

Splicing regulation through biomolecular condensates and membraneless organelles

Jimena Giudice^{1,2}  & Hao Jiang³ 

Abstract

Biomolecular condensates, sometimes also known as membraneless organelles (MLOs), can form through weak multivalent intermolecular interactions of proteins and nucleic acids, a process often associated with liquid–liquid phase separation. Biomolecular condensates are emerging as sites and regulatory platforms of vital cellular functions, including transcription and RNA processing. In the first part of this Review, we comprehensively discuss how alternative splicing regulates the formation and properties of condensates, and conversely the roles of biomolecular condensates in splicing regulation. In the second part, we focus on the spatial connection between splicing regulation and nuclear MLOs such as transcriptional condensates, splicing condensates and nuclear speckles. We then discuss key studies showing how splicing regulation through biomolecular condensates is implicated in human pathologies such as neurodegenerative diseases, different types of cancer, developmental disorders and cardiomyopathies, and conclude with a discussion of outstanding questions pertaining to the roles of condensates and MLOs in splicing regulation and how to experimentally study them.

Sections

Introduction

Functions of biomolecular condensates in splicing regulation

Spatial organization of splicing and its regulation through membraneless organelles

Diseases associated with splicing dysregulation through condensates

Conclusion and outstanding questions

¹Department of Cell Biology and Physiology, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA. ²McAllister Heart Institute, School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA. ³Department of Biochemistry and Molecular Genetics, University of Virginia School of Medicine, Charlottesville, VA, USA. ✉e-mail: jimena_giudice@med.unc.edu; hj8d@virginia.edu

Introduction

Precursor mRNA (pre-mRNA) splicing enables the formation of mRNA by removing the introns and joining the exons, a process known as constitutive splicing. Alternative splicing was first demonstrated in 1977, in a study that showed differential splicing of a pre-mRNA into multiple mature mRNAs, each with different exon combinations^{1,2}. We now know that >95% of human genes are alternatively spliced^{3,4}, thereby greatly expanding proteome diversity.

Splicing is carried out mainly by the spliceosome, which recognizes and binds to consensus sequences at the 5' and 3' ends of the introns – the 5' splice site (5'ss) and 3'ss, respectively – removes the intron and covalently joins the adjacent exons. Generally, when the sequence of the splice site is very similar to the consensus sequence, the splice site is typically considered 'strong' and constitutive splicing occurs. By contrast, when the splice site sequence diverges from the consensus sequence, the splice site is usually considered 'weak', is less efficiently recognized and is suboptimally used by the spliceosome, generating different transcripts through alternative splicing (Fig. 1a). However, in some conditions, 'strong' splice sites undergo alternative splicing and, similarly, in some contexts 'weak' splice sites are constitutively used. This variability is possible because, in addition to the strength of the splice sites, the binding of *trans*-acting factors – mostly RNA-binding proteins (RBPs) – to *cis*-regulatory sequences within the pre-mRNAs also influences splicing outcomes: RBP binding to exonic splicing enhancer (ESE) or intronic splicing enhancer (ISE) sequences favours exon inclusion, whereas binding to exonic splicing silencer (ESS) or intronic splicing silencer (ISS) sequences favours exon skipping (Fig. 1b). RBP expression levels, activity and intracellular localization also influence splicing outcomes in different contexts (for example, specific tissues or cell types, developmental stages, or in health versus disease). Moreover, splicing often occurs co-transcriptionally^{5,6}, and thus kinetics of transcription by RNA polymerase II (Pol II), chromatin structure, nucleosome occupancy and epigenetic marks all regulate splicing outcomes^{7–12}.

Biomolecular condensates have various functions, and their most prominent role is modulating the rate of biochemical reactions¹³. Whereas the biochemistry of splicing regulation has been extensively studied, how splicing is spatio-temporally regulated within the cell is less understood. Although splicing has been long associated with certain nuclear bodies, the functional and mechanistic significance of these associations remains unclear. In the past few years, our understanding of the spatio-temporal regulation of splicing within the cell has been reshaped by the emergent concept of biomolecular condensates, which are membraneless organelles (MLOs) that organize cellular components and biochemical reactions^{14,15} (Box 1). Biomolecular condensates form through weak multivalent intermolecular interactions of proteins and nucleic acids, a process often associated with liquid–liquid phase separation^{14–16}. Numerous excellent reviews have covered liquid–liquid phase separation^{14,17,18}, biomolecular condensates^{15,16} and their regulation and function^{13,19–24}. Biomolecular condensates can adopt a broad and continuous spectrum of material properties, from highly dynamic, liquid-like droplets through less fluid gels with reduced dynamicity to solid amyloid aggregates^{16,25–28} (Box 1). The liquid-like state allows random collision and sampling of reactants in chemical reactions, and is compatible with cellular regulatory functions¹⁴. Importantly, disease-causing mutations can facilitate a high degree of aggregation that underlies several degenerative diseases^{26,29–31}.

Phase-separation research is very new and evolving; consequently, some concepts are still being defined. There is prominent criticism of

overuse of the term 'phase separation' for every MLO and of the lack of in vivo evidence that MLOs are crucial for cell biology and development³² (Box 2). Although in this Review we acknowledge some of these controversies, we do not aim to discuss them in detail. Thus, we use the term 'biomolecular condensates' to adopt the most neutral, appropriate language. Biomolecular condensates in eukaryotic cells are defined as micrometre-scale membraneless compartments that function to concentrate proteins and nucleic acids¹⁶.

Many biomolecular condensates in the nucleus are involved in spatio-temporal regulation of gene expression at multiple steps including chromatin structure changes, transcription, splicing and other RNA maturation processes^{33,34}. Most nuclear condensates interact with natural long polymers in the form of RNA, including numerous long non-coding RNAs (lncRNAs), which can function as scaffolds and regulatory platforms of nuclear condensates³⁴. In the context of co-transcriptional splicing, enhancers and promoters provide many binding sites for transcription factors and co-activators to reach a high concentration for forming co-condensates³⁵. As shown in a recent preprint (not peer-reviewed), nascent RNAs from densely transcribed genomic regions attract binding by numerous RBPs in local proximity and, thus, facilitate the formation of RNA-processing condensates, prominently including nuclear speckles³⁶. Moreover, diverse and dynamic modifications of DNA³⁷, histones^{38,39} and RNAs^{40,41} enable complex regulation of nuclear condensation and associated nuclear processes. It should be noted that the mobility and coalescence of nuclear condensates are often restricted by their specific interaction with chromatin, which is largely immobile⁴², and with RNAs, many of which are tethered to or associated with chromatin. Such a restriction helps maintain the distinct identity and function of these condensates^{34,43}.

In this Review, we discuss the different manners in which biomolecular condensates function in splicing: condensates can organize pre-mRNAs and splicing factors – specific RBPs can condensate at introns or exons – and in this manner actively regulate splicing site choice and outcomes. Condensates can also serve as dynamic storage and concentration modules for multiple mRNAs and RBPs. Finally, biomolecular condensates can function similar to aggregates that sequester or inactivate splicing factors.

In the following sections, we first discuss the functional roles of biomolecular condensates in splicing regulation. Second, we discuss the spatial connection between splicing regulation, nuclear speckles and genome organization. Third, we comprehensively describe studies that revealed how splicing regulation through biomolecular condensates is implicated in human diseases. Last, we present open questions on the roles of condensates and MLOs in splicing regulation and suggest how to experimentally study them.

Functions of biomolecular condensates in splicing regulation

In this section, we discuss the consequences for splicing of dysregulation of biomolecular condensates. We begin with the molecular basis of RBP-rich condensate formation and then discuss how these condensates are shaped by alternative splicing, and how their formation and biophysical properties regulate splicing.

Roles of RNA-binding proteins and their low-complexity domains in phase separation

RBPs are main regulators of splicing and are enriched in intrinsically disordered regions (IDRs) and low-complexity (LC) domains in comparison with the whole proteome^{44–47}. The capacity of RBPs to undergo

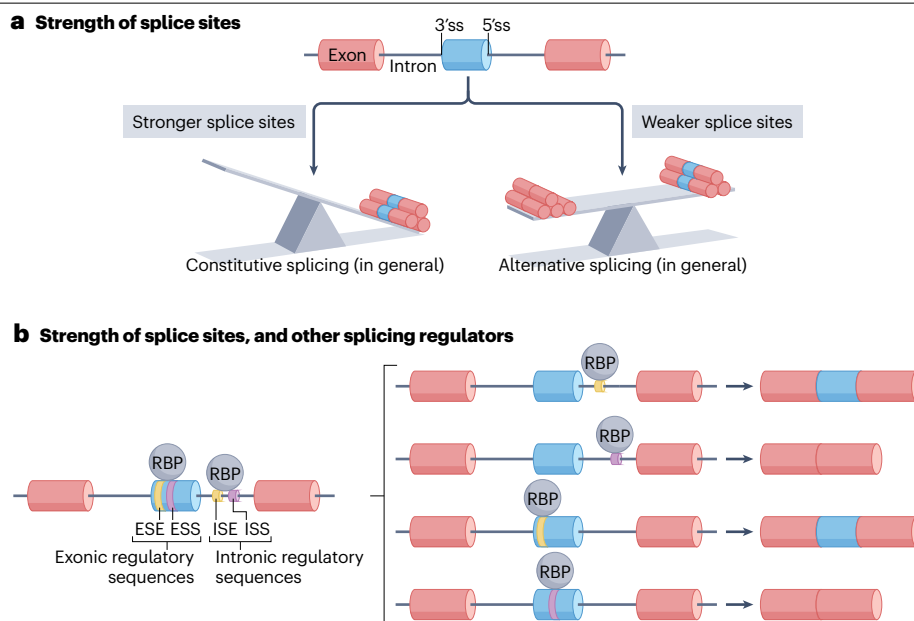


Fig. 1 | Fundamentals of splicing regulation. **a**, The spliceosome (not shown) recognizes and binds to splicing consensus sequences at the 5' and 3' ends of the introns, known respectively as the 5' splice site (5'ss) and 3'ss, thereby allowing the removal of the intron and the joining of the adjacent exons. When the sequences of the splice sites are very similar to a consensus sequence, the splice sites are usually considered 'strong' and constitutive splicing occurs (left). When the sequences of the splice sites differ from the consensus, the splice sites are often considered 'weak' and can be suboptimally used, resulting in alternative

splicing and the formation of different mRNA transcripts (right). **b**, In some conditions, 'strong' splice sites undergo alternative splicing and 'weak' splice sites are constitutively used. This is possible because, in addition to the strength of the splice sites, *trans*-acting factors (RNA-binding proteins (RBPs) and splicing factors) that bind to *cis*-regulatory sequences within the pre-mRNA influence splicing outcomes: binding to exonic splicing enhancer (ESE) or intronic splicing enhancer (ISE) sequences favours exon inclusion, whereas binding to exonic splicing silencer (ESS) and intronic splicing silencer (ISS) sequences favours exon skipping.

phase separation is mainly driven by their IDRs and LC domains, by their (often multivalent) RNA-binding domains and by post-translational modifications (PTMs) that can alter their hydrophobicity, charge and size (Fig. 2a). RNA–RNA interactions also contribute to the formation of RNA–protein condensates^{48–52}. The IDRs and LC domains facilitate the reversible concentration of RBPs within biomolecular condensates, where they can either exert their functions or be sequestered from their roles in other parts of the cell. Ultimately, these dynamics affect cytoplasmic mRNA metabolism^{25,53–55}. In the nucleus, RBPs are heavily involved in splicing regulation and their capacity to form biomolecular condensates is emerging as a key modulator of these functions^{46,56–58} (Table 1).

Heterogeneous nuclear ribonucleoproteins (HNRNPs) constitute a large family of RBPs involved in multiple aspects of nucleic acid metabolism, including alternative splicing, mRNA stability, transcription and translation regulation in health and disease⁵⁹. The LC domains of HNRNPs and other RBPs can favour the formation of liquid-like droplets or amyloid-like polymers through phase separation^{25,60–63} (Table 1). Cryo-electron microscopy revealed the structures of the amyloid fibrils formed by LC domains of HNRNPA1 and HNRNPA2 (refs. 64,65). HNRNPH1 contains DNA-recognition motifs at its amino-terminal half and two carboxy-terminal LC domains (LC1 and LC2)⁵⁸. HNRNPH1 phase separation through its LC1 domain influences its interaction with other RBPs and its functions in alternative splicing⁵⁸. By contrast, the LC2 domain is not necessary for phase separation but is required for aberrant transcription activation of fusion oncoproteins during cancer transformation⁵⁸. Thus, distinct LC domains might confer different functions to HNRNPH1 (ref. 58).

Another important family of RBPs involved in splicing regulation are the RNA-binding fox-1 homologue (RBFOX) proteins (RBFOX1, RBFOX2 and RBFOX3). RBFOX proteins regulate splicing during embryonic development and postnatally in the brain, heart and muscle⁶⁶. Through its carboxy-terminal domain (CTD), RBFOX1 interacts with a protein complex known as large assembly of splicing regulators (LASR)⁶⁷, which is essential for its functions in splicing regulation⁵⁷. A Tyr-rich LC domain within the CTD mediates RBFOX1–LASR high-order assemblies and is required for splicing activation by RBFOX1⁵⁷ (Table 1 and Fig. 2b). Mutations in these Tyr residues block the high-order assembly of RBFOX1, but not its interaction with LASR; however, these mutations abolish proper RBFOX1 functions in splicing regulation, suggesting a link between RBFOX1 capacity to form high-order assemblies and its function in splicing⁵⁷ (Fig. 2b).

A-kinase anchoring protein 95 kDa (AKAP95; also known as AKAP8) is involved in different nuclear processes including transcription and splicing regulation^{68,69}. Through its first 100 residues, AKAP95 interacts with the MLL2 (also known as KMT2D) complex, Pol II and different splicing factors, whereas its two zinc-finger domains bind to pre-mRNA introns and thus are involved in splicing regulation^{68,69} (Table 1 and Fig. 2c). An IDR comprising residues 101–210 allows AKAP95 to form dynamic, liquid-like droplets that have an essential role in regulating splicing and supporting tumorigenesis⁷⁰ (Table 1 and Fig. 2c).

In summary, it is becoming clear that the organization and activation of RBPs within condensates are a crucial layer of splicing regulation. Interestingly, alternative splicing on its own can influence phase separation, thereby forming a feedback regulatory mechanism.

Box 1

Formation and characteristics of biomolecular condensates

Biomolecular condensates formed by specific proteins are assembled through weak, multivalent and dynamic intermolecular interactions. Intrinsically disordered regions (IDRs) are a prominent feature of proteins that form condensates, especially of human nuclear proteins^{16,34}. The lack of strong engagement of intramolecular interactions between amino acid side chains in IDRs allows them to be readily available to engage in interactions with different molecules¹⁸. The driving forces underlying IDR-mediated condensation often include electrostatic interactions, π - π interactions, cation- π interactions, hydrogen bonding and hydrophobic interactions^{16,34,205}. These relatively weak and short-lived interactions promote break-reform cycles between the same and different molecules. Furthermore, the flexibility and the large amplitude of conformational fluctuations of IDRs enhance spatial freedom at the structural level and permit rapid sample interactions with other molecules, thereby forming the basis of the dynamicity and liquidity of the condensates²⁰⁶. In addition to IDRs, structured protein domains that can form multivalent interactions and other biopolymers (for example, RNA) in cells are also important mediators of biomolecular condensation^{16,207}.

