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Perspective

Phase Separation as a Missing Mechanism for Interpretation of Disease Mutations

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SUMMARY

It is unclear how disease mutations impact intrinsically disordered protein regions (IDRs), which lack a stable folded structure. These mutations, while prevalent in disease, are frequently neglected or annotated as variants of unknown significance. Biomolecular phase separation, a physical process often mediated by IDRs, has increasingly appreciated roles in cellular organization and regulation. We find that autism spectrum disorder (ASD)- and cancer-associated proteins are enriched for predicted phase separation propensities, suggesting that IDR mutations disrupt phase separation in key cellular processes. More generally, we hypothesize that combinations of small-effect IDR mutations perturb phase separation, potentially contributing to "missing heritability" in complex disease susceptibility.

INTRODUCTION

Proteins exhibit a continuum of structures ranging from fully folded to proteins that do not contain any fixed tertiary structure, also referred to as intrinsically disordered proteins. In the human proteome, fully folded proteins (~37%) or entirely intrinsically disordered proteins (~5%) only represent the two extremes of the continuum (Figure 1). The majority of human proteins (~58%) contain both folded protein domains and intrinsically disordered protein regions (IDRs) (Figure 1). Many of these proteins contain disease mutations in both their folded regions and IDRs, including the notable example of tumor suppressor protein p53 (Joerger and Fersht, 2008). However, studies tend to focus on mutations in folded regions while disregarding mutations in IDRs or annotating them as variants of unknown significance, even though IDRs are enriched in disease-associated proteins (Midic et al., 2009; Uversky et al., 2008) and up to 25% of disease-associated missense mutations co-locate to IDRs (Vacic and Iakoucheva, 2012). These observations raise important questions of how to better assess and predict the functional relevance of putative mutations impacting IDRs in disease.

Current computational algorithms that attempt to predict the "pathogenicity" or clinical relevance of a specific mutation have been developed based on the characteristics of folded protein regions (Stefl et al., 2013) with little consideration of understanding the equivalent mutational-impact on IDRs. These methods involve predicting the effect of mutations based on changes in the stability of a tertiary protein structure, which relies heavily on amino acid features relevant to protein folding such as hydrogen bonding, charge states, and exposure of hydrophobic cores (Stefl et al., 2013). However, it makes little sense to use these metrics to evaluate the "stability" of IDRs since IDRs do not contain any single fixed tertiary structure. Other methods incorporate multiple sequence alignments to determine if any mutations fall into evolutionary conserved positions, which could imply a deleterious effect (Stefl et al., 2013). Although this approach is useful for folded proteins, it does not always apply well to IDRs because many IDRs have poor positional protein sequence alignments (Colak et al., 2013), even though they can have conserved features (Zarin et al., 2019). Moreover, the conceptual difficulty in understanding how mutations in IDRs can mechanistically contribute to disease is exacerbated by the long-standing simplistic view that a protein's function is predominantly dictated by a fixed structure, despite many examples contradicting such a "structure-function" paradigm (Wright and Dyson, 2015).

To accurately predict the potential for pathogenicity of mutations in IDRs, it is important to understand their biological functions. One recently appreciated and widespread function of IDRs, in particular low-complexity IDRs (i.e., little amino acid type diversity), is their role in mediating liquid-liquid phase separation, which is a physical process that forms coexisting but

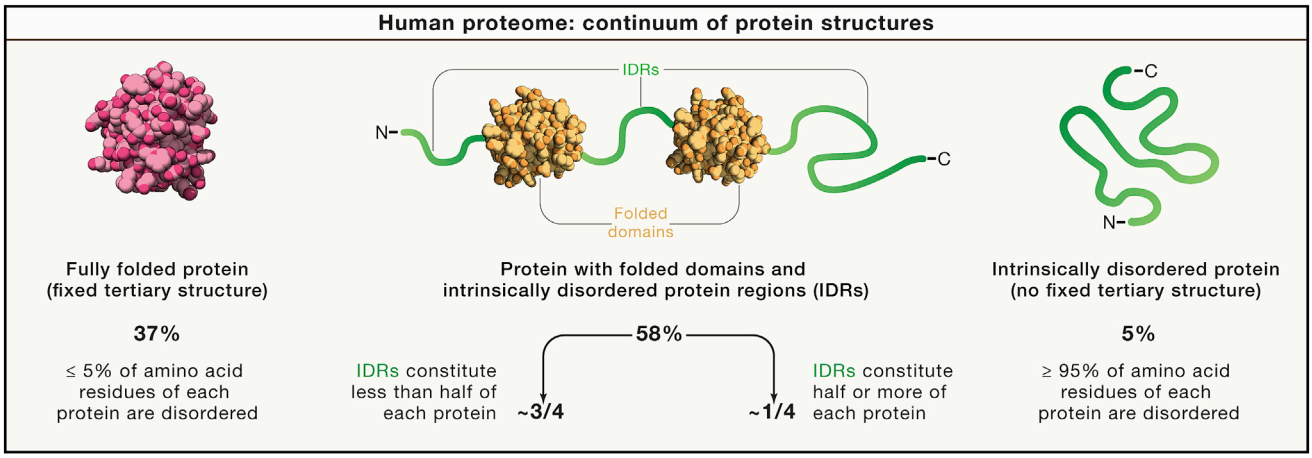

Figure 1. The Human Proteome Exhibits a Continuum of Structures with the Majority of Human Proteins Containing both Folded Protein Domains and Intrinsically Disordered Protein Regions

Continuum of protein structures in the human proteome. Left: fully folded proteins are depicted with a protein globule representing a tertiary protein structure (37%); Center: proteins containing both folded protein domains and intrinsically disordered protein regions (58%). Within this category, around three quarters of the proteins have less than half of their amino acids within IDRs, while the remaining quarter have half or more of their amino acids within IDRs; Right: intrinsically disordered proteins which contain no fixed tertiary protein structure (5%). Disorder predictions of canonical isoforms were calculated using SPOT-Disorder on the human proteome dataset (Uniprot, Proteome ID: UP000005640, Species ID: 9606, downloaded: Aug 2, 2019). Predicted disorder regions separated by seven or less "ordered" residues were concatenated together. Fully ordered or intrinsically disordered is defined as ≥95% of their amino acid sequence predicted to be ordered or disordered, respectively. An IDR is defined as ≥ 30 consecutive amino acids predicted to be disordered.

distinct phases (compartments), similar to oil droplets separating from water (Figures 2A and 2B). To phase separate, IDRs can interact with other IDRs, folded proteins or nucleic acids through different types of multivalent interactions (Box 1). In living cells, the consequence of phase separation is the formation of membrane-less organelles, also referred to as biomolecular condensates (or condensates for short). Some examples of condensates include the nucleolus and transcription factories in the nucleus, stress granules, P-bodies and neuronal granules in the cytoplasm, and synaptic densities at neuronal synapses [extensively reviewed elsewhere (Banani et al., 2017; Gomes and Shorter, 2019)]. Here, we use the word "condensate" to represent any higher-order membrane-less assembly that, regardless of its size, can exclude or specifically concentrate biomolecules (proteins or nucleic acids) into compartments or provide a unique environment to regulate biological processes. We use the phrase "liquid information flow" to underscore the role of condensates formed by liquid-liquid phase separation in organizing and regulating key biological processes, from chromosome condensation, transcription, splicing, and translation, to synaptic activity or receptor activation and then downstream signaling (Gueroussov et al., 2017; Hnisz et al., 2017; Su et al., 2016; Tsang et al., 2019). While interactions of folded protein domains and nucleic acids can also mediate phase separation, with a recent review showing general links between perturbations of phase separation and disease (Alberti and Dormann, 2019), the focus here is on IDR-regulated phase separation given the potential for illuminating the consequences of mutations within IDRs.

