates are highlighted in green and those where condensates remained intact are highlighted in red. Only 1,6-hexanediol dissolves PR_{25} condensates at high salt. Scale bars in all images are 20 μm.

To understand how salt modulation impacts phase behavior more generally, we probed the response of the highly positively-charged PR_{25} peptide, which is formed by 25 repeats of the proline–arginine dipeptide (Figure 4A). Unlike FUS, PR_{25} does not exhibit LLPS at low salt

concentrations because, in this regime, its homotypic interactions are dominated by the Arg–Arg repulsion; instead PR_{25} requires the addition of co-factor polyanions such as RNA to form complex coacervates at physiological salt (*61*, *62*). However, and strikingly, at KCl concentrations of 2.7 M, we find that PR_{25} undergoes LLPS on its own (Figure 4A). Similar to FUS in the high-salt regime, 1,6-hexanediol could dissolve these high-salt PR_{25} condensates, while the addition of PolyU RNA and ATP did not elicit any observable effects (Figure 4B). These results suggest that phase separation of PR_{25} at high salt is also driven by hydrophobic interactions. Notably, this type of phase transition is not an example of reentrant phase behavior, yet it provides an additional demonstration of the occurrence of homotypically-driven LLPS at high salt, enabled by the screening of electrostatic interactions, in this case repulsion among Arg residues, and strengthening of non-charged interactions.

To further explore the role of hydrophobicity in phase separation in the high-salt regime, we systematically varied the chemical nature of the salts used along the Hofmeister series (*63*, *64*), as was previously done for the low-complexity domain of FUS at lower salt concentrations (*52*). The Hofmeister series is ordered based on the ability of ions to reduce the solubility of hydrophobic molecules in water (*i.e.*, the 'salting-out' effect). Although the exact molecular-level mechanism explaining the salting-out effect remains controversial (*65*), it has been suggested that salts higher up in the Hofmeister series increase the solubility of proteins in solution by effectively weakening the strength of hydrophobic interactions. Therefore, ascending the Hofmeister series, higher salt concentrations should be required to induce phase separation. To test this hypothesis, we mapped out the phase behavior of FUS and PR_{25} in the high-salt regime using chloride salts of various Hofmeister series cations (Figure 5A,B). Indeed, the phase boundary shifts in the order as given by the most agreed upon ranking of the series (*66*) (*i.e.*, $K^+ < Na^+ < Rb^+ < Cs^+ < Li^+ < Ca^{2+}$); hence, condensate formation is disfavored with salts higher up in the series. Similarly, for both FUS and PR_{25}, less 1,6-hexanediol is required to dissolve condensates made in solutions containing salts higher up in the Hofmeister series (Figure 5C,D). These observations support the hypothesis that the formation of FUS and PR_{25} condensates at high salt concentration is driven by hydrophobic interactions, and is thus of a different nature to the transition observed at low salt concentrations, which has a significant electrostatic contribution. Our results also demonstrate that, analogous to the salting-out effect, salts across the Hofmeister series have a different impact on modulating the

solubility limit of phase-separating proteins, and in shifting the boundary of immiscibility that determines phase separation.