Material properties of condensates

Different proteins may form condensates with distinct material and biophysical properties and biological functions. The material properties include liquidity, viscosity, elasticity and porosity, among others. The biophysical properties include molecular dynamics inside

the condensates, the exchange rate in and out of the condensates, compactness (intermolecular distance) and mobility of the condensates within cells. These condensate properties are determined by the valency, strength and/or stability of the molecular interactions among the condensate components and with the molecules in the dilute phase, which are controlled by the primary sequences and post-translational modifications (PTMs) of the scaffold and client proteins and by other cellular factors, most prominently by chaperones^{208,209}.

Composition of condensates

Although condensates are non-stoichiometric assemblies and their structure is usually not strongly dependent on precise stoichiometric ratios of its components, the composition of condensates can strongly affect their functions. Based on current models, condensate composition is controlled by both specific interactions and non-specific interactions and is influenced by the stoichiometries and valency of both scaffold and client molecules in the condensates^{210–212}. Specific interactions between IDRs of the nuclear proteins are modulated by charge blocks, and regulate the partition, composition and functionality of co-factors²¹³.

It is important to note that biophysical and composition properties may be heterogeneous inside the condensates, supporting high-order organization and the formation of compartments within condensates, and that such heterogeneity has important implications on the function of the condensates¹⁰⁹, as is very likely to be the case for condensates involved in splicing regulation^{113,140}.

Condensate regulation by alternative splicing

Alternative splicing of RBPs can influence their ability to undergo phase separation and, thus, their control of splicing decisions. IDRs are not constrained by a specific fold and are, therefore, very tolerant to mutations^{71,72}, which has facilitated both their expansion and evolution^{73–75}. Alternatively spliced exons are significantly enriched in encoding IDRs^{76–79}, prompting the hypothesis that splicing regulation of exons encoding IDRs has contributed to the complexity of higher organisms by remodelling protein-protein interaction networks and signalling pathways⁵⁶. Almost all HNRNPA and HNRNP proteins contain Gly-Tyr-rich IDRs that are encoded by exons that are constitutively included in transcripts in non-mammals but alternatively spliced in mammals⁵⁶ (Fig. 3a). In mammals, those exons can be included or skipped and thus fine-tune the capacity of these RBPs to form biomolecular condensates⁵⁶. Base-pairing between sequences that include the branch point, the polypyrimidine tract and parts of the acceptor sequence adjacent to the alternative exons results in masking these crucial regulatory elements, and thus induces exon skipping⁵⁶. Therefore, these intramolecular RNA interactions control mammalian-specific alternative splicing in these HNRNPs⁵⁶. Differential inclusion of the alternative exons modulates the formation of biomolecular condensates of HNRNPs through Tyr multivalency, which, in turn, globally regulates splicing outcomes and thus expands the gene regulation capacity⁵⁶.

In addition to the capacity to undergo phase separation, alternative splicing can fine-tune the propensity of RBPs to aggregate. HNRNPD-like (HNRNPDL) has three splice isoforms, which differ in the presence or absence of an Arg-rich IDR (at the N terminus) and a Tyr-rich IDR (at the C terminus). Following alternative splicing, when both IDRs are present, the resulting protein – HNRNPDL1 – can undergo phase separation; when only the Tyr-rich IDR is present, the resulting HNRNPDL2 is likely to form amyloid aggregates; and when both IDRs are absent, the resulting HNRNPDL3 is soluble^{80–83} (Fig. 3b). Mutations of the highly conserved HNRNPDL1 D378 residue (D378H and D378N; or D259H and D259N in HNRNPDL2 (ref. 84)), located in the Tyr-rich IDR, cause limb-girdle muscular dystrophy^{85–87} by reducing the solubility and accelerating the aggregation of the protein⁸⁰. Although this evidence suggests a genetic loss of function, the exact mechanisms by which these mutations contribute to limb-girdle muscular dystrophy phenotypes and their impact on splicing regulation are unclear.

HNRNPDL2 is the predominant isoform expressed in human tissues⁸¹. Cryo-electron microscopy has recently revealed that full-length HNRNPDL2 amyloid fibrils are stable, are non-toxic and can bind nucleic acids⁸⁴. The amyloid core consists of a single Gly-Tyr-rich and highly hydrophilic filament, and the RNA-binding domains form a solenoidal coat around the amyloid core⁸⁴. Interestingly, the amyloid fibril core is encoded by HNRNPDL2 alternative exon 6, which is absent in the soluble HNRNPDL3 isoform, indicating that alternative splicing controlling

HNRNPD assembly (through inclusion or skipping of exon 6) expands the functional diversity of HNRNPD⁸⁴.

TAR DNA-binding protein 43 (TDP43), which is encoded by the gene *TARDBP*, is another RBP whose aggregation is influenced by alternative splicing. The full-length *TDP43* mRNA isoform includes the entire exon 6, which contains a stop codon. The full-length protein (TDP43-fl) includes a Gly-rich LC domain at the C terminus, which facilitates its nuclear aggregation and splicing functions⁸⁸ (Table 1 and Fig. 3c). Use of the same 3' ss of TDP43-fl but with two alternative 5' ss before the stop codon within exon 6 gives rise to two short TDP43 isoforms (TDP43-s1 and TDP43-s2) that include only a small portion of exon 6 and the entire exon 7 within their coding sequence. Consequently, the C termini of TDP43-s1 and TDP43-s2 lack the Gly-rich LC domain of TDP43-fl, but include a putative nuclear export signal (NES) not found in TDP43-fl⁸⁸. Owing to these characteristics, TDP43-s1 and TDP43-s2 accumulate in the cytoplasm where they form aggregates that sequester TDP43-fl through N-terminal interactions, and lack splicing regulation capacity (Fig. 3c). The short TDP43 forms accumulate in neurons and glia cells of individuals with amyotrophic lateral sclerosis (ALS) and their pathological upregulation is mediated by neuronal hyperexcitability⁸⁸.

The cytoplasmic polyadenylation element binding protein 4 (CPEB4) regulates translation of specific mRNAs by modulating their poly(A) tails. The neuron-specific CPEB4 isoform includes the alternatively spliced microexon 4 (24 nucleotides), but low levels of inclusion

of this microexon cause idiopathic autism spectrum disorder⁸⁹ or schizophrenia⁹⁰. CPEB4 forms condensates driven by homotypic interactions among clusters of aromatic amino acids (especially His), and the microexon 4-encoded eight residues engage in heterotypic intermolecular interactions that compete with the homotypic interactions to regulate the reversibility of the condensates. As shown in a recent preprint (not peer-reviewed), skipping of microexon 4 leads to irreversible CPEB4 aggregation that cannot be dissolved upon neuron depolarization, and alters CPEB4 activity in translation regulation of key targets including autism spectrum disorder-risk transcripts⁹¹. This is a striking example of how tissue-specific alternative splicing ensures crucial protein activity through regulating condensate properties.

Alternative splicing of kinases can also influence condensation and contribute to feedback loops or splicing changes. For example, CDC-like kinase 1 (CLK1) is one of the two main kinases that phosphorylates serine-arginine-rich splicing factors (SR proteins), leading to their localization in the nucleus. CLK1 is itself regulated by alternative splicing through the action of SR proteins and this regulation affects the production of functional CLK1 (versus truncated non-functional proteins or transcripts that undergo nonsense-mediated decay). Hyperphosphorylation of SR proteins leads to the dispersion of nuclear speckles and the release of stored splicing factors that ultimately control global splicing of genes, including those involved in the hypoxia response^{92–96}.

Box 2

Lack of in vivo evidence that membraneless organelles are crucial for cell biology and development

One of the main barriers in studying molecular condensation is that there is still no clear in vivo evidence of their functions in cell biology and tissue development. Almost everything we know so far about biomolecular condensates comes mostly from cell culture studies or in vitro biochemical assays. This limitation has been the focus of important discussions in the field^{32,51,214}, which we cannot present in detail here, but we shall mention the key points still open for debate and future investigations.

First, rigorous definitions of phase separation come from physics and chemistry, but they are not in full agreement with how phase separation is understood in life sciences and in physiological contexts^{32,214}. Furthermore, it is not possible to manipulate key parameters that are necessary to validate phase transitions (such as concentration or temperature) in vivo, leading research to heavily rely on less rigorous, qualitative descriptions of phase separation²¹⁴. Those descriptions cannot be considered evidence of phase separation if they lack information about concentration or temperature dependence. Studies in vitro are hugely informative in determining whether a molecule undergoes phase separation, but stating that the same phenomenon occurs in vivo requires quantitative measurements within the endogenous context and at physiological concentrations^{32,214}. In general, the evidence supporting phase separation in vivo is insufficient to discriminate between phase separation and other mechanisms³².

Second, the uncertainty about the veracity of biomolecular condensates and membraneless organelles (MLOs) in vivo stems from overuse of these terms. This strong focus on phase separation, biomolecular condensates and MLOs might be masking a considerable gap in our understanding of alternative mechanisms by which molecules can be accumulated at high local concentration in the absence of a membrane³².

Third, determining condensate function is difficult because condensates are transient, and their complex composition makes them difficult to reconstitute⁴⁶. Phase separation is proposed to facilitate or sequester molecules and reactions, but the in vivo evidence of these functions is weak and subtle owing to lack of adequate tests³².

It is necessary to work on better ways to manipulate protein concentration in vivo other than by overexpression, perhaps by tagging the molecule of interest in its native genomic locus and at endogenous expression levels. Moreover, criteria such as condensate roundness and ability to fuse and high component mobility are not strong enough evidence of phase separation in vivo. Until better experimental tools are developed, we need to be cautious in data interpretation. Investigating the physiological functions of phase separation within the limitations of tissue and organ environments is an essential future goal.

In conclusion, alternative splicing of RBPs influences their capacity to undergo phase separation, and in this manner influences their functions as splicing factors, and may greatly enhance the regulatory capacity of RBPs in mammals. However, dysregulation of these regulatory networks has the potential to promote neurodegenerative diseases, muscular disorders and psychiatric conditions.

Formation of nuclear condensates regulates splicing

In this subsection we discuss the mechanisms by which RBPs regulate splicing by organizing dedicated condensates.

Phase separation of the essential splicing factor U2AF65 along with repetitive, pyrimidine-rich RNA may contribute to the selection of specific splice sites⁹⁷. U2AF65 and its related protein CAPER α (also known as RBM39) show cooperative binding to splicing factor 3b subunit 1 (SF3B1). U2AF65 forms liquid-like condensates through its SR-rich domain, and condensation is greatly enhanced by the assembly of U2AF65, CAPER α and SF3B1 together with repetitive pyrimidine-rich RNAs. Depletion of U2AF65 or CAPER α resulted in enhanced inclusion of alternative exons preceded by repetitive pyrimidine-rich sequences in the target RNAs⁹⁷. The authors reason that when the levels of U2AF65 or CAPER α are reduced, the long pyrimidine-rich regions remain preferentially bound and spliced by U2AF65 owing to their ability to enhance U2AF65 condensation relative to pyrimidine-poor regions. Future *in vivo* characterizations of U2AF65 or CAPER α condensates and their binding at transcripts with different pyrimidine features could provide more-direct evidence to

this interesting model of how condensation of a splicing factor may influence splice site choice.

Serine/arginine repetitive matrix 2 (SRRM2) and SON DNA-binding and RNA-binding protein (SON) are scaffold proteins essential for the formation of nuclear speckles⁹⁸. SRRM2 regulates alternative splicing, especially by facilitating the inclusion of cassette exons flanked by short introns and weak splice sites⁹⁹. SRRM2 IDRs mediate the formation of condensates, which is important for regulating most, but not all, SRRM2-mediated alternative splicing⁹⁹. It is unclear which features of the transcripts – the exon–intron junction sequence or the RNA structure – determine whether their alternative splicing depends on SRRM2 condensation. More mechanistic insight will be gained by determining how SRRM2 condensation controls the formation of nuclear speckles and their properties and structural organization.

An important question is how RBP condensation regulates splicing at the molecular level. Condensates provide dynamic micro-compartments that selectively recruit specific molecules, thereby generating local enrichment of splicing regulators and substrates. This selective compartmentalization effectively reduces the search space required for molecules to find their interacting partners, and decreases their interactions with molecules that are irrelevant or harmful for splicing. Nuclear condensates including those related to splicing may have internal structures that coordinate the spatial distribution of splicing factors and their RNA substrates to regulate splicing (see below).

Recent work has shed light on the mechanism through which RBP condensation may control RNA binding, a prerequisite for splicing

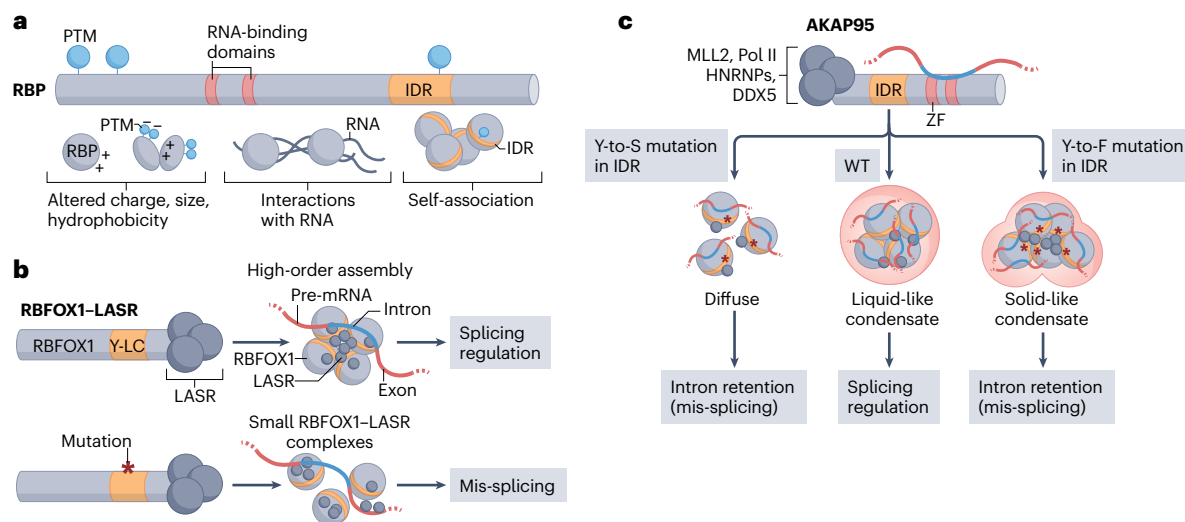


Fig. 2 | Splicing regulation through phase separation and its physiological relevance. **a**, Molecular properties of RNA-binding proteins (RBPs) that influence their condensation propensity include post-translational modifications (PTMs), which can alter their charge, size and hydrophobicity; RNA-binding domains, which mediate their interaction with RNA; and intrinsically disordered regions (IDRs), which mediate protein–protein associations. Note that IDRs are enriched in PTMs owing to their high accessibility and that these PTMs can, in turn, regulate condensation. **b**, RNA-binding fox-1 homologue 1 (RBFOX1) interacts with the large assembly of splicing regulators (LASR) complex through its carboxy terminus and together they assemble into organelle-like, high-order protein complexes. A low-complexity (LC) sequence contains a Tyr-rich domain (Y-LC) that mediates RBFOX–LASR high-order assembly and is required for splicing activation by RBFOX1 (ref. 57). Mutations (red asterisk) in these Tyr

residues block the capacity of RBFOX1 for high-order assembly, but not its interaction with LASR; however, these mutations do prevent proper RBFOX1 functions in splicing regulation⁵⁷. **c**, A-kinase anchoring protein 95 kDa (AKAP95) interacts with the MLL2 complex, RNA polymerase II (Pol II) and different splicing factors; its two zinc-finger (ZF) domains are involved in binding to pre-mRNA introns and splicing regulation⁶⁸. An IDR allows AKAP95 to form dynamic and liquid-like droplets that function in splicing regulation. Both a Tyr-to-Ser (Y-to-S) mutation that disrupts AKAP95 condensation and a Tyr-to-Phe (Y-to-F) mutation that enhances AKAP95 phase separation but reduces the dynamicity of the condensates significantly impair AKAP95 activity in regulating splicing and promoting cancer⁷⁰. DDX5, probable ATP-dependent RNA helicase; HNRNP, heterogeneous nuclear ribonucleoprotein; WT, wild type.