In this perspective, we begin by discussing how different putative mutations impacting IDRs might affect the general properties of phase separation. We then illustrate how widespread phase separation is in disease and the utility of using phase separation as a framework to study the wealth of mutational data coming from genome sequencing projects. In particular, we focus our discussion on mutations found by genetic studies of common disorders, including autism spectrum disorder (ASD) and cancer.

For ASD, phase separation is especially relevant because many of the susceptibility genes identified have a role in biological processes involving phase separation, such as RNA processing and synapse function. Moreover, while a growing fraction of ASD can be explained by penetrant "loss-of-function" mutations typically affecting folded protein regions, these high-impact mutations, in an otherwise complex disorder, explain perhaps 5%–25% of the families depending on the severity of symptoms (Fernandez and Scherer, 2017; Satterstrom et al., 2020); it could be that at least some of the "missing heritability" in ASD and other neurodevelopmental disorders (Sanders et al., 2019) may be explained by other mutations such as missense events in IDRs. Such subtle DNA sequence changes, which are readily detected by genome sequencing, can most certainly alter IDRs in known ASD susceptibility or other ASD-associated pathways, but they are not yet regularly assessed in standard medical annotation experiments and genotype-phenotype studies (Geisheker et al., 2017; Schaaf et al., 2020).

Similarly, there are a large number of genetic variants that can increase cancer risk, yet the majority of these variants are infrequently observed and explain a small fraction of the risk (Sud et al., 2017; Taipale, 2018). The key biological processes impacted in ASD are also implicated in cancer, with synapse organization more broadly characterized by receptor activation and subsequent downstream signaling. Numerous genes associated with ASD are also involved in cancer, supporting dysfunction in

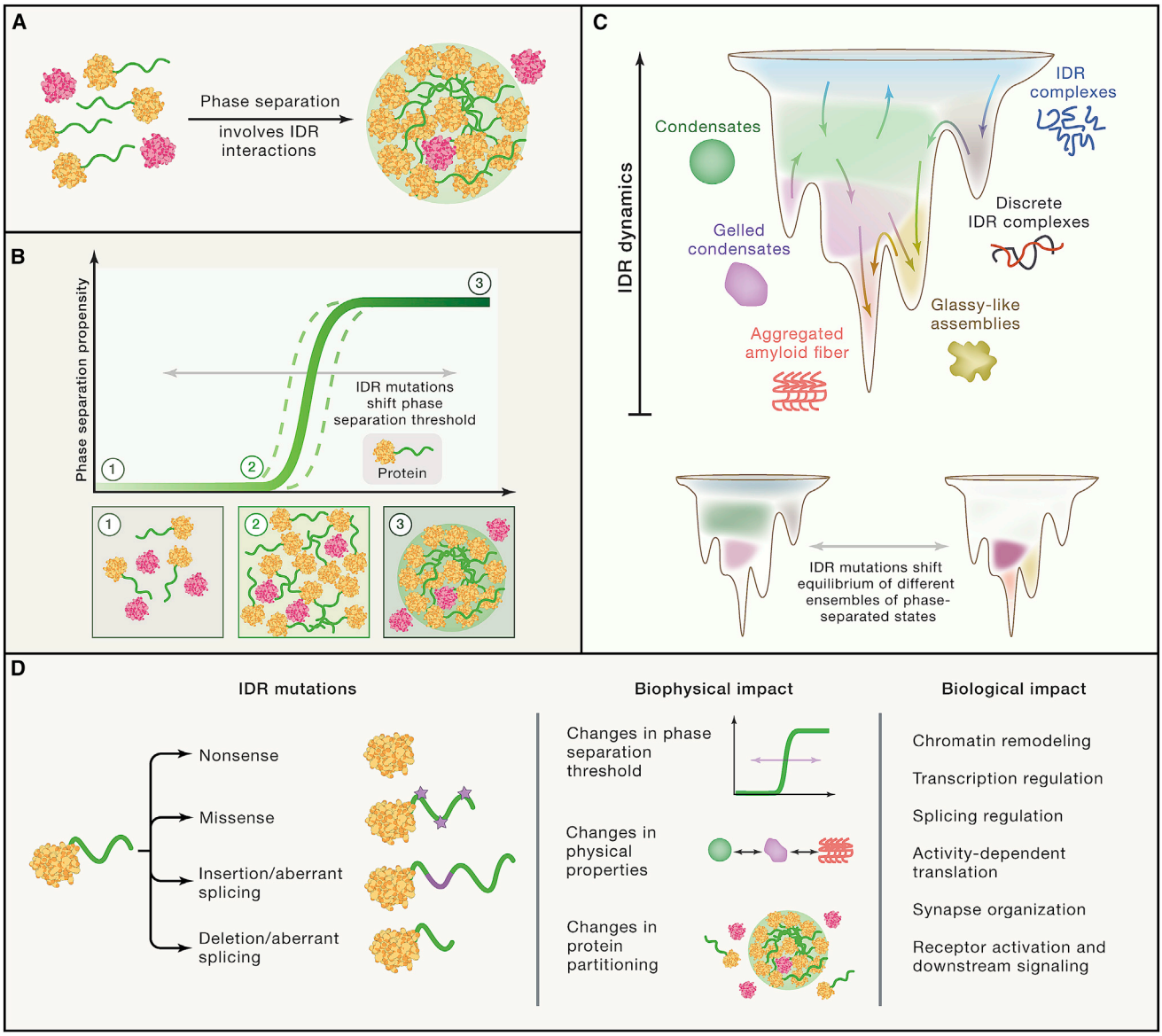

Figure 2. Mutations in IDRs can Change the Propensity for Phase Separation, the Material Properties of Condensates and Partitioning of Interacting Partners

(A) Cellular phase separation driven by IDRs (green tails) leads to the formation of condensed membrane-less droplets that preferentially include or exclude specific molecules.

(B) Phase separation has a sharp cooperative concentration-dependent threshold which may exhibit a range of physiological threshold concentrations shown as green dotted bar lines. Mutations in IDRs can cause aberrant shifts of threshold concentration outside of normal ranges. Square 1 shows minimal protein while Square 2 shows a drastic increase in protein concentration that is at the cusp of phase separation. Square 3 shows green proteins phase separating.

(C) Conceptual model of a free energy landscape showing that ensembles of interacting IDRs can sample a diverse range of states with distinct dynamics and condensate properties.

(D) Mutations in IDRs may change dynamics and sampling of the ensemble of interactions from one state (e.g., liquid condensates) to another (e.g., gelled condensates) or directly change the energy landscape.

(E) Summary of how different mutations in IDRs may lead to biophysical perturbations and biological dysregulation.

the overlapping mechanistic processes. Given these parallel observations between common disorders such as ASD and cancer, we propose a new phase separation "framework" in which IDR mutations may have synergistic effects that contribute to the underlying genetic complexity of disorders for which single genetic variations do not explain most of the heritability.

HOW CAN MUTATIONS IN IDRs IMPACT PHASE SEPARATION?

As noted before, IDRs are important for regulating phase separation; but not all IDRs contribute to phase separation and so not every IDR mutation will necessarily affect phase separation.

Box 1. IDR Interactions Driving Phase Separation

There are multiple mechanisms that IDRs, nucleic acids, or folded proteins use to phase separate to form condensates, but here we focus our attention on phase separation driven by IDR interactions. IDRs that phase separate typically contain low-complexity sequences, i.e., little amino acid diversity, that mediate different multivalent interactions to facilitate phase separation.

(i) Pi interactions: Aromatic residues have been demonstrated to be important for facilitating phase separation, with mutations disrupting phase separation in numerous examples in vitro and in cells (Lin et al., 2015). Mechanistically, these aromatic residues facilitate cation-pi or long-range pi-pi contacts (Vernon et al., 2018). For cation-pi mediated interactions, the number of aromatic and charged residues within a sequence can be predictive of phase separation (Wang et al., 2018). Pi-pi interactions are more widespread than simple aromatic-aromatic stacking contacts. Any chemical groups with linked sp2-hybridized atoms, including the peptide backbone and arginine guanidinium group, can participate in pi-pi interactions and thus potentially contribute to phase separation (Vernon et al., 2018), enabling the development of a valuable predictive algorithm for IDR phase separation based on long-range planar pi-pi interactions. This tool, called PScore (Vernon et al., 2018), returns a score reflecting the Z-score "distance" from values for folded protein sequences, with values >=4 providing a strong prediction for phase separation, and has been validated for an increasing number of proteins. Since it focuses only on planar pi-pi interactions of IDRs, it is not a comprehensive predictor and significantly under-predicts phase separation.