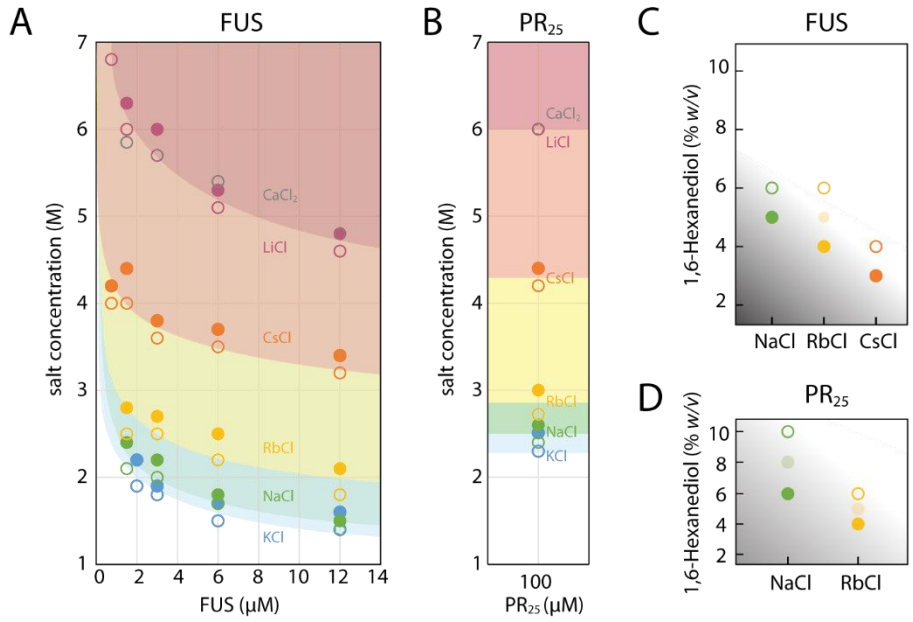

Figure 5. Hofmeister series effects on high-salt phase separation behavior of FUS and PR_{25}. (A) Phase diagram for FUS as a function of salt concentration of various salts of the Hofmeister series. Open circles indicate cases where phase separation did not occur, closed circles indicate where phase separation did occur. Each curve depicts the apparent phase boundary for the particular salt named next to it and is only meant as a guide for the eyes. Even at the saturation concentration of $CaCl_2$ (grey), the hydrophobic effect is weakened to the extent such that phase separation cannot occur, indicated by the presence of open circles and absence of closed ones in the phase diagram. **(B)** Phase behavior of PR_{25} as a function of ionic strength of various salts. The trend is consistent with panel A. **(C)** Comparison of the amount of 1,6-hexanediol required to dissolve FUS condensates in solutions of various salts along the Hofmeister series. In each solution, the final salt concentration was 4 M and the final FUS concentration was 2 μM. **(D)** Comparison of the amount of 1,6-hexanediol required to dissolve PR_{25} as a function of salts along the Hofmeister series. The final salt concentration at each point was 4 M and the PR_{25} concentration was 100 μM.

To further assess the molecular driving forces behind reentrant protein phase behavior, we developed a multiscale modeling approach, combining molecular dynamics (MD) simulations at two complementary levels of resolution (see Supplementary Materials). The advantage of our multiscale approach is that it allows us to first assess how the relative contributions of electrostatic and hydrophobic interactions among the proteins studied experimentally change as a function of salt and then determine whether such changes are consistent with protein reentrant phase behavior.