Table 1 | Protein condensates that regulate splicing and are associated with disease

Protein(s)	Features associated with phase-separation capacity	Physiological implications	Refs.
AKAP95	The balance between Phe and Tyr residues in the IDR appears to be essential for proper liquid–liquid phase separation	The capacity of AKAP95 to form liquid-like dynamic condensates is required for splicing regulation and tumorigenesis	70
HNRNPA and HNRNPD proteins	Gly-Tyr-rich IDR-encoding exons are alternatively spliced in mammals but constitutively included in non-mammals	Evolution of exon skipping in HNRNPs has expanded their regulatory capacity in mammals compared with non-mammals	56
HNRNPA1 HNRNPA2B1	The IDRs include prion-like domains	Normally these RBPs self-assemble into fibrils, but this process is exacerbated by mutations within the prion-like domains in ALS and in multisystem proteinopathies	31
HNRNPDL	An Arg-rich IDR (amino terminus) and a Tyr-rich IDR (carboxy terminus)	Both IDRs mediate phase separation, but the Tyr-rich IDR has a stronger effect; mutations in this domain cause limb-girdle muscular dystrophy	80
HNRNPH1	LC1 domain	LC1 influences interaction with RBPs and roles in splicing regulation	58
RBFOX1	LC Tyr-rich domain within the CTD	The LC domain mediates high-order assembly of RBFOX1 and LASR and is required for the capacity of RBFOX1 to activate exon inclusion	57
MeCP2	Methyl-CpG-binding domain and an intervening domain mediate binding and co-condensation with RBFOX proteins	Rett syndrome-causing mutations in MeCP2 disrupt its condensation with RBFOX proteins and result in mis-splicing of synaptic plasticity transcripts	170
TDP43	A Gly-rich LC domain encoded by exon 6 (C terminus)	This LC domain facilitates TDP43 nuclear aggregation and functions in splicing regulation, and is absent in ALS, causing TDP43 to pathologically accumulate in the cytoplasm	88
USP42	Positively charged C-terminal IDR	USP42 forms condensates to regulate splicing that supports cancer growth	173
RBM10	Unclear, but the V354M mutation in the RRM enhances RBM10 condensation	RBM10 regulates alternative splicing required for cell proliferation and apoptosis, and is frequently mutated in cancers with splicing dysregulation; the V354M mutation is found in colon cancer, and germ-line mutations cause TARP syndrome	177,178, 182,192
WASP	Unclear, likely multiple IDRs	WASP forms co-transcriptional splicing condensates with SRSF2 and Pol II; WASP deficiency alters the properties of these condensates and their function in gene expression, thereby contributing to Wiskott–Aldrich syndrome	157
RBM20	Likely RNA-binding domains and IDRs	RBM20 normally forms nuclear splicing foci that mediate alternative splicing regulation; serine-arginine-domain mutants mis-localize to the sarcoplasm and form aberrant condensates that sequester biomolecules, causing cardiomyopathy	184,186,187

AKAP95, A-kinase anchoring protein 95 kDa; ALS, amyotrophic lateral sclerosis; CTD, carboxy-terminal domain; HNRNP, heterogeneous nuclear ribonucleoprotein; HNRNPDL, HNRNPD-like; IDR, intrinsically disordered region; LASR, large assembly of splicing regulators; LC, low-complexity; MeCP2, methyl-CpG-binding protein 2; Pol II, RNA polymerase II; RBFOX, RNA-binding fox-1 homologue; RBM10, RNA-binding motif protein 10; RBP, RNA-binding protein; RRM, RNA-recognition motif; SRSF2, serine-arginine-rich splicing factor 2; TDP43, TAR DNA-binding protein 43; USP42, ubiquitin specific peptidase 42; WASP, Wiskott–Aldrich syndrome protein.

regulation¹⁰⁰. Quantitative analysis of the condensation capacity of TDP43 CTD mutants in vitro and in cells was correlated with their RNA-binding capacity, and revealed that changes in condensation capacity have differential effects on TDP43 binding to RNA depending on sequence features¹⁰⁰. Reduced propensity for condensation selectively affects TDP43 binding to RNA regions that tend to be >100 nucleotides long and contain dispersed UG-rich motifs, and impairs 3'-end processing of the transcripts¹⁰⁰. It is possible that the splicing of diverse RNAs is also differentially regulated by condensation of TDP43 (and other RBPs). The sequence and likely secondary structure¹⁰¹ of the target RNAs can determine whether and to what extent condensation propensity has a role in their binding and processing by RBPs. This model helps explain other findings, for example that TDP43 phase separation is dispensable for its splicing activity on a few tested targets¹⁰² and for repressing certain cryptic exons¹⁰³. An important difference exists between the TDP43 mutants study¹⁰⁰ and the U2AF65 study discussed above⁹⁷. In the TDP43 mutants study¹⁰⁰, the activities

on the RNA sites normally bound by the TDP43 condensates are selectively affected when the capacity for condensation is perturbed. In the U2AF65 study⁹⁷, the condensate-bound sites remain preferentially protected from splicing impairment when the overall protein level is reduced, indirectly suggesting an advantage to being able to form condensates at those sites.

Biophysical properties of nuclear condensates that regulate splicing

Condensates are not simple binary on–off switches of protein states or functions. Splicing activity is likely influenced by numerous physico-chemical properties of the condensates beyond their formation and disruption. Recent studies have investigated how the biophysical properties of condensates regulate splicing, with important biological consequences.

The dynamic biophysical properties of AKAP95 condensates control its activity in regulating both constitutive and alternative splicing⁷⁰

(Table 1). The Tyr-to-Phe mutation in the IDR enhances AKAP95 aggregation and hardens its condensates both in vitro and in cells⁷⁰ (Fig. 2c). This altered material state is reflected experimentally by reduced

fluorescence recovery after photobleaching, reduced diffusion⁷⁰ and reduced intermolecular distances¹⁰⁴ in the condensates. This mutation significantly impairs AKAP95 activity in regulating splicing, including

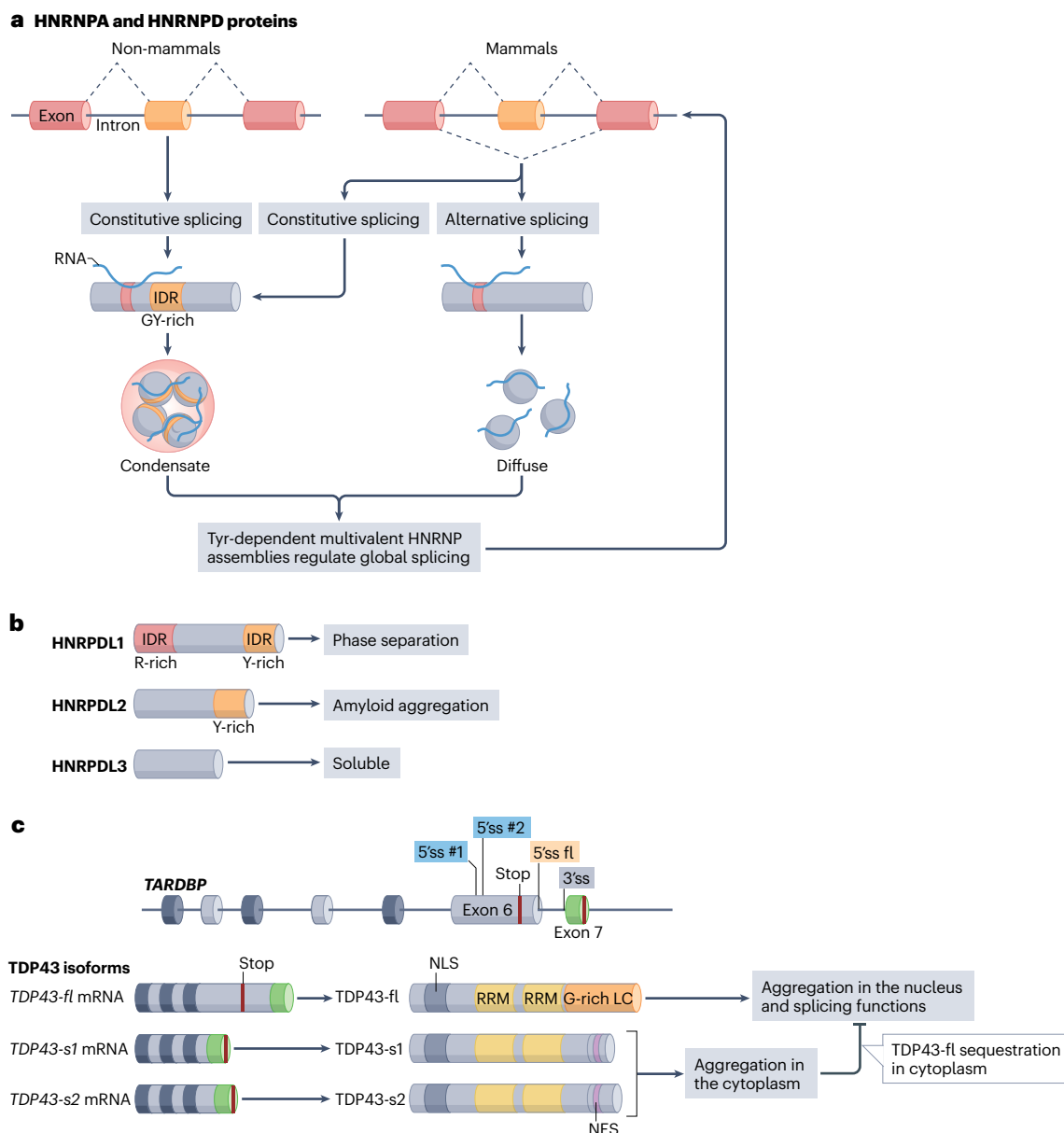


Fig. 3 | Regulation of condensate formation and properties through alternative splicing. **a**, Most heterogeneous nuclear ribonucleoprotein A (HNRNPA) and HNRNPD proteins contain Gly-Tyr (GY)-rich intrinsically disordered regions (IDRs) that are encoded by exons that are constitutively spliced in non-mammals but alternatively spliced in mammals⁵⁶. In mammals, those exons can be included or skipped and, in this way, fine-tune the propensity of these RNA-binding proteins (RBPs) to form biomolecular condensates and expand their capacity to regulate gene expression. **b**, HNRNPDL-like (HNRNPDL) has three splice isoforms, which differ in the presence or absence of an Arg (R)-rich IDR at the amino terminus and a Tyr (Y)-rich IDR at the carboxy terminus. HNRNPDL1 includes both IDRs and can undergo phase separation; HNRNPDL2 only includes the Tyr-rich IDR and forms amyloid aggregates; and HNRNPDL3 lacks both IDRs and is soluble^{80–83}. **c**, TAR DNA-binding protein 43 (TDP43) is

encoded by the *TARDBP* gene. The full-length *TDP43* mRNA isoform includes the entire exon 6, which contains a translation stop codon. The full-length protein (TDP43-fl) contains a Gly (G)-rich low-complexity (LC) domain at the C terminus, which facilitates nuclear aggregation and mediates its functions in splicing regulation⁸⁸. Usage of the same 3' splice site (3'ss) as the TDP43-fl, but with two alternative 5'ss within exon 6 before the stop codon, gives rise to two short TDP43 isoforms (TDP43-s1 and TDP43-s2) that include only a small portion of exon 6 and the entire exon 7 within their coding sequence. Consequently, the C termini of TDP43-s1 and TDP43-s2 lack the Gly-rich LC domain present in TDP43-fl, but include a putative nuclear export signal (NES) not found in TDP43-fl (ref. 88). Thus, the short TDP43 variants aggregate in the cytoplasm, where they sequester TDP43-fl, and lack splicing regulation capacity. NLS, nuclear localization signal; RRM, RNA-recognition motif.

intron removal, and alternative splicing, and in supporting tumour growth⁷⁰. This study highlights the requirement for RBP condensates with proper biophysical features for controlling their biochemical functions in splicing regulation.

In response to metabolic changes, arginyl-tRNA synthetase (ArgRS) can modulate splicing by interacting with SRRM2 in the nucleus and impeding its mobility¹⁰⁵. Depletion of ArgRS or arginine enhanced the rates of recovery after photobleaching of endogenous SRRM2, and depletion of SRRM2 and ArgRS led to mis-splicing of a subset of genes in opposite directions¹⁰⁵. These results are consistent with the notion that high SRRM2 mobility (following ArgRS depletion) favours SRRM2 activity in alternative splicing. These splicing alterations lead to the expression of different protein isoforms, which might influence cellular metabolism and peptide presentation to immune cells, ultimately contributing to the cellular response to the inflammation-induced Arg depletion¹⁰⁵. Although how ArgRS regulates SRRM2 mobility is unclear, this study has revealed how regulating the properties of the splicing condensates can serve as a response to metabolic changes.

Nuclear speckle proteins are highly enriched in mixed-charge domains, especially those containing Arg residues¹⁰⁶. Arg has a crucial role (that cannot be performed by Lys) in condensation of proteins (including many RBPs) containing mixed-charge domains and in their incorporation into nuclear speckles. The RNA-recognition motif (RRM) of these RBPs synergizes with the mixed-charge domains to promote their specific incorporation into nuclear speckles instead of into the nucleolus. Increasing the negative charge of these mixed-charge domains (by increasing the number of negatively charged amino acids) abolishes their condensation and incorporation into speckles; by contrast, increasing the positive charge enhances protein condensation, slows condensate dynamics and impairs mRNA export from the nucleus¹⁰⁶. Although the authors did not test splicing, we speculate that tuning the material properties of speckles through the mixed-charge domains might influence splicing activity, which would be consistent with other studies showing a requirement for proper condensate dynamics for their activity in gene regulation^{33,70,107}.

It is difficult to definitively attribute splicing alterations to changes in a specific property of the condensates, as multiple condensate features are likely connected through their shared physico-chemical basis and are often co-affected when one property is perturbed. For example, mutations in a scaffold protein A that enhance homotypic intermolecular interactions (A–A) among the scaffold protein molecules can lead to enhanced condensation and increased local concentration (which may promote reaction rates). However, the same mutations can also lead to a transition of the material property from liquid-like to solid-like (which may reduce reaction rates). Moreover, enhanced homotypic interactions may compete with heterotypic interactions (A–B) of this scaffold protein with different molecules (client proteins), and thus disfavour the incorporation of other components (clients) of the condensates. This possibility has been nicely demonstrated by the “unblending of transcriptional condensates” as a consequence of repeat expansion in the transcription factors¹⁰⁸. Therefore, alterations in composition can co-occur with changes in material properties and condensation capacity. It is hard to attribute the effects on a biological activity such as splicing to changes in either of these properties of the condensates.

Spatial organization of splicing and its regulation through membraneless organelles

In this section, MLOs refers to nuclear bodies that have been linked to splicing, including splicing condensates, nuclear speckles and

transcriptional condensates. MLOs often have an internal organization¹⁰⁹, which is important for their function¹¹⁰. The structural organization of MLOs is governed by a network of homotypic and heterotypic molecular interactions and influenced by different physico-chemical properties of the condensate proteins (for example, hydrophobicity, micropolarity)^{110,111}. We discuss, from a spatial perspective, how splicing is regulated by the structure of nuclear speckles (and a few other nuclear bodies) and by genome organization.