(ii) Electrostatics: Positively and negatively charged stretches of IDRs can facilitate multivalent interaction. IDRs often contain regions enriched in positively charged residues that can interact and phase separate with negatively charged residues on another IDR (Pak et al., 2016) or negatively charged nucleic acids (DNA/RNA) (Zhang et al., 2015). Some IDRs have charge-based sequence patterning with blocks of net positive and net negative charges that can mediate multivalent interactions, such as in DDX4, a germ granule protein (Nott et al., 2015), and FMRP, a translational regulator and RNA-binding protein (Tsang et al., 2019). Scrambling the charge blocks in DDX4 disrupted phase separation (Nott et al., 2015), while enhancement of the negative charge blocks in FMRP increased phase separation (Tsang et al., 2019), supporting the role of charge block patterning.

(iii) Hydrophobicity: The hydrophobic effect is an entropically driven process that originates from the relationship between the protein and its solvent environment. Increasing the temperature is predicted to enhance phase separation that is primarily directed by the hydrophobic effect. Numerous IDR-containing proteins/peptides with a relatively high percentage of hydrophobic residues phase separate in response to increasing temperatures (Reichheld et al., 2017).

(iv) Sequence motifs: Motifs within IDRs can facilitate phase separation. For instance, arginine glycine-rich (RG/RGG) motifs are often found in tandem in IDRs of RNA-binding proteins and can form both pi and charge interactions with RNA (Chong et al., 2018). Phase-separation to hydrogels is associated with short motifs referred to as LARKS (low-complexity, aromatic-rich, kinked segments). LARKS provide weak reversible interactions, described as "Velcro-like", in the form of labile amyloid-like cross-beta interactions (Hughes et al., 2018). These motifs have been identified in numerous low-complexity IDRs, suggestive of their widespread function (Hughes et al., 2018). LARK-like interactions can lead to ordered fibrillar structures, but are dynamic and reversible, consistent with their contribution to liquid phase separation.

(v) Linkers: Computational studies have predicted that increasing the solvation of IDR linkers by forming favorable interactions with solvent disfavors phase separation (Harmon et al., 2017). In contrast, having some compaction due to intra-linker self-attractive forces favors phase separation (Harmon et al., 2017). Moreover, the compactness of these linkers can regulate inter-domain distances that may play a role in their binding and multivalent interactions. In terms of intermolecular linker interactions, linkers may interact, repel, or entangle. Consequently, this may have positive or negative cooperative effects on the binding affinities of tethered modular interaction domains and thus phase separation. Inter-linker entanglement can keep binding domains close for interaction, while inter-linker repulsion would result in a higher energy barrier to interaction. Perturbations of IDR linkers may have profound effects on condensate properties and formation, with mutations or post-translational modifications changing the nature of linkers by modifying compactness and entanglement. Aberrant splicing can affect the length and properties of the linker. These perturbations can affect the total avidity of scaffold protein interactions and thus change the threshold concentrations and potential valency for phase separation.

Thus, the first step is to determine if an IDR of interest linked to disease will phase separate, either experimentally or by using recently established predictive tools such as PScore (see Box 1 for more information) (Vernon and Forman-Kay, 2019; Vernon et al., 2018). If an IDR is not predicted to phase separate, it still likely has other important regulatory functions, extensively reviewed elsewhere (Wright and Dyson, 2015). If phase separation is predicted or experimentally demonstrated for an IDR of interest, then how could different mutations in that IDR affect its phase separation? Given the cooperative threshold properties of phase separation, mutations that affect IDR protein concentrations or even a single amino acid change in an IDR can perturb the threshold concentration for phase separation, i.e., condensate formation (Figure 2B). Similarly, IDR mutations may also change the material properties of condensates ranging from dynamic liquids to aberrant fibrils (Figures 2C and 2D).

Based on the IDR interactions that regulate phase separation, different classes of mutations may have different effects (Figure 2E). For example, nonsense mutations that truncate large IDR segments will eliminate potential multivalent protein-protein interactions facilitating phase separation and cause anomalous shifts in threshold protein concentrations necessary

for condensate formation (Figure 2B). Threshold concentrations are certainly not fixed since cells can exhibit a range of physiological thresholds depending on the types and strengths of protein interactions (homotypic and heterotypic) (Riback et al., 2020), but we emphasize that mutations can cause aberrant shifts from physiologically relevant to pathological threshold concentrations. Nonsense mutations typically lead to haploinsufficiency, and the underlying molecular mechanism may be from the perturbation of phase separation due to the abnormal increase in threshold protein concentration required to phase separate, with similar effects due to mutations that delete genes encoding proteins IDRs. Truncations of IDRs could also affect protein stability (Babu et al., 2011), which would result in lower protein concentrations. IDR truncations may also disrupt specific protein-protein interactions that affect protein partitioning into condensates (Miyake et al., 2020) and alter protein-protein interactions that change normal threshold concentrations for phase separation (Riback et al., 2020). Missense mutations in IDRs can change the strength of the underlying physicochemical interactions (Box 1) that give rise to material properties of a condensate, varying from a dynamic liquid (readily assembling and disassembling) to an aberrant gelled or fibrillar state in disease (Molliex et al., 2015; Murakami et al., 2015; Patel et al., 2015). These mutations have the potential to produce dominant-negative effects by altering the wild-type properties of condensates. For example, pathological gelled condensates have been functionally associated with the trapping of proteins and nucleic acids and perturbing their transport or disassembly required for translational regulation (Gopal et al., 2017; Murakami et al., 2015). Missense mutations in IDRs may also disrupt specific protein interactions that affect protein or nucleic acid partitioning into condensates causing mis-localization and associated gain or loss-of-function effects (Hofweber et al., 2018; Niaki et al., 2020). Conformations of IDRs may also be affected by missense mutations that promote intramolecular interactions (inhibiting phase separation) instead of expanded multivalent intermolecular interactions (favoring phase separation) (Sanders et al., 2020). Moreover, because IDRs are the predominant sites of post-translational modifications (PTM) (Tompa et al., 2014), missense mutations that alter PTM sites can lead to dysregulation of phase separation (Monahan et al., 2017).

Insertions or deletions that lead to frameshifts can act as nonsense mutations. In-frame insertions or deletions can have more significant biophysical effects than missense mutations depending on how many residues and which ones. Repeat expansion mutations can add sizeable low-complexity phase-separating regions that often have high propensity to phase separate (Alberti and Dormann, 2019) mRNA regions that code for IDRs are frequently sites of alternative splicing, thereby enabling modulation of specific protein interactions, phase separation propensities and cellular signaling pathways (Buljan et al., 2013; Ellis et al., 2012; Romero et al., 2006). Different alternative splice isoforms that incorporate or remove different multivalent elements in IDRs (Box 1) have been shown to produce different phase separation propensities and changes in function, with effects mimicking those for insertion and deletion mutations. For example, different protein isoforms can disrupt or modulate phase separation (Smith et al., 2020) or cause irreversible protein aggregation in disease (Batlle et al., 2020). Moreover, changing the multivalent patterns in IDRs may lead to gain-of-toxic function by introducing new interaction partners and activating aberrant pathways (Han et al., 2020). Importantly, regulation of phase separation by alternative splicing may be a mechanism to allow proteins to "escape" specific condensates to diversify their function, and mis-splicing will dysregulate this process (Gueroussov et al., 2017). In summary, mutations in IDRs can perturb function by altering phase separation propensities, the physical features of condensates and the partitioning and interactions of other proteins (Figure 2E).