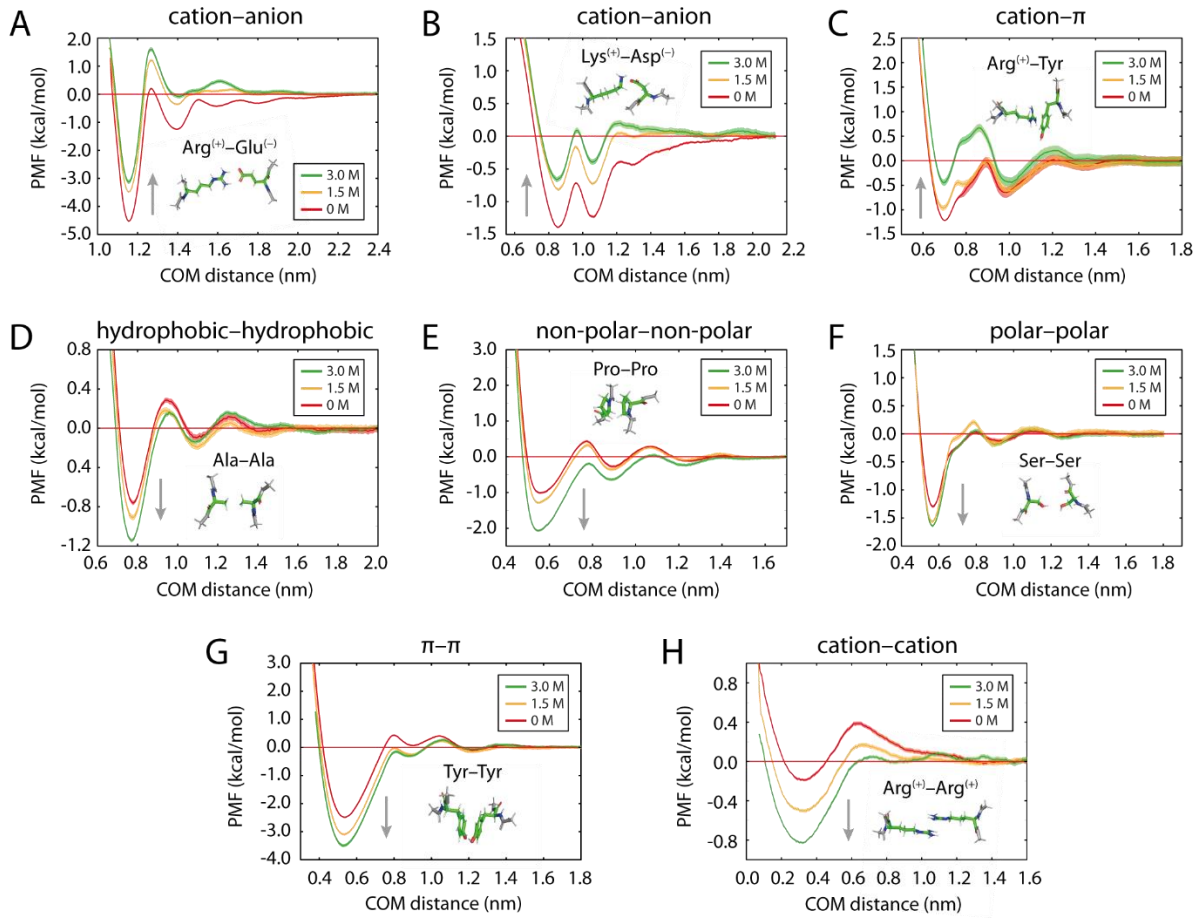

Figure 6. Effect of salt on the potential of mean force (PMF) between selected amino acid pairs in explicit solvent and NaCl ions as a function of the center-of-mass (COM) distance. (A,B) cation–anion, **(C)** cation–π, **(D)** hydrophobic–hydrophobic, **(E)** non-polar–non-polar, **(F)** polar–polar, **(G)** π–π, (**H**) cation–cation. The second well in (B) emerges from the interaction of Asp with an additional H atom in the Lys sidechain, which is displaced by about 1.7 Å from the two H atoms that contribute to the first well. To evaluate (C) a model for the polarized cation–π system was developed (see Methods). The grey arrows in each panel highlight the general shift direction of the PMF minimum as salt concentration is raised. Upward arrows show weakening of cation–anion and cation–π interactions upon increasing salt, and downward arrows show strengthening of non-ionic interactions and cation–cation interactions (with π-orbitals). Error bars are shown as bands and represent the standard deviations obtained by bootstrapping the results from three independent simulations.

First, at high resolution, using all-atom umbrella sampling MD simulations of amino acid pairs with different chemical identities (*e.g.*, charged, uncharged, aromatic groups) in explicit solvent and ions, we calculated the potential of mean force (PMF) as a function of the center-of-mass (COM) distance between amino acids at three different salt concentrations (0 M, 1.5 M and 3 M NaCl), while verifying that the correct ion solubilities in water were observed (see

Supplementary Materials). The PMF curves (Figure 6) show that the attractive interaction energies at short inter-molecular distances among oppositely charged amino acids and among positive and aromatic amino acids (cation–π) decrease monotonically with increasing NaCl concentration (Figure 6A–C). Conversely, those involving only uncharged residues, including polar ones, increase significantly (*i.e.*, Ala–Ala, Pro–Pro, Ser–Ser, and Tyr–Tyr) (Figure 6D–G). This observation supports our hypothesis that, at low salt, protein condensation is most strongly stabilized by electrostatic forces, while at high salt the hydrophobic contributions become dominant.