Regulation of splicing by the internal organization of nuclear speckles

Nuclear speckles are one of the most prominent nuclear bodies associated with splicing regulation. Multiple biological processes have been linked to nuclear speckles; however, the exact physiological function of nuclear speckles and whether they are sites of active splicing reactions are still under debate^{112,113}. Some studies proposed that nuclear speckles are storage or assembly sites of pre-mRNA processing factors, based on the observation that they do not contain DNA and exhibit only a low level of transcriptional activity inside them^{112,114}. By contrast, others have shown that a group of genes can cluster around nuclear speckles upon activation of their transcription, explaining why these mRNAs co-localize with speckles. In this manner, nuclear speckles might act as hubs that coordinate gene transcription and pre-mRNA processing^{115–120}. A wide variety of studies have suggested that proximity to nuclear speckles is associated with higher gene transcription, co-transcriptional splicing and post-transcriptional splicing (reviewed elsewhere^{121,122}).

Nuclear speckles exhibit the key properties of phase-separated MLOs, including liquid-like behaviours^{123–126} and dynamic exchange of RBPs and RNAs with the surrounding nucleoplasm^{123–129}. Nuclear speckles are highly enriched in polyadenylated RNA, factors required for splicing such as small nuclear ribonucleoproteins and splicing factors (SR proteins such as SRRM1 and SRRM2, SON and others), and cleavage and polyadenylation proteins; therefore, they are good candidates for spatially regulating the splicing reaction¹²¹. Some splicing regulators exhibit different localization relative to nuclear speckles: certain SR proteins are enriched within nuclear speckles^{130,131}, whereas certain HNRNPs are not enriched in or are depleted from speckles^{132,133}. SR proteins and HNRNPs often have antagonistic effects on splicing¹³⁴, whereas SR-binding motifs tend to be more enriched in exons, HNRNP-binding motifs are more enriched in introns¹³⁵. However, some SR proteins bind also introns and some HNRNPs bind also exons^{136,137}.

An ‘interface splicing’ model was recently proposed for serine-arginine-rich splicing factor 1 (SRSF1) and HNRNPA1¹³⁸. In a simplified view, nuclear speckles contain an inner layer surrounded by an outer layer, and the contact area between them is the speckle–nucleoplasm interface region¹³⁸ (Fig. 4). According to the model, positioning of RNA within the speckle–nucleoplasm interface coordinates splicing regulation: whereas exons are sequestered inside the nuclear speckles by SRSF1, introns are bound by HNRNPA1, which is excluded from the inner layer of the nuclear speckles¹³⁸. In this manner, the exon–intron junctions are positioned at the speckle–nucleoplasm interface, where the spliceosomes are known to be localized¹¹³ (Fig. 4).

The model further proposes a certain order for the regulation of splicing: first, the RNA is positioned at the speckle–nucleoplasm interface based on the recognition and binding of splicing regulatory elements by SRSF1 and HNRNPA1; then the spliceosome assembles;

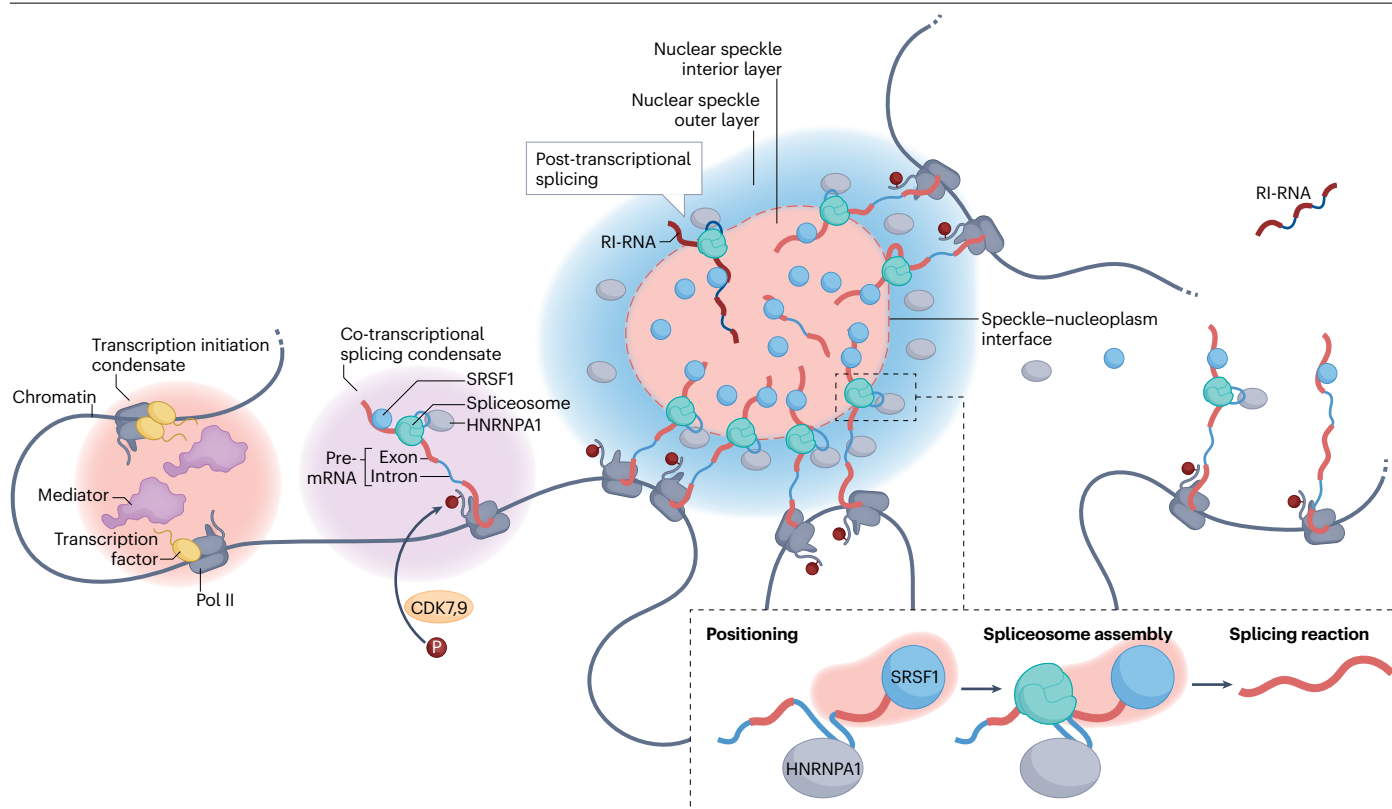


Fig. 4 | Spatial regulation of splicing through nuclear membraneless organelles. Genomic regions with transcribed genes concentrated in 3D space are in close proximity to nuclear speckles, which support concentration of splicing factors, and the pre-mRNAs of these genes are spliced efficiently; genes that are farther away from speckles (right-hand side) are spliced less efficiently owing to the reduced local concentration of splicing factors^{36,121,122,142,152}. The ‘interface splicing’ model was recently proposed for serine-arginine-rich splicing factor 1 (SRSF1) and heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1)¹³⁸. The outer layer of the speckle is enriched in HNRNPA1, which binds to introns of pre-mRNAs, whereas the interior layer of the speckle is enriched in SRSF1, which binds to exons of the pre-mRNAs^{138,140}. This distribution facilitates the localization of the exon–intron boundary at the interface of the two layers,

where active spliceosomes are located and execute the splicing reaction^{138,140}. The model proposes a certain order for the regulation of splicing: first, the RNA is positioned at the nuclear speckle interface based on the recognition of binding motifs by SRSF1 and HNRNPA1; then the spliceosome assembles; and, finally, the splicing reaction takes¹³⁸ (inset). Some retained-intron RNAs (RI-RNAs) in nuclear speckles can be efficiently spliced post-transcriptionally, whereas RI-RNAs far from speckles (top-right corner) are spliced less efficiently and may undergo decay¹²¹. The transition from transcription initiation condensates, which include transcription factors and the Mediator complex, to elongation and co-transcriptional splicing condensates is regulated by phosphorylation (P) of the carboxy-terminal domain (CTD) of RNA polymerase II (Pol II)¹⁵⁵ (left). CDK7, cyclin-dependent kinase 7.

and, finally, the splicing reaction takes place at the interface¹³⁸ (Fig. 4). Therefore, based on this model, the regulatory elements in the pre-mRNAs first determine the positioning of the pre-mRNAs across the speckle–nucleoplasm interface (exon is recognized by SRSF1, and thus is positioned inside the speckle; intron is recognized by HNRNPA1, and thus is positioned at the speckle–nucleoplasm interface). Only after this positioning does assembly of the spliceosome occur, and the splicing reaction takes place. This temporal order is consistent with what other studies have observed¹³⁹.

A recent study supports the interface splicing model using super-resolution imaging¹⁴⁰. As predicted by the model, transcripts containing regions enriched in SRSF1-binding motifs and another region enriched in HNRNPA1-binding motifs localize at the speckle–nucleoplasm interface with the SRSF1-binding motif closer to the centre of the speckle than the HNRNPA1-binding motif¹⁴⁰ (Fig. 4). This specific RNA position and orientation is referred to as ‘intra-organellar RNA organization’¹⁴⁰. SRSF1 depletion induced the migration of the transcripts

towards the speckle periphery, whereas HNRNPA1 depletion caused them to move towards the speckle interior¹⁴⁰. Importantly, the strength of the RNA–protein interactions is what drives this intra-speckle RNA orientation, thereby organizing the RNA substrate for the splicing reaction. These results show how the structure of the nuclear speckle may facilitate splicing through organizing pre-mRNA substrates, although more studies are needed to show the functional importance of the organization in regulating splicing activity. Moreover, intra-organellar RNA organization likely exists in other MLOs enriched in certain RBPs but depleted for others¹⁴⁰.

The interface of nuclear speckles is not the only location where splicing takes place¹³⁸: co-transcriptional splicing can occur outside nuclear speckles at nascent transcripts¹⁴¹ and some eukaryotes lack nuclear speckles altogether¹⁴². Therefore, the interface splicing model leaves some important questions open. We still do not know how unspliced transcripts localize to the nuclear speckles or how spliced transcripts are released from the nuclear speckles¹³⁸. Moreover, given

that numerous HNRNP proteins undergo phase separation on their own, how are HNRNP phase-separation properties linked to their location outside nuclear speckles and their activities in splicing regulation.

Regulation of splicing by other nuclear bodies

Several other nuclear bodies, including nuclear stress bodies (nSBs), the perinucleolar compartment and paraspeckles, have been implicated in splicing regulation, often for sequestering splicing-regulating factors rather than for being sites of active splicing. The perinucleolar compartment, which is localized at the periphery of the nucleolus, contains several non-coding RNAs and RBPs. The lncRNA pyrimidine-rich non-coding transcript (*PNCTR*)¹⁴³ binds to multiple copies of polypyrimidine tract binding protein 1 (PTBP1), an RBP that regulates different aspects of RNA metabolism, including splicing. *PNCTR* is highly expressed in cancer and is important for tumour growth, likely through sequestering PTBP1 in the perinucleolar compartment and thus blocking its function in regulating splicing of apoptosis genes¹⁴⁴.

In response to heat shock, nSBs form around highly repetitive satellite III (*HSATIII*) lncRNAs, which are transcribed from pericentromeric *HSATIII* repeats of several human chromosomes¹⁴⁵. During stress recovery, CLK1 is recruited to nSBs to re-phosphorylate SRSF9, thereby causing accumulation of the intron-retaining RNAs in the nucleus¹⁴⁶. During heat stress recovery, nSBs recruit the m⁶A-methyladenosine (m⁶A) RNA methyltransferase complex to modify *HSATIII*, which in turn recruits m⁶A reader proteins, resulting in reduced m⁶A modification of pre-mRNAs in the nucleoplasm and repression of m⁶A-dependent splicing¹⁴⁷. Therefore, nSBs serve as platforms for splicing regulation in preparing cells for recovery from heat stress.

Paraspeckles form around the lncRNA nuclear paraspeckle assembly transcript 1 and are generally thought to regulate cellular processes, including splicing, by acting as a molecular sponge¹⁴⁸. However, paraspeckles were recently shown to interact with the SWI/SNF chromatin-remodelling complex and regulate the transcription and alternative splicing of a specific set of genes¹⁴⁹.

Chromatin organization and activity is crucial for the spatial organization of discrete nuclear bodies that do not normally fuse together. The localization of different types of nuclear bodies is consistent with their association with different chromatin regions and activity: nuclear speckles and paraspeckles are both associated with actively transcribed chromatin; perinucleolar compartments are associated with Pol I-transcribed lncRNAs at the periphery of the nucleolus; and nSBs are found at specific pericentromeric regions, as described above. Therefore, transcription activity from specific chromatin regions likely governs the formation of distinct nuclear bodies. This notion is supported by the disorganization and fusion of the different nuclear bodies upon transcription inhibition by actinomycin¹⁵⁰.

Transcription and splicing converge at membraneless organelles

Most splicing occurs co-transcriptionally¹⁵¹; thus, discussing splicing regulation in the context of transcription is important.

If we ignore spatial organization and consider the direct recruitment of the splicing machinery by Pol II and/or nascent pre-mRNAs, we might reason that the splicing efficiency (ratio of spliced RNA to total transcribed RNA) would be similar for transcripts with different expression levels. However, splicing efficiency increases with the gene transcription rate, exhibiting an 'economy of scale' behaviour¹⁵², which reflects the importance of spatial organization in regulating gene expression and suggests that highly expressed genes gain

disproportionate allocation of the splicing machinery in the 3D nuclear space. Key components of the spliceosome are more enriched on pre-mRNAs of genes close to nuclear speckles than those far from nuclear speckles, even if transcribed at similar levels^{36,121,122,142,152} (Fig. 4). Moreover, the splicing efficiency is higher for transcripts that are closer to nuclear speckles, which is not merely a correlation because directing a reporter pre-mRNA to nuclear speckles is sufficient to increase the efficiency of its splicing. However, considering how splicing is regulated by the organization of the nuclear speckle, the reporter used in that study may not have had the most optimal configuration to set the exon–intron junction at the speckle interface enriched with spliceosomes¹³⁸. It would be interesting to investigate how splicing is altered when the intron is directed to the outer layer of the nuclear speckle or when the exon is directed to the interior of the nuclear speckle.

Proximity to nuclear speckles enhances the efficiency not only of co-transcriptional splicing but, probably, also of post-transcriptional splicing, as polyadenylated transcripts in the proximity of their genomic loci have better chances to diffuse into the nearby nuclear speckles and get spliced post-transcriptionally than transcripts that are far from the speckles¹²¹ (Fig. 4). Proximity to nuclear speckles might be important in determining the fate of the many RNAs that retain or detain introns, known as retained-intron RNAs (RI-RNAs) or RNAs with detained introns¹⁵³. RI-RNAs are either further spliced post-transcriptionally to produce mRNAs for export or degraded by the RNA exosome^{121,154}. Proximity to nuclear speckles may allow RI-RNAs to be spliced post-transcriptionally, and often alternatively, as a rapid regulatory response to developmental cues¹²¹. RI-RNAs that are far from speckles are inefficiently processed and fated for decay¹²¹.