COMPLEX DISEASES EXAMINED THROUGH A NEW FRAMEWORK

How widespread and relevant is phase separation in medical conditions involving genes that code for proteins enriched with IDRs? By analyzing different databases, we find significant enrichment for phase separation (as predicted by PScore) of disease-associated proteins including ASD ($p = 2.46 \times 10^{-10}$), cancer ($p = 3.08 \times 10^{-14}$) and neurological disorders in general ($p = 8.75 \times 10^{-5}$) (see Figures 3A and 3B for methods and databases used). There is no significant difference in PScore for proteins associated with type 2 diabetes, consistent with the smaller sample size for type 2 diabetes, but also suggestive of some specificity of phase separation to disease mechanisms. This result highlights the potential functional importance of phase separation in a range of diseases. Importantly, PScore predicts only proteins expected to phase separate due to planar pi-pi interactions in their IDRs, which does not account for the many other interactions contributing to IDR-driven phase separation (see Box 1). Thus, our predictions based on PScore are considered conservative estimates that are expected to be substantial under-predictions of phase separation (Vernon and Forman-Kay, 2019; Vernon et al., 2018).

From our pilot analysis, we selected ASD as a significant exemplar for studying IDR mutations and phase separation for multiple reasons. First, genes associated with ASD demonstrate a strong enrichment for predicted phase separation propensity (Figure 3B). Moreover, because the PScore of ASD-associated proteins is significantly greater than the PScore of other proteins encoded by highly abundant brain genes ($p = 2.27 \times 10^{-7}$) (Figure 3B), this result strongly suggests that phase separation is not a baseline property of highly expressed neuronal proteins; rather, phase separation may specifically be involved in biological processes underlying ASD. Of equal importance is the wealth of high-quality genome sequence data available for ASD and the growing body of literature testing the myriad of putative mutations of all classes for their functional and clinical impact through population genetic studies, and model systems (de la Torre-Ubieta et al., 2016; Vorstman et al., 2017). We use cancer as a second example, given the highly significant enrichment for phase separation (as predicted by PScore) of cancer-associated proteins ($p = 3.08 \times 10^{-14}$). The substantial overlap of cellular processes implicated in ASD and cancer also hints at common mechanisms and pathways.

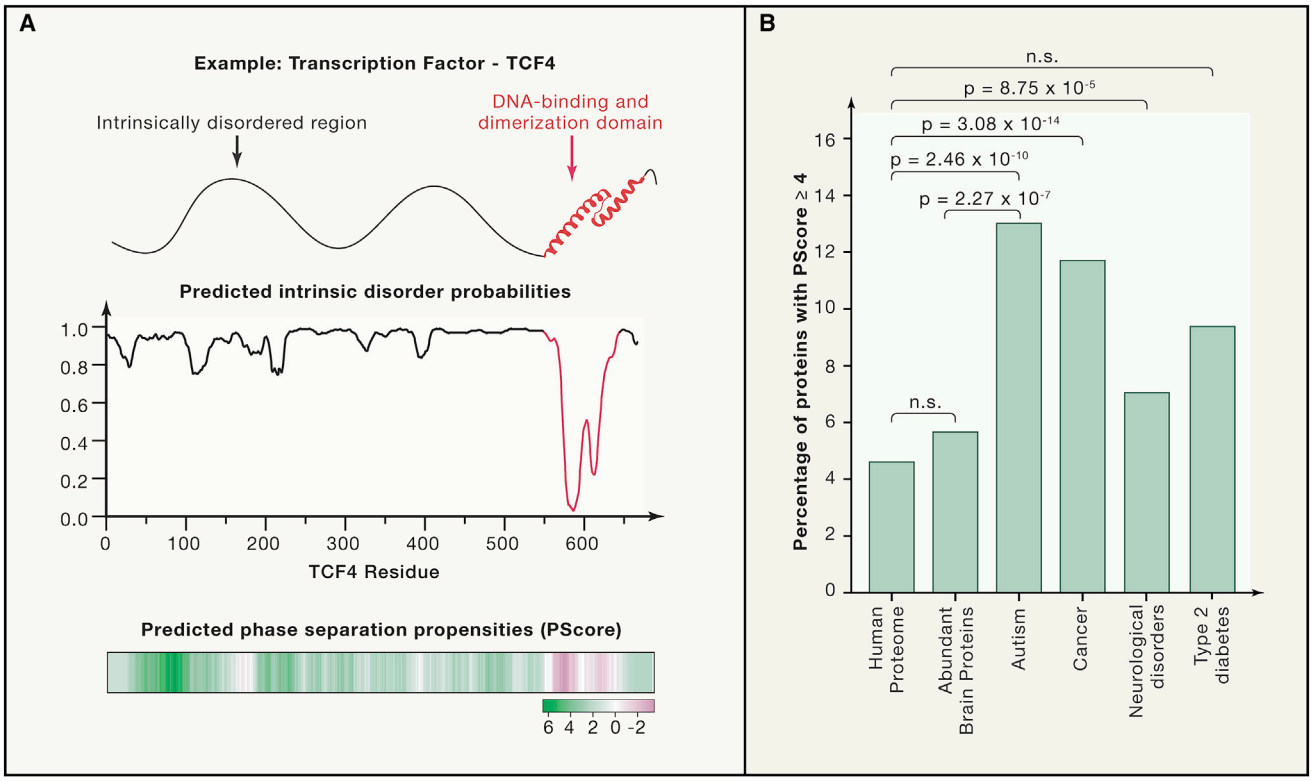

Figure 3. Proteins Encoded by Genes Implicated in Numerous Diseases are Enriched for Predicted Phase Separation Propensity

(A) As an example, Transcription factor 4 (SFARI Score: 1; PScore: 5.77) contains a long predicted intrinsically disordered tail shown in black with a C-terminal DNA-binding and dimerization domain shown in red [PDB:60D3]. Predicted phase separation propensities (PScore) is shown below. A score of ≥ 4 is considered a confident phase separation prediction and a score of 0 represents the PDB average.

(B) Percentage of proteins with predicted phase-separation propensity (PScore ≥ 4). The human proteome retrieved from UNIPROT (ID UP000005640) (n = 20401). Highly expressed brain genes represent the 75th percentile from BrainSpan (n = 4667). Genes associated with Autism retrieved from the SFARI database including high confidence or strong ASD candidates "SFARI 1, 2" (n = 369). Genes associated with cancer retrieved from COSMIC cancer gene consensus (n = 735). Genes associated with neurological disease retrieved from a subset from OMIM (n = 1390). Genes associated with type 2 diabetes retrieved from the Type 2 Diabetes Knowledge Portal including only 'CAUSAL' and 'STRONG' categories (n = 69). PScore is enriched in autism relative to the human proteome ($p = 2.46 \times 10^{-10}$) or highly expressed brain genes ($p = 2.27 \times 10^{-7}$). P values derived from Fisher's exact test.

In the subsequent sections, we discuss existing data and evidence-guided hypotheses regarding the impact of IDR mutations on phase separation in established ASD- and cancer-associated cellular processes, namely, (1) chromatin regulation, (2) transcription, (3) splicing, (4) translation, and more specifically for each (5) synapse organization or (6) receptor activation and signaling. We tabulate all mentioned (and additional) ASD-associated candidate genes (proteins) with their PScore, evidence for phase separation, the strength of autism association (e.g., SFARI scores) (Larsen et al., 2016), and annotated missense or truncation mutations within IDRs along with clinical annotations (e.g., ClinVar) in Table S1. The examples we cite are for genes having mutations in their IDRs and, for clarity, these IDR mutations are also mapped to corresponding canonical protein isoforms in Table S1. IDR variants and PScore values for selected cancer-associated proteins with mutations in their IDRs are provided in Table S2. The need to understand consequences of IDR mutations is underscored by the high percentages of ASD and cancer variants mapping to IDRs, with Table 1 showing the average percentages, 69% and 50%, respectively, for the selected genes we highlight in Tables S1 and S2.