Importantly, the Arg–Arg pair transitions from exhibiting mainly a charge–charge repulsive interaction at low salt to a dominant attractive π–π interaction (*67*) at high NaCl concentration (Figure 6H). This transition occurs because once the repulsion among positive guanidinium groups is screened, the sp^2-hybridized planar guanidiniums can interact via their π–orbitals. Glu and Asp also have charged sidechains and π-orbitals, and hence, their homotypic interaction could similarly exhibit this striking transition from repulsion to attraction as salt increases. These unexpected results further illuminate the differences in the molecular interactions stabilizing protein condensates at high salt versus low salt, and put forward Arg–Arg as a key driving force behind homotypic LLPS of PR_{25} in the high-salt regime.

Next, using a coarse-grained modelling approach, we investigated whether salt-modulation of intermolecular interactions, observed atomistically, is indeed responsible for protein LLPS at high salt. For this purpose, we conducted direct coexistence simulations of tens to hundreds of interacting FUS and PR_{25} polypeptide chains using the amino-acid resolution coarse-grained model of the Mittal group (*16*, *68*, *69*) which considers sequence-dependent electrostatic and hydrophobic interactions, plus a modification (see Supplementary Materials) that incorporates our atomistic results (Figure 7A). Consistent with our experiments at low salt, we observe LLPS for FUS (due to strong attractive electrostatic cation–anion and cation–π interactions), but not for PR_{25}, which is highly enriched in positively charged amino acids that repel each other more strongly in this regime (Figure 7B, all interactions). To confirm the dependence of LLPS on electrostatic forces at low salt, we turned off all interactions involving charged residues (both charged–charged and cation–π interactions) and, as expected, observed melting of the FUS condensates (Figure 7C; no electrostatics); this finding corroborates the key role of electrostatic interactions in stabilizing protein condensates at low salt. Finally, to recapitulate reentrant phase

behavior at high salt, we moderately increased the strength of the hydrophobic interactions (by 30%, as suggested by our PMF calculations), while keeping the electrostatic interactions turned off. Indeed, we observe that this change is sufficient to yield a reentrant phase transition for FUS and induce phase separation for PR_{25} (Figure 7D; no electrostatics + increased hydrophobicity). Overall, these results show that protein LLPS in the high-salt regime is driven by hydrophobic interactions; thereby, providing a molecular explanation for our experimental observations.

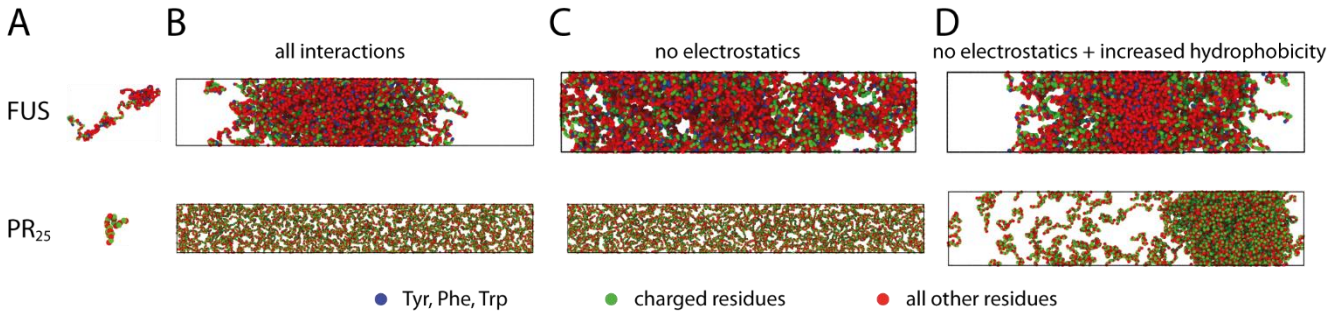

Figure 7. Dependence of LLPS on electrostatic versus hydrophobic forces for FUS and PR_{25} from direct coexistence simulations using a sequence-dependent protein coarse-grained model. (A) Illustration of the coarse-grained models for the different proteins with one bead representing each amino acid. Amino acids are colored according to their chemical identity (aromatics in blue, charged residues in green, all other residues in red; color code shown at the bottom). Snapshots for simulations with **(B)** 'all interactions', **(C)** 'no electrostatics', and **(D)** 'no electrostatics + increased hydrophobicity' for FUS (24 proteins) and PR_{25} (400 peptides).