So which genes are close to nuclear speckles and thus have their pre-mRNAs spliced highly efficiently? A recent preprint has shown that genes close to nuclear speckles correspond with genomic regions with high Pol II occupancy and high overall transcription activity³⁶ (Fig. 4). Moreover, proximity of a gene to nuclear speckles does not necessarily correlate with the transcription activity at the gene itself but, rather, with the overall Pol II occupancy at the genomic region around this gene. This 'regional' effect suggests that pre-mRNAs of a gene that is highly transcribed but located in an overall lowly transcribed region may not be spliced very efficiently as this genomic region is likely to be far from nuclear speckles, whereas pre-mRNAs of a lowly transcribed gene located in an overall highly transcribed region may be spliced quite efficiently. Whether gene location near nuclear speckles actually causes splicing efficiency to be high still needs to be tested.

Co-transcriptional splicing condensates

Transcription initiation is also now thought to involve condensates, specifically 'transcriptional condensates', which are composed of transcription factors, co-activators, Pol II and RNA and involve specific, and perhaps unspecific, multivalent interactions. The transition from transcriptional condensates to co-transcriptional splicing condensates is regulated by phosphorylation of the Pol II CTD – during transcription initiation, hypophosphorylated Pol II CTD II interacts with the Mediator complex¹⁵⁵ (Fig. 4). The transcription elongation complex can form phase-separated condensates that incorporate and concentrate positive transcription elongation factor b (P-TEFb), which otherwise is sequestered in a soluble complex¹⁵⁶. Phosphorylated Pol II in the elongation stage loses binding to the Mediator but gains binding to several splicing factors, effectively switching the Pol II partition from the transcription initiation condensate to the co-transcriptional splicing condensate¹⁵⁵ (Fig. 4).

It is unclear whether the elongation condensates¹⁵⁶ are identical to the co-transcriptional splicing condensates, but it is reasonable to assume that a strong overlap exists between them. Moreover, there is likely a major overlap between co-transcriptional splicing condensates and nuclear speckles. Co-transcriptional splicing condensates likely form at any actively transcribed gene body regardless of the distance of the gene to other actively transcribed regions¹⁵⁵, whereas nuclear speckles may be considered a special type of co-transcriptional splicing condensate that are prominent hubs where the pre-mRNAs of numerous active genes clustered in 3D space are undergoing splicing. Nuclear speckles also contain post-transcriptional splicing reactions, as discussed above; moreover, they may also store splicing factors not actively engaged in splicing¹¹².

AKAP95 associates with the MLL2 complexes and regulates both transcription⁶⁹ and splicing⁶⁸. AKAP95 condensates are likely involved in co-transcriptional splicing regulation. AKAP95 puncta substantially overlaps with SRSF2 signals in nuclear speckles and with the actively transcribing, CTD-phosphorylated Pol II, but not with CTD-unphosphorylated Pol II⁷⁰. However, higher resolution is needed to better understand the spatial relationship between AKAP95 and the speckle substructures. It is worth noting that the immunofluorescence antibody used to detect the nuclear speckles protein SC35 (also known as SRSF2) actually detects SRRM2, which is a key nuclear speckles protein⁹⁸. An open question is that if AKAP95 is indeed located exclusively in the outer layer (the periphery) of nuclear speckles, then how does AKAP95 condensation influence the condensation and properties of this layer of the speckles to regulate splicing.

Wiskott–Aldrich syndrome protein (WASP) was proposed to directly regulate splicing by forming co-transcriptional splicing condensates at gene bodies¹⁵⁷. Wiskott–Aldrich syndrome is a primary immunodeficiency disorder. WASP can directly regulate the expression of genes encoding splicing factors, and forms phase-separated nuclear condensates that include SRSF2, active Pol II and nascent RNAs¹⁵⁷. WASP deficiency enhanced SRSF2 mobility at certain nuclear regions, but not in others¹⁵⁷, which suggests that WASP aggregates may act as co-transcriptional splicing condensates that regulate transcription and splicing of nascent pre-mRNAs. WASP deficiency alters the properties of these condensates and their function in controlling gene expression, thereby contributing to the development of Wiskott–Aldrich syndrome¹⁵⁷.

Diseases associated with splicing dysregulation through condensates

In this section, we discuss how splicing dysregulation through perturbed phase separation promotes neurodegenerative diseases, different types of cancer, developmental disorders and cardiomyopathies.

Neurodegenerative diseases

Numerous neurodegenerative and neurodevelopmental diseases are caused by mutations in specific RBPs and exhibit splicing-relevant aggregation of these RBPs^{26,29,31,46,158–161}.

The splicing-regulating activity of TDP43 is compromised in ALS and in frontotemporal dementia^{103,162}. TDP43 can aggregate into both reversible stress granules and irreversible neuropathogenic amyloid aggregates (Fig. 5a). The irreversible aggregates were found in humans with ALS, frontotemporal lobar degeneration, Alzheimer disease, Huntington disease and Parkinson disease^{163–169}. The LC domain of TDP43 is involved in both types of aggregation. Atomic structures revealed that the TDP43 LC domain can form typical steric zipper β -sheets.

Six segments within the LC domain can form typical steric zipper β -sheets that are characteristic of pathogenic amyloid fibrils and four other segments can form labile amyloid-like interactions found in non-pathological stress granules¹⁶⁹. TDP43 mutants (A315T and A315E) present in familial ALS perturb the group of segments, suggesting that these mutations might favour the transition from reversible to irreversible pathogenic TDP43 aggregation¹⁶⁹. PTMs in TDP43 can also influence its ability to undergo phase separation. Lys84 acetylation reduces nuclear import of TDP43, and Lys136 acetylation impairs its RNA-binding and splicing capabilities¹⁶⁴ (Fig. 5a). When TDP43 fails to interact with RNA, its capacity to undergo phase separation through its LC domain is enhanced, thereby favouring the formation of pathological, insoluble aggregates that contain phosphorylated and ubiquitylated TDP43 (ref. 164). Interestingly, sirtuin 1 potentially deacetylates Lys136 and reduces the aggregation propensity of TDP135.

HNRPN1, HNRPA2, FUS and TDP43 can be mutated in ALS and the mutations enhance the capacity of these RBPs to undergo phase separation and form pathological aggregates^{26,29,31,46}. Mutations in prion-like domains of HNRNPA2B1 and HNRNPA1 exist in families with multisystem proteinopathies and in one family with ALS³¹. Multisystem proteinopathies are characterized by inherited degeneration affecting multiple tissues (brain, muscle, motor neuron, bone), but muscle degeneration is a main feature. Normally, these RBPs self-assemble into fibrils, but this process is exacerbated by the mutations in the prion-like domains³¹. Consequently, excessive aggregation of these RBPs in stress granules occurs, leading to the formation of cytoplasmic inclusions and dysregulation of the functions of the aggregated RBPs³¹. Although not yet probed, it is widely hypothesized that pathological aggregation of these RBPs that alters their splicing functions is the underlying mechanism of these diseases.

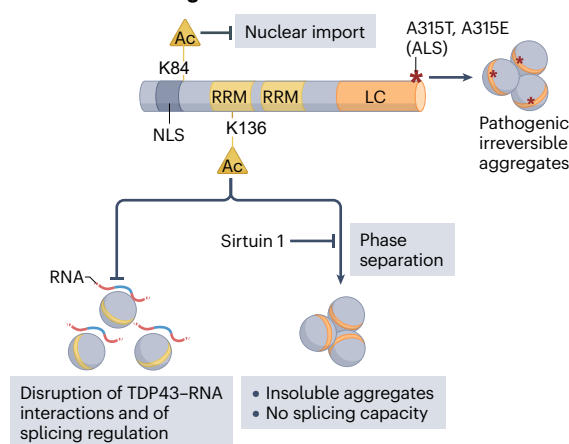
Rett syndrome

Pathological mutations in methyl-CpG-binding protein 2 (MeCP2) disrupt condensation of the RBFOX proteins and result in mis-splicing of synaptic plasticity transcripts¹⁷⁰ (Table 1 and Fig. 5b). MeCP2 is associated with the RBFOX–LASR complex, which regulates splicing and has an important role in mammalian neuronal development. Although other studies have shown that MeCP2 alone and the RBFOX–LASR complex each forms condensates or higher-order assemblies, MeCP2 and RBFOX proteins exhibit synergistic effects in forming co-condensates¹⁷⁰. Importantly, Rett syndrome-causing mutations in *MECP2* abolish this co-condensation capacity. In mouse models, MeCP2 loss or its Rett syndrome-causing mutations disrupt the assembly of the MeCP2–RBFOX–LASR complex and impair RBFOX binding to pre-mRNAs, leading to aberrant splicing of neurexin and neuregulin1 pre-mRNAs, which are crucial for synaptic plasticity¹⁷⁰. MeCP2 has been shown to regulate co-transcriptional splicing by binding to methylated DNA and modulating Pol II elongation^{171,172}. The more recent study¹⁷⁰ further shows that the assembly of the MeCP2–RBFOX–LASR complex is disrupted by inhibition of DNA methylation or transcription. Together, these results suggest that MeCP2–RBFOX co-condensates function by locally concentrating splicing factors on chromatin loci to regulate co-transcriptional splicing of nascent pre-mRNAs. Thus, MeCP2 mutations in Rett syndrome disrupt this co-condensation with splicing regulators and cause mis-splicing of neuronal transcripts.

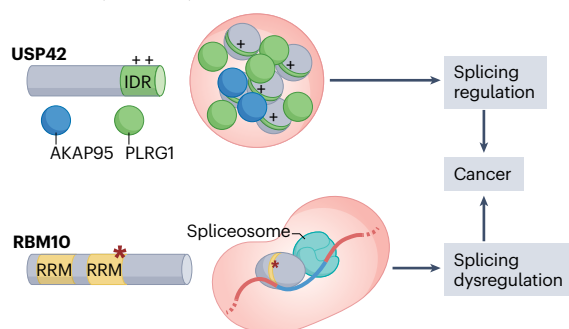
Cancer

AKAP95 is associated with human cancers and tumorigenesis, in part, through its activity in regulating splicing. To regulate splicing and

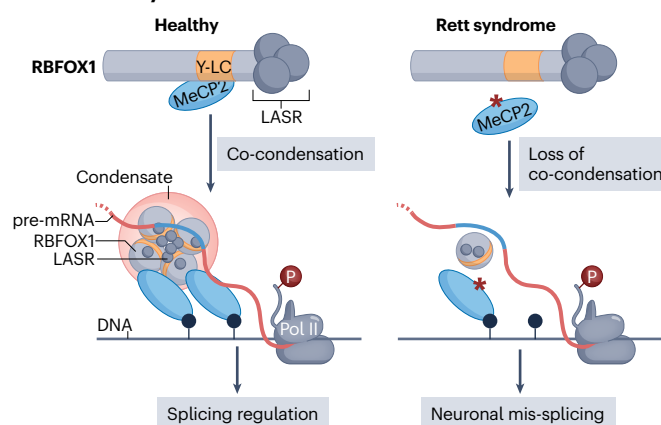
a TDP43: neurodegenerative diseases



c USP42, AKAP95, RMB10: cancer



b Rett syndrome



d RBM20: cardiomyopathy

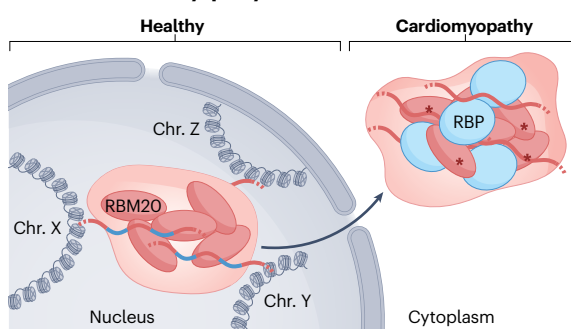


Fig. 5 | Condensate-mediated splicing regulation in health and disease. a, TAR DNA-binding protein 43 (TDP43) mutants (A315T and A315E) present in familial amyotrophic lateral sclerosis (ALS) might favour the transition from reversible to irreversible, pathogenic TDP43 aggregation¹⁶⁹. Lys84 (K84) acetylation reduces the nuclear import of TDP43. Lys136 (K136) acetylation impairs the RNA-binding and splicing capabilities of TDP43, and failure to interact with RNA enhances its capacity to undergo phase separation through its low-complexity (LC) domain, thereby favouring the formation of pathological, insoluble TDP43 aggregates¹⁶⁴. Sirtuin 1 deacetylates Lys136, thereby reducing the aggregation propensity of TDP43. **b**, Methyl-CpG-binding protein 2 (MeCP2) normally forms co-condensates and assemblies of the MeCP2–RNA-binding fox-1 homologue (RFX1)–large assembly of splicing regulators (LASR) complex that regulate co-transcriptional splicing. MeCP2 mutations (red asterisk) in Rett syndrome reduce co-condensation and impair the binding of RFX1–LASR to key neuronal pre-mRNAs, thereby affecting their alternative splicing^{171,172}. **c**, A-kinase anchoring protein 95 kDa (AKAP95) and the deubiquitinase ubiquitin specific peptidase 42

(USP42) form liquid-like condensates that regulate splicing programmes that support cancer growth^{70,173}. Through its positively charged carboxy-terminal intrinsically disordered region (IDR), USP42 condensation is important for its co-localization with pleiotropic regulator 1 (PLRG1), a spliceosome component, and nuclear speckles. Depletion of AKAP95, USP42 or PLRG1 markedly reduced cancer cell growth and caused alternative splicing changes in numerous genes, including some involved in cell growth^{70,173}. V354M, a colon cancer mutation in an RNA-recognition motif (RRM) of RNA-binding motif protein 10 (RBM10), enhances RBM10 condensation¹⁸², which may explain how this and related RBM10 mutations affect cancer-associated splicing^{177,178,182,192}. **d**, RBM20 forms nuclear foci that regulate alternative splicing of transcripts from multiple chromosomes. Mutations in the serine-arginine-rich domain of RBM20 are associated with cardiomyopathies; they alter its location to the cytoplasm, where it forms condensates that sequester mRNAs and proteins^{184,186,187}. NLS, nuclear localization signal; Pol II, RNA polymerase II; RBP, RNA-binding protein; Y-LC, Tyr-rich low-complexity domain.

cancer, AKAP95 forms condensates with specific liquidity and dynamics, which suggests that cancer inhibition might be possible by either disrupting or solidifying the liquid-like condensates that promote tumorigenesis⁷⁰. Similarly, the deubiquitinase ubiquitin specific peptidase 42 (USP42) forms liquid-like condensates that regulate splicing programmes and support cancer growth¹⁷³ (Table 1 and Fig. 5c). Through its positively charged C-terminal IDR, USP42 condensation is important for its co-localization with pleiotropic regulator 1 (PLRG1), a spliceosome component, and with nuclear speckles, and USP42 regulates the morphological properties of these SRSF2 condensates¹⁷³. Depletion of USP42 or PLRG1 markedly reduced cancer cell growth

and caused alternative splicing changes in numerous genes, including some that regulate cell growth. Moreover, both USP42 and PLRG1 are upregulated in lung cancer and high expression levels are associated with low survival¹⁷³.

Other examples include the Epstein–Barr virus protein EBNA2, which activates transcription through phase separation¹⁷⁴. EBNA2 associates with several splicing factors and regulates alternative splicing of transcripts enriched in tumorigenic pathways¹⁷⁴. RNA-binding motif protein 5 (RBM5) and RBM10 associate with spliceosomes and control alternative splicing of genes involved in cell proliferation and apoptosis^{175,176}. Somatic mutations of RBM5 and RBM10 occur in several cancers

Glossary

Amyloid aggregates

Fibrillar protein aggregates typically 7–13 nm in diameter and a β -sheet secondary structure.

Limb-girdle muscular dystrophy

A large group of genetic diseases characterized by muscle weakness and wasting.

Nuclear speckles

Dynamic, membraneless subnuclear compartments that are enriched in splicing factors and other proteins involved in transcription, RNA processing and reversible protein phosphorylation.