ASD and cancer have previously been noted to share risk genes (Crawley et al., 2016), though their relationships are unclear. Using the lens of phase separation, we identify an overlap between ASD and cancer-associated genes that are predicted to phase separate (Table S3), suggestive of overlapping affected core pathways. Importantly, introducing phase separation as a framework for understanding IDR mutations does not imply that other established or proposed mechanisms for ASD and cancer are not operative. In many cases, these are described through effects on regulation of chromatin, transcription, splicing, translation, synapse organization, receptor activation and downstream signaling. What we are proposing is biophysical insight into how IDR mutations can feed into these previously described mechanisms.

Chromatin Regulation

Transcriptional profiling studies have reported altered gene expression patterns in post-mortem brains from individuals with ASD (Gandal et al., 2018; Velmeshev et al., 2019), as well as in studies of cell-based and animal models of ASD (Tran et al., 2019). Cancer has very strong associations with

Table 1. Percentage of Variants Mapping to IDRs for Described ASD[a] and Cancer[b] Genes

	Variants in IDRs (%)	Predicted Pathogenic Missense in IDRs (%)
ASD	69	N/A
Cancer	50	48

[a]averages of the selected genes listed in Table S1 (SELECTED_GENES_SFARI)
[b]averages of the selected genes listed in Table S2 (SELECTED_GENES_COSMIC)

dysregulated chromatin (Valencia and Kadoch, 2019). Given the recent evidence supporting protein-DNA phase separation as a mechanism that can organize condensed chromatin structures at different length scales from clusters of nucleosomes to kilobase-sized and larger domains (Gibson et al., 2019; Larson et al., 2017; Sanulli et al., 2019; Strom et al., 2017; Wang et al., 2019), we can begin to speculate how ASD and cancer mutations impacting chromatin regulators could perturb phase separation and lead to transcriptional dysregulation.

For example, the Polycomb repressive complex (PRC1) is a group of proteins that perform post-translational modifications on histone tails to compact chromatin and inhibit gene expression. (Francis et al., 2004). Phase separation of PRC1 is mediated by one of its core protein components, chromobox 2 (CBX2) (Plys et al., 2019; Tatavosian et al., 2019). PRC1 can also activate gene expression by interacting with other predicted phase-separating proteins including autism susceptibility candidate 2 (AUTS2) (Gao et al., 2014). ASD-associated mutations of AUTS2 (Table S1), initially predicted to act via haploinsufficiency (Beunders et al., 2013), could decrease AUTS2 cellular concentration and potentially alter PRC1-AUTS2 condensates. In this instance, mutations in IDRs may lead to dominant-negative effects resulting from altered condensates properties or phase separation threshold concentrations.

Other histone-modifying enzymes are predicted to phase separate and contain ASD and cancer mutations in their IDRs (e.g., EP300, KDM6B, KMT2A/C). These mutations may result in mis-localization of these enzymes such that they fail to modify appropriate histones and perturb condensate formation and properties (Tables S1, S2). Chromodomain Helicase DNA-binding Protein 8 (CHD8) is a chromatin remodeler predicted to phase separate and is linked to ASD due to its regulation of other ASD candidate genes during neurodevelopment (Katayama et al., 2016). CHD8 haploinsufficiency in ASD supports the concept of achieving a certain protein concentration threshold to form condensates to regulate gene expression. Other Chromodomain Helicase DNA-binding Proteins (CHD1/2/7) and chromatin-remodeling factors (e.g., ARID1B, SMARCA4, SMARCC2) are predicted to phase separate, and we speculate that ASD mutations in their IDRs (as well as cancer-associated mutations specifically in ARID1B) could perturb their phase separation propensity (Tables S1, S2, S3). Many chromatin regulators implicated in cancer (Gibbons, 2005; Morgan and Shilatifard, 2015) are predicted to phase separate. These proteins include components of the Polycomb repressive complex (e.g., ASLX2), histone-modifying enzymes (KMT2D) and chromatin remodelling factors (e.g., ARID1A, SMARD1). We propose that these mutations impact the properties of chromatin condensates, alter protein-protein interactions and influence gene expression. In particular, changes in gene expression may alter the compositions of other condensates that may affect their function by changing their interaction partners and threshold concentrations for phase separation.

Transcription

Regulation of gene transcription in response to neuronal activity has been hypothesized to play a key role in the etiology of ASD (Ebert and Greenberg, 2013). Transcriptional dysregulation is a known core driver of cancer (Bradner et al., 2017). The dynamic properties of phase separation to readily assemble and disassemble likely contributes to transcriptional processes underlying ASD and cancer. Newer studies suggest that phase separation could provide a mechanism for concentrating co-activators, transcription factors and RNA polymerase II into dynamic transcription condensates at super-enhancers (Boehning et al., 2018; Boija et al., 2018; Cho et al., 2018; Sabari et al., 2018), although rigorous experiments testing and confirming these ideas and observations are still needed (McSwiggen et al., 2019; Mir et al., 2019).

The C-terminal IDR (CTD) of RNA polymerase II phase separates *in vitro* and clusters with chromatin in cells dependent on the number of heptapeptide repeats it harbors (Boehning et al., 2018). Phosphorylation of the CTD can modify intermolecular interactions and allow RNA polymerase II to partition into different transcriptional or splicing condensates (Guo et al., 2019; Lu et al., 2018) to facilitate the processing of ''information'' stored in mRNAs. Interestingly, a set of ASD-candidate genes includes specific kinases (*CDK13*, *CCNK*, *DYRK1A*) (Table S1) that may phase separate to capture and phosphorylate the CTD (Lu et al., 2018) and to target RNA Pol II to different condensates. The cancer-associated kinase, CDK12, also phosphorylates RNA Pol II with a potentially similar effect on RNA Pol II partitioning (Bonnal et al., 2020; Pilarova et al., 2020). Given these observations, we expect that ASD- and cancer-associated mutations in IDRs of these kinases (Table S1, S2, S3) could alter their phase separation propensities leading to aberrant localization and phospho-regulation of RNA Pol II. The functional effects of kinase or RNA Pol II phase separation could be evaluated by examining the transcriptional activity of highly transcribed synaptic genes with broad enhancer-like domains implicated in ASD, referred to as BELD genes (Zhao et al., 2018) or similarly the transcription of oncogenic-drivers.

Transcriptional regulators are predicted to phase separate with mutations affecting IDR elements. In particular, Transcription Factor 4 (TCF4) is an activity-dependent transcription factor with a significant number of binding sites found within numerous ASD candidate genes (Forrest et al., 2018), which is reflective of its direct regulatory role in synaptic plasticity and neurodevelopment (Jung et al., 2018). TCF4 organizes transcriptional networks necessary for neuronal differentiation (Quevedo et al., 2019) in a phosphorylation-regulated manner (Sepp et al., 2017), which likely plays a role in its phase separation propensities. Moreover, numerous TCF4 isoforms contain variable

C-terminal IDR lengths that we predict would alter its predicted phase separation propensity. Thus, we expect that mutations in TCF4 IDRs and mis-splicing of its C-terminal IDR will perturb its phase separation leading to dysregulated transcription.