Conclusion

This work demonstrates the existence of reentrant protein phase separation as a function of salt concentration in which protein molecules are driven by homotypic multivalent interactions to demix from a homogeneous phase in the limit of both low and high electrostatic screening. Previously, a different type of reentrant phase transition (*i.e.*, from the well-mixed to the phase-separated and back to the well-mixed state) has been described for systems that phase separate via heterotypic protein–RNA interactions at intermediate RNA concentrations exclusively (*26–29*). Additional theoretical and experimental work has predicted the ability for reentrant phase separation of proteins, peptides, and polymers to occur as a function of pH (*70*), temperature (*69*, *71*, *72*), and pressure (*73*, *74*). Our work presents a novel type of reentrant phase transition in which proteins can phase separate on their own in two distinct regimes in response to changes of ionic strength. While phase separation in the absence of charge screening at low salt concentration

is driven by the cooperation of electrostatic and hydrophobic interactions, the same process at high salt concentration is stabilized mainly by hydrophobic and non-ionic interactions, such as Ala-Ala, Pro–Pro, Tyr–Tyr, Ser–Ser, Arg–Arg (Figure 8). Thereby, our work provides a new view on hydrophobicity and non-ionic interactions as non-specific driving forces for the condensation process, and expands the molecular grammar of interactions governing LLPS of proteins.