Scaffold and client

In the context of condensates, scaffold molecules (proteins or long non-coding RNAs (lncRNAs)) are required for and drive condensate formation; client molecules are not essential for condensate formation, but partition into the condensates through their interactions with the scaffold or other client molecules.

Stress granules

Biomolecular condensates of mRNAs stalled in translation and RNA-binding proteins (RBPs), which form in the cytoplasm following physical, mechanical or chemical stress.

and cause splicing dysregulation^{177–180}. Whereas most of the truncating mutations may be loss-of-function mutations owing to transcript decay or loss of functional domains, certain missense mutations may provide mechanistic insights. For example, the V354E mutation in the RRM of RBM10 is found in lung cancer and actively disrupts RBM10-mediated alternative splicing of *NUMB*, which is involved in cell proliferation¹⁷⁹. As V354E does not affect binding to *NUMB* mRNA, it remains unclear how it disrupts RBM10 activity other than it might be related to the negative charge of Glu, as similar effects were observed for V354D (ref. 181). Another missense mutation of RBM10 Val354, V354M, which is found in colon cancer, has recently been shown to enhance RBM10 condensation in the nucleus¹⁸² (Table 1 and Fig. 5c). This finding suggests that mutations in *RBM10* (and potentially in *RBM5*) alter its functions in cancer-relevant splicing regulation by changing condensate formation and properties. Clearly, more in-depth studies are needed to link the functions of the mutants and isoforms^{180,183} of these proteins with their effects on condensation.

Not all these studies have rigorously demonstrated a functional role of condensate properties in mediating the cancer-related phenotypes. The effects on cancer growth and cancer-associated splicing were examined following USP42 depletion, but not by introducing the condensation mutations¹⁷³. The evidence that EBNA2 regulates splicing of tumour-associated transcripts is weak, as it relies only on treatment with 1,6-hexanediol¹⁷⁴, an aliphatic alcohol that can disrupt phase separation but also numerous other processes. It remains unclear whether the RBM10 V354M mutation contributes to tumorigenesis through splicing dysregulation associated with the altered condensation¹⁸².

Cardiomyopathy

RBM20 regulates tissue-specific alternative splicing by forming nuclear splicing foci that contain multiple loci from different chromosomes¹⁸⁴. Common point mutations in *RBM20* are associated with congenital dilated cardiomyopathy¹⁸⁵. These mutations are often located in the SR-rich domain, and cause defective splicing and re-localization of RBM20 from its normal location at nuclear speckles associated with chromatin to sarcoplasmic viscous liquid-like condensates analogous

to stress granules, which contain cardiac mRNAs and proteins^{186,187} (Table 1 and Fig. 5d). It is possible that the disease phenotypes result from both splicing dysregulation (caused by the absence of normal RBM20 nuclear speckles) and sequestration of other proteins into the aberrant cytoplasmic RBM20 condensates.

Nuclear speckleopathies

Nuclear speckleopathies are a growing class of developmental disorders caused by mutations in genes encoding nuclear speckle proteins, many of which regulate RNA processing¹⁸⁸. Some notable examples include ZTTK syndrome, NKAP-related syndrome, TARP syndrome and others¹⁸⁸. The common clinical feature of these disorders is a global developmental delay, especially in the brain¹⁸⁸. ZTTK syndrome is caused by mutations in SON, a crucial scaffold protein of nuclear speckles⁹⁸. De novo mutations in SON disrupt splicing of genes required for brain development and metabolism¹⁸⁹. NKAP-related syndrome is caused by mutations in NF- κ B-activating protein (NKAP)¹⁹⁰, which associates with multiple splicing-regulating proteins and small nuclear RNAs and controls RNA processing¹⁹¹. TARP syndrome is caused by germ-line mutations in *RBM10*¹⁹². However, functional connections with splicing (dys)regulation have not yet been well demonstrated for disease mutations of several of these proteins^{190,193}.

Conclusion and outstanding questions

Studies conducted in recent years suggest that splicing reactions are spatio-temporally regulated by condensation of splicing factors and RBPs into nuclear MLOs. The dynamic formation, composition and biophysical properties and the internal organization of these condensates profoundly affect the efficiency of co-transcriptional and post-transcriptional splicing, and the fate of transcripts that retain introns. Chromatin and genome organization also influence splicing by dictating the position of nuclear speckles as hubs of splicing factors and likely of splicing. Importantly, numerous human diseases are caused by splicing dysregulation associated with altered formation and properties of nuclear condensates.

Despite this advancement in our understanding of the functional relationship between splicing and phase separation, several outstanding questions and challenges remain:

- How do we rigorously link condensates to function in splicing regulation? Studies often do not provide very strong, mechanistic evidence to link RBP-condensate formation or properties to splicing function, and it remains challenging to do so, as we still lack a loss-of-function methodology that can dissolve or disrupt specific protein condensates in cells. There is no single, robust method to fully address this question. Instead, a multipronged approach is required to perturb (induce, reduce, restore or enhance) specific condensates (and not other molecular properties) and determine the effects on splicing^{194,195}. The most common strategy is to identify and mutate residues that are key for condensation, but it is sometimes difficult to avoid effects on other (including undiscovered) activities of the protein. Mutations in IDRs or PTMs of client proteins may alter splicing outcomes by affecting protein localization and dwell time in the condensate without affecting the properties of the condensate^{106,196}. For IDR-mediated condensation, it is necessary to demonstrate restoration of condensation and splicing functions by replacing the IDR with condensate-forming IDRs of unrelated proteins. Splicing modulation by chemical or optical manipulation^{197,198} of RBP condensation also strengthens the causal link between condensation and splicing.

- How are intricate MLO structures formed and how do they regulate splicing? So far, most studies have focused on homotypic condensates of single purified proteins *in vitro*, but heterotypic interactions are likely a major driving force in forming and shaping multicomponent condensates in cells¹⁹⁹. Moreover, *in vitro* reconstitution studies of RBP condensation should be performed in the presence and absence of RNA, because the formation, properties and structures of the condensates are likely affected by RNA. Advanced imaging techniques that can capture single-molecule activity in live cells would be instrumental in visualizing these features.
- How is splicing regulated through condensates in the context of dynamic changes of genome organization and activity in health and disease? A great example is the formation of RBM20 splicing foci in response to the dynamic genome reorganization occurring during human cardiogenesis¹⁸⁴. As many cardiac genes on different chromosomes are activated, some of them gain spatial proximity with each other and form inter-chromosomal contacts that allow coordinated transcription, as well as splicing in the RBM20-containing splicing foci at these contact sites¹⁸⁴. Both RBM20 and the RNAs are important for the formation of the splicing foci, exhibiting a complex and dynamic interplay of genome architecture, transcription and splicing¹⁸⁴. This evidence also suggests that splicing of these RNAs could be altered by conditions affecting any component involved in the formation of the splicing foci. As discussed¹⁸⁴, congenital heart disease-associated mutations in the transcription factor GATA4 lead to reduced transcription of a key cardiac gene²⁰⁰ that normally facilitates the formation of RBM20 splicing foci, and thus may affect splicing of other RBM20 targets. Clearly, multiple sequencing-based and imaging technologies are required to probe and integrate these dynamic spatial changes to generate mechanistic insights.

Finally, although we do not aim to review the progress in targeting biomolecular condensates for therapy (reviewed elsewhere^{201–203}), we will mention a recent report about small molecules designed to perturb condensates for splicing regulation²⁰⁴. Small molecule inhibitors targeting the dimethylarginine binding pocket in the Tudor domain of survival motor neuron domain-containing 1 (SMNDC1) can alter the phase separation behaviour and splicing functions of SMNDC1, and the architecture of nuclear speckles²⁰⁴. As a splicing factor essential for spliceosome assembly, SMNDC1 interacts with multiple nuclear speckle proteins and forms nuclear condensates through its IDR. Inhibitors targeting its Tudor domain reduced the mobility of SMNDC1 and SRRM2 in nuclear speckles without affecting the structure of the MLO, but they resulted in global splicing changes. However, because the inhibitors cause a downregulation of SMNDC1 protein, it is hard to attribute the splicing and other biological effects to inhibition of SMNDC1 condensation²⁰⁴. Clearly, a long way remains ahead for successfully modulating splicing through pharmacological regulation of biomolecular condensates.

Published online: 21 May 2024

References

- Berget, S. M., Moore, C. & Sharp, P. A. Spliced segments at the 5' terminus of adenovirus 2 late mRNA. *Proc. Natl Acad. Sci. USA* **74**, 3171–3175 (1977).
- Chow, L. T., Gelinas, R. E., Broker, T. R. & Roberts, R. J. An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. *Cell* **12**, 1–8 (1977).
- Pan, Q., Shai, O., Lee, L. J., Frey, B. J. & Blencowe, B. J. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.* **40**, 1413–1415 (2008).
- Wang, E. T. et al. Alternative isoform regulation in human tissue transcriptomes. *Nature* **456**, 470–476 (2008).
- Tilgner, H. et al. Deep sequencing of subcellular RNA fractions shows splicing to be predominantly co-transcriptional in the human genome but inefficient for lncRNAs. *Genome Res.* **22**, 1616–1625 (2012).
- Vargas, D. Y. et al. Single-molecule imaging of transcriptionally coupled and uncoupled splicing. *Cell* **147**, 1054–1065 (2011).
- Fiszbein, A. et al. Alternative slicing of G9a regulates neuronal differentiation. *Cell Rep.* **14**, 2797–2808 (2016).
- Kornblihtt, A. R. et al. Alternative splicing: a pivotal step between eukaryotic transcription and translation. *Nat. Rev. Mol. Cell Biol.* **14**, 153–165 (2013).
- Luco, R. F., Allo, M., Schor, I. E., Kornblihtt, A. R. & Misteli, T. Epigenetics in alternative pre-mRNA splicing. *Cell* **144**, 16–26 (2011).
- Neugebauer, K. M. On the importance of being co-transcriptional. *J. Cell Sci.* **115**, 3865–3871 (2002).
- Ip, J. Y. et al. Global impact of RNA polymerase II elongation inhibition on alternative splicing regulation. *Genome Res.* **21**, 390–401 (2011).
- Perales, R. & Bentley, D. “Cotranscriptionality”: the transcription elongation complex as a nexus for nuclear transactions. *Mol. Cell* **36**, 178–191 (2009).
- Lyon, A. S., Peebles, W. B. & Rosen, M. K. A framework for understanding the functions of biomolecular condensates across scales. *Nat. Rev. Mol. Cell Biol.* **22**, 215–235 (2021).
- Hyman, A. A., Weber, C. A. & Julicher, F. Liquid–liquid phase separation in biology. *Annu. Rev. Cell Dev. Biol.* **30**, 39–58 (2014).
- Shin, Y. & Brangwynne, C. P. Liquid phase condensation in cell physiology and disease. *Science* **357**, eaaf4382 (2017).
- Banani, S. F., Lee, H. O., Hyman, A. A. & Rosen, M. K. Biomolecular condensates: organizers of cellular biochemistry. *Nat. Rev. Mol. Cell Biol.* **18**, 285–298 (2017).
- Bergeron-Sandoval, L. P., Safaee, N. & Michnick, S. W. Mechanisms and consequences of macromolecular phase separation. *Cell* **165**, 1067–1079 (2016).
- Choi, J. M., Holehouse, A. S. & Pappu, R. V. Physical principles underlying the complex biology of intracellular phase transitions. *Annu. Rev. Biophys.* **49**, 107–133 (2020).
- Alberti, S. & Dormann, D. Liquid–liquid phase separation in disease. *Annu. Rev. Genet.* **53**, 171–194 (2019).
- Snead, W. T. & Gladfelter, A. S. The control centers of biomolecular phase separation: how membrane surfaces, PTMs, and active processes regulate condensation. *Mol. Cell* **76**, 295–305 (2019).
- Alberti, S. & Hyman, A. A. Biomolecular condensates at the nexus of cellular stress, protein aggregation disease and ageing. *Nat. Rev. Mol. Cell Biol.* **22**, 196–213 (2021).
- Boeynaems, S. et al. Protein phase separation: a new phase in cell biology. *Trends Cell Biol.* **28**, 420–435 (2018).
- Uversky, V. N. Protein intrinsic disorder-based liquid–liquid phase transitions in biological systems: complex coacervates and membrane-less organelles. *Adv. Colloid Interface Sci.* **239**, 97–114 (2017).
- Mitrea, D. M. & Kriwacki, R. W. Phase separation in biology: functional organization of a higher order. *Cell Commun. Signal.* **14**, 1 (2016).
- Kato, M. et al. Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels. *Cell* **149**, 753–767 (2012).
- Patel, A. et al. A liquid-to-solid phase transition of the ALS protein FUS accelerated by disease mutation. *Cell* **162**, 1066–1077 (2015).
- Alberti, S., Gladfelter, A. & Mittag, T. Considerations and challenges in studying liquid–liquid phase separation and biomolecular condensates. *Cell* **176**, 419–434 (2019).
- Woodruff, J. B., Hyman, A. A. & Boke, E. Organization and function of non-dynamic biomolecular condensates. *Trends Biochem. Sci.* **43**, 81–94 (2018).
- Molliex, A. et al. Phase separation by low complexity domains promotes stress granule assembly and drives pathological fibrillization. *Cell* **163**, 123–133 (2015).
- Ramaswami, M., Taylor, J. P. & Parker, R. Altered ribostasis: RNA-protein granules in degenerative disorders. *Cell* **154**, 727–736 (2013).
- Kim, H. J. et al. Mutations in prion-like domains in hnRNP A2B1 and hnRNP A1 cause multisystem proteinopathy and ALS. *Nature* **495**, 467–473 (2013).
- McSwiggen, D. T., Mir, M., Darzacq, X. & Tjian, R. Evaluating phase separation in live cells: diagnosis, caveats, and functional consequences. *Genes. Dev.* **33**, 1619–1634 (2019).
- Li, W. & Jiang, H. Nuclear protein condensates and their properties in regulation of gene expression. *J. Mol. Biol.* **434**, 167151 (2021).
- Sabari, B. R., Dall'Agnese, A. & Young, R. A. Biomolecular condensates in the nucleus. *Trends Biochem. Sci.* **45**, 961–977 (2020).
- Shrinivas, K. et al. Enhancer features that drive formation of transcriptional condensates. *Mol. Cell* **75**, 549–561.e7 (2019).
- Bhat, P. et al. 3D genome organization around nuclear speckles drives mRNA splicing efficiency. Preprint at *bioRxiv* <https://doi.org/10.1101/2023.01.04.522632> (2023).
- Li, C. H. et al. MeCP2 links heterochromatin condensates and neurodevelopmental disease. *Nature* **586**, 440–444 (2020).
- Gibson, B. A. et al. Organization of chromatin by intrinsic and regulated phase separation. *Cell* **179**, 470–484.e21 (2019).
- Wang, L. et al. Histone modifications regulate chromatin compartmentalization by contributing to a phase separation mechanism. *Mol. Cell* **76**, 646–659.e6 (2019).
- Ries, R. J. et al. m⁶A enhances the phase separation potential of mRNA. *Nature* **571**, 424–428 (2019).
- Lee, J. H. et al. Enhancer RNA m⁶A methylation facilitates transcriptional condensate formation and gene activation. *Mol. Cell* **81**, 3368–3385.e9 (2021).