Ligand-dependent transcriptional regulators have been shown to have tunable transcriptional activities correlated with altered condensate properties (Nair et al., 2019). Acute estrogen stimulation forms transcriptionally active and reversible estrogen receptor alpha (ERα) condensates while chronic stimulation forms stabilized gelled ERα condensates that are transcriptionally less active (Nair et al., 2019). The potential link between these findings and the finding that elevated prenatal estrogen levels significantly modulate developmental trajectories in ASD (Baron-Cohen et al., 2020) might be worth considering. Moreover, an ASD-associated C-terminal IDR truncation of the transcription factor β-catenin (CTNNB1) is predicted to abolish its phase separation capacity (Zamudio et al., 2019) (Table S1), and potentially disrupt Wnt/β-catenin-dependent signaling strongly implicated in ASD (Kwan et al., 2016). In cancer, phosphorylation of the disordered N terminus of β-catenin leads to its degradation, likely via phase separation of a multi-protein destruction complex (Kim et al., 2010; Schaefer and Peifer, 2019). This region is a cancer "mutation hotspot" with 9 mutations in the Online Mendelian Inheritance in Man (OMIM) database of genes and genetic disorders (Kim and Jeong, 2019) (Table S3); these mutations may prevent phosphorylation, stabilize β-catenin, and enhance expression of specific target genes to promote cellular proliferation in various cancers (Morin et al., 1997). Therefore, phase separation of transcription factors like ERα and β-catenin can act as sensors to specific intracellular and extracellular changes and respond to the "information" flow from the environment by modulating appropriate gene expression programs.

Splicing

Transcriptome-wide dysregulation of mRNA splicing levels has been observed in post-mortem brains of people with ASD (Gandal et al., 2018; Irimia et al., 2014; Voineagu et al., 2011). Aberrant mRNA splicing has also been shown to be a driver of cancer (Sveen et al., 2016). These findings raise questions about the properties of spliced regions and their mechanistic contributions to disease. We postulate that given the abnormal splicing patterns and isoform usage reported in ASD and cancer, and the tendency of IDRs to undergo alternative splicing, it is likely that alternative splicing modulates the phase-separation propensities of spliced proteins. Moreover, IDR mutations can affect the formation and function of phase-separated splicing assemblies and directly cause mis-splicing of proteins which perturbs their biological and regulatory functions. For example, cancer-associated mutations are found in IDRs of a number of splicing regulators predicted to phase separate (e.g., HNRNPA2B1, RBM10, SFPQ, Table S2), and we suspect that these mutations perturb their phase separation propensities and splicing function.

The hnRNPA/D protein family forms distinct multivalent assemblies, regulated by phase separation with additional hnRNPs, to globally control alternative splicing (Gueroussov et al., 2017). Note that HNRNPA2B1 is overexpressed in breast cancers (Hu et al., 2017). Splicing of alternative exons overlapping glycine-tyrosine rich (GY) motifs within hnRNPA/D C-terminal IDRs can regulate their phase separation propensity and splicing functions (Batlle et al., 2020; Gueroussov et al., 2017). Fibrillization can be enhanced in liquid condensed phases (Murakami et al., 2015; Patel et al., 2015), and missense mutations in the IDR of hnRNPA1 leading to pathological fibrillization have been reported (Molliex et al., 2015). This raises the possibility that mutations in other hnRNP gene families linked to ASD (e.g., *hnRNPU* and *hnRNPH2*) (Table S1) may have similar pathological mechanisms via phase separation to disrupt splicing function.

The splicing factor protein RBFOX1 regulates alternative splicing of neuronal gene networks enriched with ASD candidate genes (Lee et al., 2016; Weyn-Vanhentenryck et al., 2014). Intriguingly, the splicing activity of RBFOX1 was shown to be correlated with the propensity of its C-terminal IDRs to form higher-order splicing protein assemblies (Ying et al., 2017). Mutations in RBFOX1-IDR disrupted the formation of splicing assemblies and led to aberrant splicing and inclusion (Ying et al., 2017). Thus, truncation mutations affecting the C-terminal IDR of RBFOX1 (Lee et al., 2016) (Table S1) may perturb protein-protein interactions and shift the phase separation threshold of splicing assemblies resulting in the dysregulated splicing observed in ASD.

Highly conserved neuronal microexons (3-27 nt) are most frequently mis-spliced in ASD (Irimia et al., 2014; Voineagu et al., 2011). Microexon splicing events generally lead to the skipping of one to nine amino acids, which would be sufficient to remove specific motifs in IDRs important for phase separation including short linear motif (SLiM), multivalent repeats, or a stretch of low-complexity sequence (Irimia et al., 2014) (Box 1). For example, splicing of an ASD-associated microexon in the N-terminal IDR of the cap-binding initiation factor eIF4G modulated its ability to phase separate which coincides with dysregulation of synaptic protein synthesis and altered synaptic plasticity in mouse models (Gonatopoulos-Pournatzis et al., 2020). More studies of the transcriptomes in ASD and cancer are needed to probe the link between alternative splicing patterns and pathology for these diseases.

Translation

ASD-relevant genes encode proteins such as FMRP, CAPRIN1 (also known as FMR2), and TSC1/2 that regulate localized translation in a neuronal activity-dependent manner (Jung et al., 2014). Disruption of activity-dependent translation has been proposed to be linked to ASD (Gkogkas et al., 2013; Kelleher and Bear, 2008). Dysregulation of RNA processing (e.g., LSM14A) and translational control (DROSHA) are also linked to cancer (Anderson et al., 2015; Jansson and Lund, 2012) and have a clear relationship to phase separation (Table S2). Specific signaling systems such as the mammalian target of rapamycin (mTOR) pathway can regulate activity-dependent translation with several proteins in this pathway implicated in ASD (Winden et al., 2018) and regulation of translation by mTOR has very strong links to cancer and tumor cell proliferation (Mossmann et al., 2018).

For ASD, activity-dependent translation involves packaging and transporting mRNAs toward distal sites of a neuron via

phase separation of RNA-binding proteins that form neuronal granules (Jung et al., 2014). An ASD truncation mutation that removes the C-terminal IDR of the neuronal granule protein CAPRIN1 is predicted to impede its phase separation (Table S1). This mutation may impair CAPRIN1 mediated localization and translation of mRNAs. Moreover, phase separation may facilitate localized translation in axons and dendrites in response to neuronal activity. Phosphorylation of the C-terminal IDR of the RNA-binding protein FMRP promotes its phase separation which correlates with *in vitro* translation inhibition (Tsang et al., 2019). This result raises the interesting hypothesis that loss of FMRP expression in Fragile X syndrome, a monogenic disorder often exhibiting ASD (Kelleher and Bear, 2008), may dysregulate translation in part due to perturbed phase separation properties of neuronal granules. Thus, in neurons, mRNAs are first packaged and transported within condensates in a translationally silent state; after arriving at their cellular destination (axon/synapse), signaling dependent post-translational modifications modulate condensate properties to release mRNAs for translation.

Cytoplasmic polyadenylation element-binding, encoded by the CPEB1-4 genes, regulates the stability and translation of CPEB targeted mRNAs via their polyA tails (Richter, 2007). Mis-splicing of CPEB4 causes an aberrant shortening in polyA tail length (de-adenylation) of known ASD candidate genes and a corresponding reduction in their protein levels (Parras et al., 2018). It would be interesting to investigate the predicted phase separation of CPEB4 and its isoforms and their mechanistic impact on polyA tail regulation and translation. Moreover, phase separation may fine-tune de-adenylation and translation rates (Kim et al., 2019). Modulating the PTM states of FMRP and CAPRIN1 produced different FMRP-CAPRIN1 condensate environments which corresponded to different *in vitro* de-adenylation or translation rates (Kim et al., 2019). It remains to be tested if IDR mutations in other ASD-associated deadenylases (Table S1) can alter phase separation propensities and contribute to the abnormal de-adenylation and translation activity reported in ASD.

Synapse Organization

An imbalance in excitatory and inhibitory synaptic activity is hypothesized to underlie ASD (Nelson and Valakh, 2015). Recently, *in vitro* phase separation of ASD-associated synaptic receptors and scaffolding proteins (Table S1) were shown to form pre and postsynaptic condensates, which mimicked the organization of synapses (Milovanovic et al., 2018; Zeng et al., 2016, 2018). These experimental findings have led to proposals for the role of phase separation in regulating the organization of excitatory and inhibitory synapses.