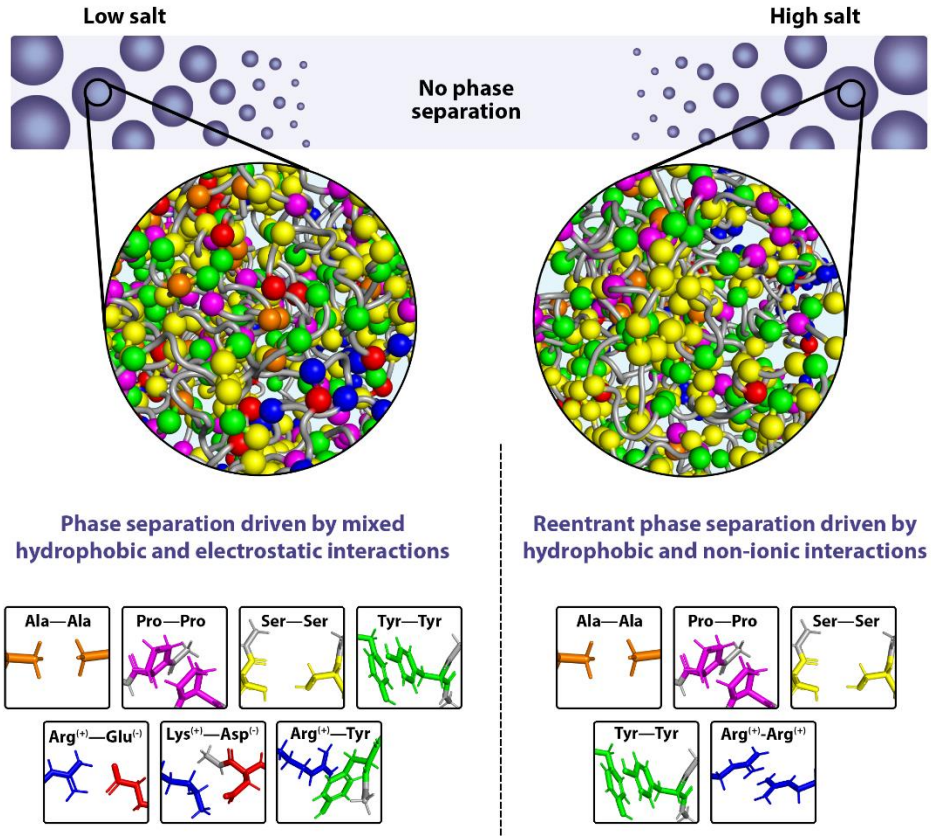

Figure 8. Schematic illustration of the different molecular forces that stabilize condensates in the low-salt versus the high-salt reentrant regime. While phase separation in the low-salt regime is driven by both electrostatic and hydrophobic interactions, the condensation process in the reentrant high-salt regime is governed by hydrophobic and non-ionic interactions.

The observed modulation of LLPS underscores that the molecular driving forces for phase separation are not only dictated by the protein sequence but are also highly sensitive to changes in solution conditions. Solution conditions are essential to LLPS because they regulate the competition between the free-energy reduction stemming from favorable protein–protein interactions in conjunction with the release of water molecules to the bulk, and the entropic cost of demixing (*1*, *14*). The tuneability of LLPS with salt also highlights the complex interplay of

specific intermolecular interactions that commit proteins to particular phases with the condensate environment, and demonstrates the ability for reentrant phase transitions to occur in biomolecular systems under conditions of varying electrostatic screening.

Electrostatically screened conditions have been suggested to be relevant in the pH-driven phase separation of yeast proteins in response to thermal stress, where a drop in pH induces LLPS of Sup35 by promoting the protonation of glutamates (*75*). Such behavior is consistent with a scenario where protonation screens the electrostatic repulsion among Sup35 and, subsequently, enables LLPS via hydrophobic contacts. Similar stress-induced phase separation of the yeast protein Pab1 was shown to be highly dependent on a large hydrophobic domain (*76*). Overall, these observations show that hydrophobically driven protein phase separation plays a critical role in cellular stress response, and thus serve to indicate that such interactions may be a phenomenon of general relevance driving LLPS within the cell.

In addition, aberrant phase separation of FUS, TDP-43, and Annexin A11, the proteins we study here, has been associated with neurodegenerative diseases, such as amyotrophic lateral sclerosis, frontotemporal dementia, and Alzheimer's disease (*7*). The discovery of salt-mediated reentrant phase separation for these systems suggests a crucial feature of protein LLPS behavior that may be important when developing therapies for phase-separation implicated diseases (*77*). For example, drugs that are designed to prevent or reverse phase separation, yet induce high electrostatic screening, could in turn trigger reentrant transitions. Thus, therapies must be aimed at transitioning pathological condensates into an intermediate and not a high electrostatic screening regime.

Moreover, pathological liquid-to-solid phase transitions in FUS are thought to emerge purely from hydrophobic interactions among "low-complexity aromatic-rich kinked segments" (LARKS) that assemble into protofilaments (*78*). LARKS-containing peptides devoid of charged residues interact weakly under physiological conditions (*i.e.*, salt and temperature), as shown by their phase-separation being enhanced at higher salt concentrations (*29*). This observation suggests that, under physiological conditions, LARKS-driven pathological transitions are disfavored. However, in the reentrant high-salt regime, or conditions in cells that give rise to similar electrostatic screening, our work suggests that pathological LARKS–LARKS interactions would be significantly enhanced due to strengthening of hydrophobic interactions, thereby enabling pathological transitions.

Taken together our study identifies a novel salt-mediated reentrant protein LLPS behavior that is enabled by hydrophobic and non-ionic interactions. The discovery of high-salt protein phase separation provides a compelling view of the plasticity of the molecular driving forces for protein phase separation, and emphasizes that these forces are not only defined by the amino acid sequence, but also critically influenced by the condensate environment. These findings highlight the importance of considering solution conditions to which condensates are exposed when aiming at predicting, rationalizing, or modulating protein phase behavior, and when designing therapies to bypass phase separation-related pathologies.

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Supplementary Materials

Materials and Methods
Supplementary Material for atomistic PMF calculations
Figure S1