42. Strickfaden, H. et al. Condensed chromatin behaves like a solid on the mesoscale in vitro and in living cells. *Cell* **183**, 1772–1784.e13 (2020).
43. Bhat, P., Honson, D. & Guttman, M. Nuclear compartmentalization as a mechanism of quantitative control of gene expression. *Nat. Rev. Mol. Cell Biol.* **22**, 653–670 (2021).
44. Castello, A. et al. Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. *Cell* **149**, 1393–1406 (2012).
45. Michelitsch, M. D. & Weissman, J. S. A census of glutamine/asparagine-rich regions: implications for their conserved function and the prediction of novel prions. *Proc. Natl Acad. Sci. USA* **97**, 11910–11915 (2000).
46. Wiedner, H. J. & Giudice, J. It's not just a phase: function and characteristics of RNA-binding proteins in phase separation. *Nat. Struct. Mol. Biol.* **28**, 465–473 (2021).
47. Calabretta, S. & Richard, S. Emerging roles of disordered sequences in RNA-binding proteins. *Trends Biochem. Sci.* **40**, 662–672 (2015).
48. Aumiller, W. M. Jr., Pir Cakmak, F., Davis, B. W. & Keating, C. D. RNA-based coacervates as a model for membraneless organelles: formation, properties, and interfacial liposome assembly. *Langmuir : ACS J. Surf. Colloids* **32**, 10042–10053 (2016).
49. Van Treec, B. et al. RNA self-assembly contributes to stress granule formation and defining the stress granule transcriptome. *Proc. Natl Acad. Sci. USA* **115**, 2734–2739 (2018).
50. Bevilacqua, P. C., Williams, A. M., Chou, H. L. & Assmann, S. M. RNA multimerization as an organizing force for liquid–liquid phase separation. *RNA* **28**, 16–26 (2022).
51. Putnam, A., Thomas, L. & Seydoux, G. RNA granules: functional compartments or incidental condensates? *Genes. Dev.* **37**, 354–376 (2023).
52. Ma, W., Zhen, G., Xie, W. & Mayr, C. In vivo reconstitution finds multivalent RNA–RNA interactions as drivers of mesh-like condensates. *eLife* **10**, e64252 (2021).
53. Wallace, E. W. et al. Reversible, specific, active aggregates of endogenous proteins assemble upon heat stress. *Cell* **162**, 1286–1298 (2015).
54. Courchaine, E. M., Lu, A. & Neugebauer, K. M. Droplet organelles? *EMBO J.* **35**, 1603–1612 (2016).
55. Kroschwald, S. et al. Promiscuous interactions and protein disaggregases determine the material state of stress-inducible RNP granules. *eLife* **4**, e06807 (2015).
56. Gueroussou, S. et al. Regulatory expansion in mammals of multivalent hnRNP assemblies that globally control alternative splicing. *Cell* **170**, 324–339.e23 (2017).
57. Ying, Y. et al. Splicing activation by Rbfox requires self-aggregation through its tyrosine-rich domain. *Cell* **170**, 312–323.e10 (2017).
58. Kim, G. H. & Kwon, I. Distinct roles of hnRNP1 low-complexity domains in splicing and transcription. *Proc. Natl Acad. Sci. USA* **118**, e2109668118 (2021).
59. Geuens, T., Bouhy, D. & Timmerman, V. The hnRNP family: insights into their role in health and disease. *Hum. Genet.* **135**, 851–867 (2016).
60. Kwon, I. et al. Phosphorylation-regulated binding of RNA polymerase II to fibrous polymers of low-complexity domains. *Cell* **155**, 1049–1060 (2013).
61. Xiang, S. et al. The LC domain of hnRNP2 adopts similar conformations in hydrogel polymers, liquid-like droplets, and nuclei. *Cell* **163**, 829–839 (2015).
62. Lin, Y., Protter, D. S., Rosen, M. K. & Parker, R. Formation and maturation of phase-separated liquid droplets by RNA-binding proteins. *Mol. Cell* **60**, 208–219 (2015).
63. Kato, M. & McKnight, S. L. A solid-state conceptualization of information transfer from gene to message to protein. *Annu. Rev. Biochem.* **87**, 351–390 (2018).
64. Lu, J. et al. CryoEM structure of the low-complexity domain of hnRNP2 and its conversion to pathogenic amyloid. *Nat. Commun.* **11**, 4090 (2020).
65. Sun, Y. et al. The nuclear localization sequence mediates hnRNP1 amyloid fibril formation revealed by cryoEM structure. *Nat. Commun.* **11**, 6349 (2020).
66. Conboy, J. G. Developmental regulation of RNA processing by Rbfox proteins. *Wiley Interdiscip. Rev. RNA* **8**, e1398 (2017).
67. Damianov, A. et al. Rbfox proteins regulate splicing as part of a large multiprotein complex LASR. *Cell* **165**, 606–619 (2016).
68. Hu, J. et al. AKAP95 regulates splicing through scaffolding RNAs and RNA processing factors. *Nat. Commun.* **7**, 13347 (2016).
69. Jiang, H. et al. Regulation of transcription by the MLL2 complex and MLL complex-associated AKAP95. *Nat. Struct. Mol. Biol.* **20**, 1156–1163 (2013).
70. Li, W. et al. Biophysical properties of AKAP95 protein condensates regulate splicing and tumorigenesis. *Nat. Cell Biol.* **22**, 960–972 (2020).
71. van der Lee, R. et al. Classification of intrinsically disordered regions and proteins. *Chem. Rev.* **114**, 6589–6631 (2014).
72. Holehouse, A. S. & Kragelund, B. B. The molecular basis for cellular function of intrinsically disordered protein regions. *Nat. Rev. Mol. Cell Biol.* **25**, 187–211 (2024).
73. Van Roey, K. et al. Short linear motifs: ubiquitous and functionally diverse protein interaction modules directing cell regulation. *Chem. Rev.* **114**, 6733–6778 (2014).
74. Ward, J. J., Sodhi, J. S., McGuffin, L. J., Buxton, B. F. & Jones, D. T. Prediction and functional analysis of native disorder in proteins from the three kingdoms of life. *J. Mol. Biol.* **337**, 635–645 (2004).
75. Xue, B., Dunker, A. K. & Uversky, V. N. Orderly order in protein intrinsic disorder distribution: disorder in 3500 proteomes from viruses and the three domains of life. *J. Biomol. Struct. Dyn.* **30**, 137–149 (2012).
76. Barbosa-Morais, N. L. et al. The evolutionary landscape of alternative splicing in vertebrate species. *Science* **338**, 1587–1593 (2012).
77. Buljan, M. et al. Tissue-specific splicing of disordered segments that embed binding motifs rewires protein interaction networks. *Mol. Cell* **46**, 871–883 (2012).
78. Ellis, J. D. et al. Tissue-specific alternative splicing remodels protein–protein interaction networks. *Mol. Cell* **46**, 884–892 (2012).
79. Romero, P. R. et al. Alternative splicing in concert with protein intrinsic disorder enables increased functional diversity in multicellular organisms. *Proc. Natl Acad. Sci. USA* **103**, 8390–8395 (2006).
80. Battle, C. et al. hnRNPDL phase separation is regulated by alternative splicing and disease-causing mutations accelerate its aggregation. *Cell Rep.* **30**, 1117–1128.e5 (2020).
81. Akagi, T. et al. Molecular characterization of a mouse heterogeneous nuclear ribonucleoprotein D-like protein JKTBP and its tissue-specific expression. *Gene* **245**, 267–273 (2000).
82. Kamei, D., Tsuchiya, N., Yamazaki, M., Meguro, H. & Yamada, M. Two forms of expression and genomic structure of the human heterogeneous nuclear ribonucleoprotein D-like JKTBP gene (HNRPD). *Gene* **228**, 13–22 (1999).
83. Kawamura, H. et al. Identification of the nucleocytoplasmic shuttling sequence of heterogeneous nuclear ribonucleoprotein D-like protein JKTBP and its interaction with mRNA. *J. Biol. Chem.* **277**, 2732–2739 (2002).
84. Garcia-Pardo, J. et al. Cryo-EM structure of hnRNPDL-2 fibrils, a functional amyloid associated with limb-girdle muscular dystrophy D3. *Nat. Commun.* **14**, 239 (2023).
85. Vieira, N. M. et al. A defect in the RNA-processing protein HNRPD causes limb-girdle muscular dystrophy 1G (LGMD1G). *Hum. Mol. Genet.* **23**, 4103–4110 (2014).
86. Starling, A., Kok, F., Passos-Bueno, M. R., Vainzof, M. & Zatz, M. A new form of autosomal dominant limb-girdle muscular dystrophy (LGMD1G) with progressive fingers and toes flexion limitation maps to chromosome 4p21. *Eur. J. Hum. Genet.* **12**, 1033–1040 (2004).
87. Sun, Y. et al. Limb girdle muscular dystrophy D3 HNRNPDL related in a Chinese family with distal muscle weakness caused by a mutation in the prion-like domain. *J. Neurol.* **266**, 498–506 (2019).
88. Weskamp, K. et al. Shortened TDP43 isoforms upregulated by neuronal hyperactivity drive TDP43 pathology in ALS. *J. Clin. Invest.* **130**, 1139–1155 (2020).
89. Parras, A. et al. Autism-like phenotype and risk gene mRNA deadenylation by CPEB4 mis-splicing. *Nature* **560**, 441–446 (2018).
90. Ollá, I. et al. Pathogenic mis-splicing of CPEB4 in schizophrenia. *Biol. Psychiatry* **94**, 341–351 (2023).
91. Garcia-Cabau, C. et al. Kinetic stabilization of translation-repression condensates by a neuron-specific microexon. Preprint at bioRxiv <https://doi.org/10.1101/2023.03.19.532587> (2023).
92. Bowler, E. et al. Hypoxia leads to significant changes in alternative splicing and elevated expression of CLK splice factor kinases in PC3 prostate cancer cells. *BMC Cancer* **18**, 355 (2018).
93. de Oliveira Freitas Machado, C. et al. Poison cassette exon splicing of SRSF6 regulates nuclear speckle dispersal and the response to hypoxia. *Nucleic Acids Res.* **51**, 870–890 (2023).
94. Hochberg-Laufer, H. et al. Availability of splicing factors in the nucleoplasm can regulate the release of mRNA from the gene after transcription. *PLoS Genet.* **15**, e1008459 (2019).
95. Jakubauskiene, E., Vily, L., Makino, Y., Poellinger, L. & Kanopka, A. Increased serine-arginine (SR) protein phosphorylation changes pre-mRNA splicing in hypoxia. *J. Biol. Chem.* **290**, 18079–18089 (2015).
96. Jakubauskienė, E., Vily, L., Pečiulienė, I. & Kanopka, A. The role of hypoxia on Alzheimer's disease-related APP and Tau mRNA formation. *Gene* **766**, 145146 (2021).
97. Tari, M. et al. U2AF⁶⁵ assemblies drive sequence-specific splice site recognition. *EMBO Rep.* **20**, e47604 (2019).
98. Ilik, I. A. et al. SON and SRRM2 are essential for nuclear speckle formation. *eLife* **9**, e60579 (2020).
99. Xu, S. et al. SRRM2 organizes splicing condensates to regulate alternative splicing. *Nucleic Acids Res.* **50**, 8599–8614 (2022).
100. Hallegger, M. et al. TDP-43 condensation properties specify its RNA-binding and regulatory repertoire. *Cell* **184**, 4680–4696.e22 (2021).
101. Langdon, E. M. et al. mRNA structure determines specificity of a polyQ-driven phase separation. *Science* **360**, 922–927 (2018).
102. Schmidt, H. B., Barreau, A. & Rohatgi, R. Phase separation-deficient TDP43 remains functional in splicing. *Nat. Commun.* **10**, 4890 (2019).
103. Ling, J. P., Pletnikova, O., Troncoso, J. C. & Wong, P. C. TDP-43 repression of nonconserved cryptic exons is compromised in ALS-FTD. *Science* **349**, 650–655 (2015).
104. Palomba, F. et al. Fluorescence methods for the biophysical characterization of protein phase separated condensates. *Biophys. J.* **121**, 280a (2022).
105. Cui, H. et al. Arg-tRNA synthetase links inflammatory metabolism to RNA splicing and nuclear trafficking via SRRM2. *Nat. Cell Biol.* **25**, 592–603 (2023).
106. Greig, J. A. et al. Arginine-enriched mixed-charge domains provide cohesion for nuclear speckle condensation. *Mol. Cell* **77**, 1237–1250.e4 (2020).
107. Shi, B. et al. UTX condensation underlies its tumour-suppressive activity. *Nature* **597**, 726–731 (2021).
108. Basu, S. et al. Unblending of transcriptional condensates in human repeat expansion disease. *Cell* **181**, 1062–1079.e30 (2020).
109. Fare, C. M., Villani, A., Drake, L. E. & Shorter, J. Higher-order organization of biomolecular condensates. *Open. Biol.* **11**, 210137 (2021).
110. Feric, M. et al. Coexisting liquid phases underlie nucleolar subcompartments. *Cell* **165**, 1686–1697 (2016).
111. Ye, S. et al. Micropolarity governs the structural organization of biomolecular condensates. *Nat. Chem. Biol.* **20**, 443–451 (2023).
112. Spector, D. L. & Lamond, A. I. Nuclear speckles. *Cold Spring Harb. Perspect. Biol.* **3**, a000646 (2011).