For example, ASD-associated proteins SynGAP and PSD95 formed post-synaptic condensates with a sharp concentration-dependency (Zeng et al., 2016). This result supports the haploinsufficiency reported for SynGAP in ASD since condensate formation occurs only when a threshold concentration is reached. Moreover, because PSD95 is an interaction hub connecting numerous synaptic proteins (Feyder et al., 2010), perturbations to PSD95 phase separation would be expected to disrupt post-synaptic condensate formation and cause mis-localization of regulatory components (Table S1). In particular, the *SHANK1*, *SHANK2*, and *SHANK3* genes encode scaffolding proteins that interact with PSD95 and contain numerous ASD mutations in their long IDR linkers (Leblond et al., 2014) (Table S1). Mutations in IDR linkers can affect molecular interactions that modulate phase separation properties (Box 1), making them targets for further investigation.

Both genetic and functional studies have linked numerous mutations in voltage-gated calcium channels to ASD (Heyes et al., 2015). For example, pre-synaptic Calcium Voltage-Gated Channel Subunit alpha 1A (CACNA1A) is predicted to phase separate and is involved in calcium signaling, with mutations implicated in Timothy Syndrome, a condition characterized by cognitive impairments and ASD symptoms (Splawski et al., 2004). The cytoplasmic C-terminal IDR of CACNA1A exhibits polyQ expansions (Zhuchenko et al., 1997) that may rigidify postsynaptic condensates and impair their regulatory function, as shown previously for other phase-separating proteins (Peskett et al., 2018). Other protein repeat expansions have also been shown to alter protein-protein interactions and condensate compositions, leading to dysregulated function and disease (Basu et al., 2020). Moreover, cytosolic loops and IDR tails of other membrane receptors (e.g., SCN4A and HCN1, Table S1) have mutations linked to ASD; valuable insights may come from establishing the impact of these ASD-associated variants on post-synaptic condensates and their effect on clustering receptors to regulate synaptic transmission.

Reception Activation and Signaling

Predicted phase separation of transmembrane NOTCH receptors (e.g., NOTCH1/2, Table S2) may represent a crucial mechanism of information transfer from cellular surfaces to the nucleus via cell-to-cell contacts and downstream signaling (Nowell and Radtke, 2017). Phase separation of activity-dependent transcription factors is implicated in the relay of cellular stimuli back to the nucleus (e.g., NF-κB), and its dysregulation may result in oncogenic cellular proliferation and survival (Hoesel and Schmid, 2013). Oncogenic disruption of signaling pathways can dysregulate protein phase separation resulting in aberrant downstream signaling and protein regulation including actin filament organization (e.g., WASP) implicated in cancer (Yamaguchi and Condeelis, 2007). Thus, both receptor activation and downstream signaling in cancer can involve phase separation.

A PHASE SEPARATION FRAMEWORK FOR UNDERSTANDING DISEASE-ASSOCIATED MUTATIONS IN IDRs

The formation and regulation of condensates driven through IDR-mediated phase separation is central in organizing the dynamic "liquid" flow of information from DNA to RNA to protein and back (Figure 4). Beginning in the nucleus of a cell, different condensates can regulate various aspects of chromatin regulation, transcription, and alternative splicing. Formation of transport condensates moves mRNAs from the nucleus to sites of local translation, including the synapse for activity-dependent translation in neurons. The dynamic nature of phase separation allows condensates to regulate translation in response to various stimuli, including synaptic transmission. Translated synaptic

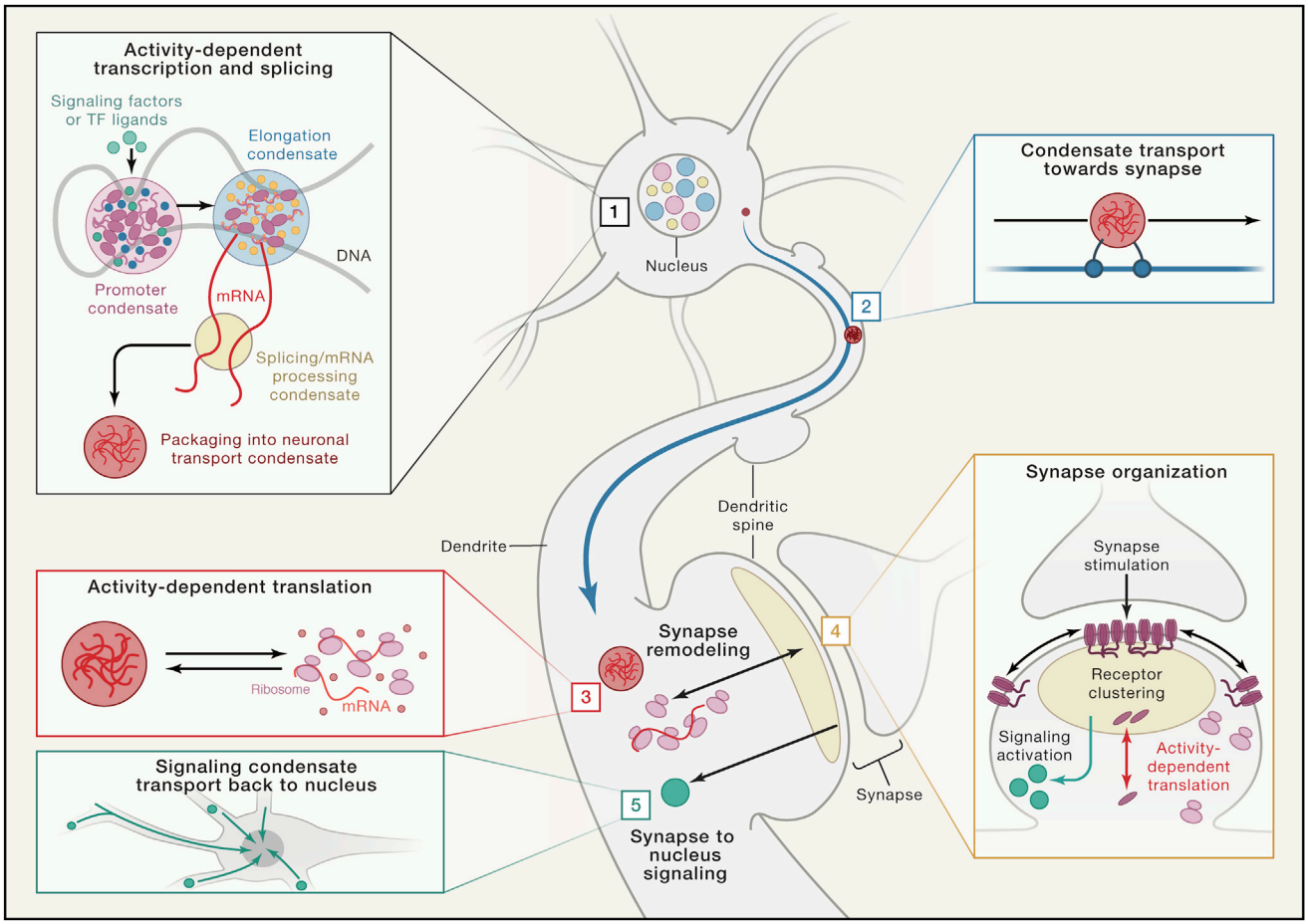

Figure 4. Schematic Model for How Synaptic Plasticity or Cell Growth may be Regulated via Liquid Information Flow from DNA to RNA to Protein in a Feedback-Dependent Manner

1. Beginning in the nucleus, specific promoter condensates are formed in response to phase separation of specific signaling factors, transcription factors and ligands. RNA Pol II is shuttled between promoter and elongation condensates based on its phosphorylation state. Transcribed mRNAs are processed and spliced in specific condensates and packaged into specific condensates for transport. **2.** mRNA transport condensates transport translationally silenced mRNAs toward sites of local translation, including dendrites and axons in neurons. **3.** At synaptic sites, neuronal condensates can reversibly disassemble to release mRNAs for translation or reassemble to capture mRNAs for translational silencing in response to stimuli. Proteins synthesized here include synaptic scaffolding and adhesion proteins and receptor subunits that remodel post-synaptic condensates. Note localized translation occurs in pre-synaptic compartments, as well (not shown). **4.** Post-synaptic condensates cluster receptors to facilitate synaptic transmission and signaling processes. Pre-synaptic condensates (not shown) can also cluster receptors and synaptic vesicles to facilitate signal transmission. Receptor clustering is associated with activation of growth factor receptors in all cells. **5.** Activation of signaling processes promotes the formation of signaling condensates that are trafficked from synapses or from the growth factor receptors of cells back to the nucleus to modulate transcription and splicing.

proteins build and organize the synapse to facilitate appropriate synaptic transmission. Translation in cells in general enables cell growth, a key factor in cancer. Activation of different signaling pathways can send information back to the nucleus via activity-dependent transcription factors. Phase separation of these transcription factors helps organize different signaling and activity-dependent transcription/splicing programs by changing condensate properties, molecular compositions or phase separation propensities. This effectively controls mRNA isoform expression and protein translation, which in turn can further impact phase separation processes involving genes and proteins. This general understanding of biology in terms of phase separation is relevant to normal cellular processes as well as understanding how mutations in these proteins cause disease.

In the framework of phase separation, mutations that profoundly alter IDR properties might cause a drastic effect on phase separation thresholds resulting in pathological condensates disrupting processes related to synaptic plasticity or cell growth (Figure 5A). However, the formation of condensates and their characteristics could also be impacted by a combination of IDR mutations that have lower impact on IDR properties. Due to the sensitivity of phase separation to local protein concentrations, these variants, which individually may have smaller functional effects, could collectively cause drastic shifts in phase separation thresholds. In this way, individual mutations affecting IDRs that may appear benign, i.e., variants that would not be expected to have a major gain or loss-of-function individually, may together have a substantial and aberrant effect on phase

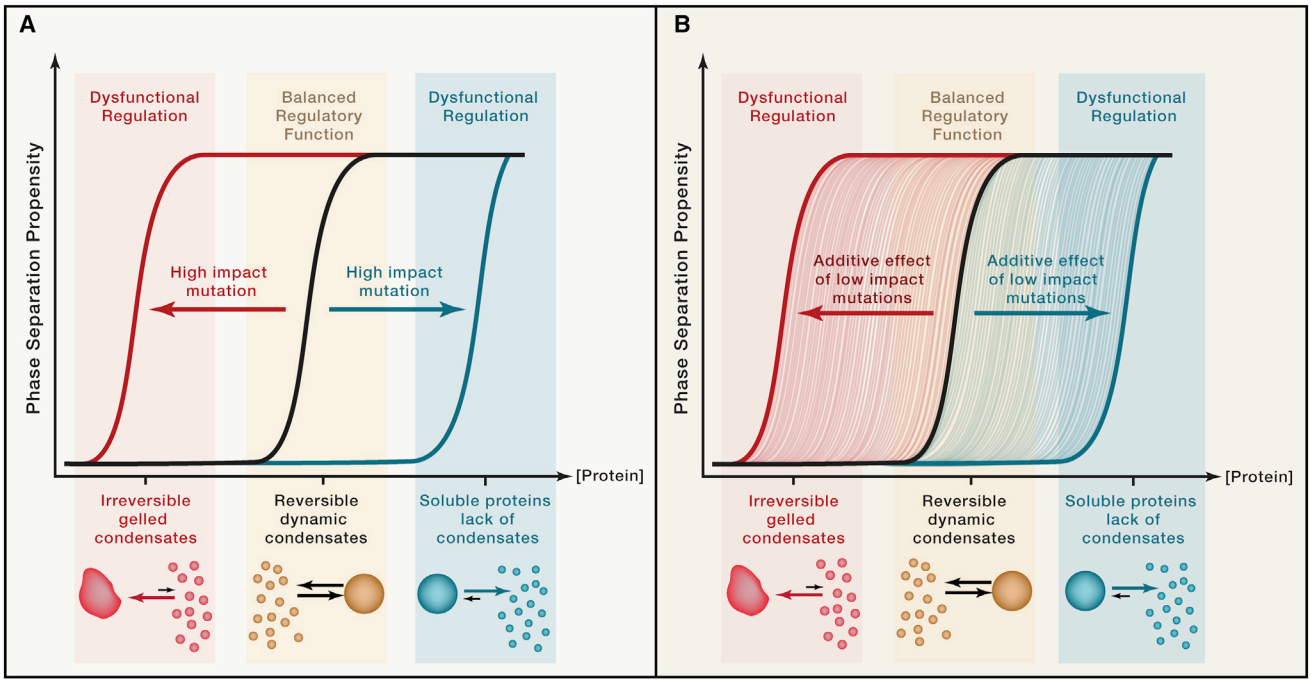

Figure 5. Pathogenic Perturbations to Phase Separation Thresholds as an Overarching Mechanism Underlying Disease

(A) A single or limited number of mutations that profoundly alter IDR properties (high impact) may cause dramatic aberrant shifts to the normal cellular phase separation threshold (represented by black line in middle). This results in pathological or decreased propensities of condensate formation in related cellular pathways.

(B) Mutations causing small alterations in IDR properties (low impact) may have subtle effects on the normal cellular phase separation threshold (represented by black line in middle). Accumulation of specific IDR mutations may cause aberrant shifts to the phase separation similar to high impact IDR mutations. Additive effects between high and low impact IDR mutations may result in pathological or decreased propensities for condensate formation in related cellular pathways.

separation in related pathways (Figure 5B). Thus, the 'right' combination of high and low impact mutations in IDRs may provide a sufficient threshold for "ASD susceptibility" by causing anomalous shifts in phase separation propensities of individual proteins in related pathways, potentially explaining "missing heritability." Mutations in IDRs, including their impact on cellular phase separation, represent a largely unexplored area of research; however, their roles in biological processes linked to phase separation, from regulation of chromatin, transcription, splicing, and other mRNA processing, translation, to receptor activation and downstream signaling, demonstrate the generality of this phase separation framework for understanding complex diseases.

Box 2. Approaches to Study the Impact of IDR Disease Variants on Phase Separation

We can examine the collective properties of IDRs as condensates to provide insights into their function and the effects of mutation using a variety of tools (Mitrea et al., 2018). Phase separation can be induced in the test-tube using purified proteins (Alberti et al., 2018). The biophysical properties and partitioning into reconstituted condensates and mutational effects can be examined using microscopy. IDR interactions within condensates can also be studied using NMR spectroscopy (Brady et al., 2017; Kim et al., 2019). In vitro reconstitution of receptor clustering and synaptic scaffolding proteins can be performed on 2D lipid membranes with mutation that can be used to evaluate partition coefficients and specific biochemical assays (Feng et al., 2019). High resolution microscopy and single molecule tracking can characterize condensates in cells and track diffusion of proteins through condensates (Cho et al., 2018). Engineered optogenetic approaches have been designed to induce phase separation with light in both the nucleus and cytoplasm (Bracha et al., 2019). With the development of new tools coupled with established methods, the mutational impact on phase separation can be evaluated to link mutations to functional effects.

CONCLUSION

Current bioinformatics tools are not well-suited for examining and predicting a putative mutation's impact concerning IDRs. Here, we demonstrate phase separation as a general property of proteins associated with common diseases. We provide a range of testable hypotheses involving IDR-mediated phase separation that can provide mechanistic insights into genetically complex disorders such as autism and cancer (Tables S1, S2, and S3). Numerous tools are being developed to study phase separation and IDRs, which we recommend be built into standard genotype-phenotype algorithms of analysis, and in particular for autism and cancer (Box 2). Ultimately, measuring the impact of DNA mutation on phase separation may provide a leap forward in understanding complex genetic disease.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cell.2020.11.046.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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