113. Fei, J. et al. Quantitative analysis of multilayer organization of proteins and RNA in nuclear speckles at super resolution. *J. Cell Sci.* **130**, 4180–4192 (2017).
114. Fakan, S. & van Driel, R. The perichromatin region: a functional compartment in the nucleus that determines large-scale chromatin folding. *Semin. Cell Dev. Biol.* **18**, 676–681 (2007).
115. Brown, J. M. et al. Association between active genes occurs at nuclear speckles and is modulated by chromatin environment. *J. Cell Biol.* **182**, 1083–1097 (2008).
116. Hall, L. L., Smith, K. P., Byron, M. & Lawrence, J. B. Molecular anatomy of a speckle. *Anat. Rec. A Discov. Mol. Cell. Evol. Biol.* **288**, 664–675 (2006).
117. Han, J., Xiong, J., Wang, D. & Fu, X. D. Pre-mRNA splicing: where and when in the nucleus. *Trends Cell Biol.* **21**, 336–343 (2011).
118. Hu, Y., Plutz, M. & Belmont, A. S. Hsp70 gene association with nuclear speckles is Hsp70 promoter specific. *J. Cell Biol.* **191**, 711–719 (2010).
119. Shopland, L. S., Johnson, C. V. & Lawrence, J. B. Evidence that all SC-35 domains contain mRNAs and that transcripts can be structurally constrained within these domains. *J. Struct. Biol.* **140**, 131–139 (2002).
120. Smith, K. P., Moen, P. T., Wydner, K. L., Coleman, J. R. & Lawrence, J. B. Processing of endogenous pre-mRNAs in association with SC-35 domains is gene specific. *J. Cell Biol.* **144**, 617–629 (1999).
121. Gordon, J. M., Phizicky, D. V. & Neugebauer, K. M. Nuclear mechanisms of gene expression control: pre-mRNA splicing as a life or death decision. *Curr. Opin. Genet. Dev.* **67**, 67–76 (2021).
122. Faber, G. P., Nadav-Eliyahu, S. & Shav-Tal, Y. Nuclear speckles—a driving force in gene expression. *J. Cell Sci.* **135**, jcs259594 (2022).
123. Misteli, T., Caceres, J. F. & Spector, D. L. The dynamics of a pre-mRNA splicing factor in living cells. *Nature* **387**, 523–527 (1997).
124. Kim, J., Han, K. Y., Khanna, N., Ha, T. & Belmont, A. S. Nuclear speckle fusion via long-range directional motion regulates speckle morphology after transcriptional inhibition. *J. Cell Sci.* **132**, jcs226563 (2019).
125. Monneron, A. & Bernhard, W. Fine structural organization of the interphase nucleus in some mammalian cells. *J. Ultrastruct. Res.* **27**, 266–288 (1969).
126. Lamond, A. I. & Spector, D. L. Nuclear speckles: a model for nuclear organelles. *Nat. Rev. Mol. Cell Biol.* **4**, 605–612 (2003).
127. Boronenkov, I. V., Loijens, J. C., Umeda, M. & Anderson, R. A. Phosphoinositide signaling pathways in nuclei are associated with nuclear speckles containing pre-mRNA processing factors. *Mol. Biol. Cell* **9**, 3547–3560 (1998).
128. Ishihama, Y., Tadakuma, H., Tani, T. & Funatsu, T. The dynamics of pre-mRNAs and poly(A)⁺ RNA at speckles in living cells revealed by iFRAP studies. *Exp. Cell Res.* **314**, 748–762 (2008).
129. Politz, J. C. et al. Rapid, diffusional shuttling of poly(A) RNA between nuclear speckles and the nucleoplasm. *Mol. Biol. Cell* **17**, 1239–1249 (2006).
130. Tripathi, V. et al. SRSF1 regulates the assembly of pre-mRNA processing factors in nuclear speckles. *Mol. Biol. Cell* **23**, 3694–3706 (2012).
131. Mintz, P. J., Patterson, S. D., Neuwald, A. F., Spahr, C. S. & Spector, D. L. Purification and biochemical characterization of interchromatin granule clusters. *EMBO J.* **18**, 4308–4320 (1999).
132. Dopie, J., Sweredoski, M. J., Moradian, A. & Belmont, A. S. Tyramide signal amplification mass spectrometry (TSA-MS) ratio identifies nuclear speckle proteins. *J. Cell Biol.* **219**, e201910207 (2020).
133. Saitoh, N. et al. Proteomic analysis of interchromatin granule clusters. *Mol. Biol. Cell* **15**, 3876–3890 (2004).
134. Erkelenz, S. et al. Position-dependent splicing activation and repression by SR and hnRNP proteins rely on common mechanisms. *RNA* **19**, 96–102 (2013).
135. Fairbrother, W. G., Yeh, R. F., Sharp, P. A. & Burge, C. B. Predictive identification of exonic splicing enhancers in human genes. *Science* **297**, 1007–1013 (2002).
136. Martinez-Contreras, R. et al. hnRNP proteins and splicing control. *Adv. Exp. Med. Biol.* **623**, 123–147 (2007).
137. Slišković, I., Eich, H. & Müller-McNicoll, M. Exploring the multifunctionality of SR proteins. *Biochem. Soc. Trans.* **50**, 187–198 (2022).
138. Liao, S. E. & Regev, O. Splicing at the phase-separated nuclear speckle interface: a model. *Nucleic Acids Res.* **49**, 636–645 (2021).
139. Rosenberg, A. B., Patwardhan, R. P., Shendure, J. & Seelig, G. Learning the sequence determinants of alternative splicing from millions of random sequences. *Cell* **163**, 698–711 (2015).
140. Paul, S. et al. RNA molecules display distinctive organization at nuclear speckles. *iScience* **27**, 109603 (2024).
141. Potashnikov, I. A., Derby, R. J. & Spector, D. L. Differential distribution of factors involved in pre-mRNA processing in the yeast cell nucleus. *Mol. Cell. Biol.* **10**, 3524–3534 (1990).
142. Girard, C. et al. Post-transcriptional spliceosomes are retained in nuclear speckles until splicing completion. *Nat. Commun.* **3**, 994 (2012).
143. Makeyev, E. V. & Huang, S. The perinuclear compartment: structure, function, and utility in anti-cancer drug development. *Nucleus* **15**, 2306777 (2024).
144. Yap, K. et al. A short tandem repeat-enriched RNA assembles a nuclear compartment to control alternative splicing and promote cell survival. *Mol. Cell* **72**, 525–540.e13 (2018).
145. Biamonti, G. & Vourc'h, C. Nuclear stress bodies. *Cold Spring Harb. Perspect. Biol.* **2**, a000695 (2010).
146. Ninomiya, K. et al. lncRNA-dependent nuclear stress bodies promote intron retention through SR protein phosphorylation. *EMBO J.* **39**, e102729 (2020).
147. Ninomiya, K. et al. m⁶A modification of HSATIII lncRNAs regulates temperature-dependent splicing. *EMBO J.* **40**, e107976 (2021).
148. Nakagawa, S., Yamazaki, T. & Hirose, T. Molecular dissection of nuclear paraspeckles: towards understanding the emerging world of the RNP milieu. *Open Biol.* **8**, 180150 (2018).
149. Reddy, D. et al. Paraspeckles interact with SWI/SNF subunit ARID1B to regulate transcription and splicing. *EMBO Rep.* **24**, e55345 (2023).
150. Shav-Tal, Y. et al. Dynamic sorting of nuclear components into distinct nucleolar caps during transcriptional inhibition. *Mol. Biol. Cell* **16**, 2395–2413 (2005).
151. Carrocci, T. J. & Neugebauer, K. M. Pre-mRNA splicing in the nuclear landscape. *Cold Spring Harb. Symp. Quant. Biol.* **84**, 11–20 (2019).
152. Ding, F. & Elowitz, M. B. Constitutive splicing and economies of scale in gene expression. *Nat. Struct. Mol. Biol.* **26**, 424–432 (2019).
153. Boutz, P. L., Bhutkar, A. & Sharp, P. A. Detained introns are a novel, widespread class of post-transcriptionally spliced introns. *Genes. Dev.* **29**, 63–80 (2015).
154. Fu, X. D. Exploiting the hidden treasure of detained introns. *Cancer Cell* **32**, 393–395 (2017).
155. Guo, Y. E. et al. Pol II phosphorylation regulates a switch between transcriptional and splicing condensates. *Nature* **572**, 543–548 (2019).
156. Guo, C. et al. ENL initiates multivalent phase separation of the super elongation complex (SEC) in controlling rapid transcriptional activation. *Sci. Adv.* **6**, eaay4858 (2020).
157. Yuan, B. et al. Wiskott-Aldrich syndrome protein forms nuclear condensates and regulates alternative splicing. *Nat. Commun.* **13**, 3646 (2022).
158. Nedelsky, N. B. & Taylor, J. P. Bridging biophysics and neurology: aberrant phase transitions in neurodegenerative disease. *Nat. Rev. Neurol.* **15**, 272–286 (2019).
159. Taylor, J. P., Brown, R. H. Jr & Cleveland, D. W. Decoding ALS: from genes to mechanism. *Nature* **539**, 197–206 (2016).
160. Conlon, E. G. & Manley, J. L. RNA-binding proteins in neurodegeneration: mechanisms in aggregate. *Genes. Dev.* **31**, 1509–1528 (2017).
161. Mathieu, C., Pappu, R. V. & Taylor, J. P. Beyond aggregation: pathological phase transitions in neurodegenerative disease. *Science* **370**, 56–60 (2020).
162. Brown, A. L. et al. TDP-43 loss and ALS-risk SNPs drive mis-splicing and depletion of UNC13A. *Nature* **603**, 131–137 (2022).
163. Amador-Ortiz, C. et al. TDP-43 immunoreactivity in hippocampal sclerosis and Alzheimer's disease. *Ann. Neurol.* **61**, 435–445 (2007).
164. Garcia Morato, J. et al. Sirtuin-1 sensitive lysine-136 acetylation drives phase separation and pathological aggregation of TDP-43. *Nat. Commun.* **13**, 1223 (2022).
165. Higashi, S. et al. Concurrence of TDP-43, tau and alpha-synuclein pathology in brains of Alzheimer's disease and dementia with Lewy bodies. *Brain Res.* **1184**, 284–294 (2007).
166. Nakashima-Yasuda, H. et al. Co-morbidity of TDP-43 proteinopathy in Lewy body related diseases. *Acta Neuropathol.* **114**, 221–229 (2007).
167. Neumann, M. et al. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* **314**, 130–133 (2006).
168. Schwab, C., Arai, T., Hasegawa, M., Yu, S. & McGeer, P. L. Colocalization of transactivation-responsive DNA-binding protein 43 and huntingtin in inclusions of Huntington disease. *J. Neuropathol. Exp. Neurol.* **67**, 1159–1165 (2008).
169. Guenther, E. L. et al. Atomic structures of TDP-43 LCD segments and insights into reversible or pathogenic aggregation. *Nat. Struct. Mol. Biol.* **25**, 463–471 (2018).
170. Jiang, Y. et al. Rett syndrome linked to defects in forming the MeCP2/Rbfox/LASR complex in mouse models. *Nat. Commun.* **12**, 5767 (2021).
171. Maunakea, A. K., Chepelev, I., Cui, K. & Zhao, K. Intragenic DNA methylation modulates alternative splicing by recruiting MeCP2 to promote exon recognition. *Cell Res.* **23**, 1256–1269 (2013).
172. Wong, J. J. et al. Intron retention is regulated by altered MeCP2-mediated splicing factor recruitment. *Nat. Commun.* **8**, 15134 (2017).
173. Liu, S. et al. USP42 drives nuclear speckle mRNA splicing via directing dynamic phase separation to promote tumorigenesis. *Cell Death Differ.* **28**, 2482–2498 (2021).
174. Peng, Q. et al. Epstein-Barr virus EBNA2 phase separation regulates cancer-associated alternative RNA splicing patterns. *Clin. Transl. Med.* **11**, e504 (2021).
175. Wang, Y. & Wang, Z. Targeting dysregulated splicing factor in cancer: lessons learned from RBM10 deficiency. *J. Mol. Cell Biol.* **15**, mjad063 (2023).
176. Damianov, A. et al. The splicing regulators RBM5 and RBM10 are subunits of the U2 snRNP engaged with intron branch sites on chromatin. *Mol. Cell* **18**, 1496–1511 (2024).
177. Zhao, J. et al. Functional analysis reveals that RBM10 mutations contribute to lung adenocarcinoma pathogenesis by deregulating splicing. *Sci. Rep.* **7**, 40488 (2017).
178. Bao, Y. et al. RBM10 loss promotes EGFR-driven lung cancer and confers sensitivity to spliceosome inhibition. *Cancer Res.* **83**, 1490–1502 (2023).
179. Bechara, E. G., Sebestyén, E., Bernardis, I., Eyras, E. & Valcárcel, J. RBM5, 6, and 10 differentially regulate NUMB alternative splicing to control cancer cell proliferation. *Mol. Cell* **52**, 720–733 (2013).
180. Inoue, A. RBM10: structure, functions, and associated diseases. *Gene* **783**, 145463 (2021).
181. Hernández, J. et al. Tumor suppressor properties of the splicing regulatory factor RBM10. *RNA Biol.* **13**, 466–472 (2016).
182. Banani, S. F. et al. Genetic variation associated with condensate dysregulation in disease. *Dev. Cell* **57**, 1776–1788.e8 (2022).
183. Loisel, J. J. & Sutherland, L. C. RBM10: harmful or helpful—many factors to consider. *J. Cell. Biochem.* **119**, 3809–3818 (2018).
184. Bertero, A. et al. Dynamics of genome reorganization during human cardiogenesis reveal an RBM20-dependent splicing factory. *Nat. Commun.* **10**, 1538 (2019).

185. Koelemen, J., Gotthardt, M., Steinmetz, L. M. & Meder, B. RBM20-related cardiomyopathy: current understanding and future options. *J. Clin. Med.* **10**, 4101 (2021).
186. Schneider, J. W. et al. Dysregulated ribonucleoprotein granules promote cardiomyopathy in RBM20 gene-edited pigs. *Nat. Med.* **26**, 1788–1800 (2020).
187. Wang, C. et al. RBM20^{S639G} mutation is a high genetic risk factor for premature death through RNA–protein condensates. *J. Mol. Cell. Cardiol.* **165**, 115–129 (2022).
188. Regan-Fendt, K. E. & Izumi, K. Nuclear speckleopathies: developmental disorders caused by variants in genes encoding nuclear speckle proteins. *Hum. Genet.* **143**, 529–544 (2023).
189. Kim, J. H. et al. De novo mutations in SON disrupt RNA splicing of genes essential for brain development and metabolism, causing an intellectual-disability syndrome. *Am. J. Hum. Genet.* **99**, 711–719 (2016).
190. Fiordaliso, S. K. et al. Missense mutations in NKAP cause a disorder of transcriptional regulation characterized by marfanoid habitus and cognitive impairment. *Am. J. Hum. Genet.* **105**, 987–995 (2019).
191. Burgute, B. D. et al. NKAP is a novel RS-related protein that interacts with RNA and RNA binding proteins. *Nucleic Acids Res.* **42**, 3177–3193 (2014).
192. Johnston, J. J. et al. Massively parallel sequencing of exons on the X chromosome identifies *RBM10* as the gene that causes a syndromic form of cleft palate. *Am. J. Hum. Genet.* **86**, 743–748 (2010).
193. Imagawa, E. et al. Functional insight into a neurodevelopmental disorder caused by missense variants in an RNA-binding protein, RBM10. *J. Hum. Genet.* **68**, 643–648 (2023).
194. Li, W. & Jiang, H. Analysis of phase-separated biomolecular condensates in cancer. *Methods Mol. Biol.* **2660**, 345–356 (2023).
195. Banerjee, P. R. et al. Dissecting the biophysics and biology of intrinsically disordered proteins. *Trends Biochem. Sci.* **49**, 101–104 (2023).
196. Miyagi, T. et al. The patterning and proportion of charged residues in the arginine-rich mixed-charge domain determine the membrane-less organelle targeted by the protein. *Int. J. Mol. Sci.* **23**, 7658 (2022).
197. Bracha, D., Walls, M. T. & Brangwynne, C. P. Probing and engineering liquid-phase organelles. *Nat. Biotechnol.* **37**, 1435–1445 (2019).
198. Nakamura, H., DeRose, R. & Inoue, T. Harnessing biomolecular condensates in living cells. *J. Biochem.* **166**, 13–27 (2019).
199. Riback, J. A. et al. Composition-dependent thermodynamics of intracellular phase separation. *Nature* **581**, 209–214 (2020).
200. Ang, Y. S. et al. Disease model of GATA4 mutation reveals transcription factor cooperativity in human cardiogenesis. *Cell* **167**, 1734–1749.e22 (2016).
201. Conti, B. A. & Oppikofer, M. Biomolecular condensates: new opportunities for drug discovery and RNA therapeutics. *Trends Pharmacol. Sci.* **43**, 820–837 (2022).
202. Mitrea, D. M., Mittasch, M., Gomes, B. F., Klein, I. A. & Murcko, M. A. Modulating biomolecular condensates: a novel approach to drug discovery. *Nat. Rev. Drug Discov.* **21**, 841–862 (2022).
203. Patel, A. et al. Principles and functions of condensate modifying drugs. *Front. Mol. Biosci.* **9**, 1007744 (2022).
204. Enders, L. et al. Pharmacological perturbation of the phase-separating protein SMNDC1. *Nat. Commun.* **14**, 4504 (2023).
205. Vernon, R. M. et al. Pi–pi contacts are an overlooked protein feature relevant to phase separation. *eLife* **7**, e31486 (2018).
206. Fuxreiter, M. Fuzzy protein theory for disordered proteins. *Biochem. Soc. Trans.* **48**, 2557–2564 (2020).
207. Li, P. et al. Phase transitions in the assembly of multivalent signalling proteins. *Nature* **483**, 336–340 (2012).
208. Schuster, B. S. et al. Biomolecular condensates: sequence determinants of phase separation, microstructural organization, enzymatic activity, and material properties. *J. Phys. Chem. B* **125**, 3441–3451 (2021).
209. Li, D. & Liu, C. Spatiotemporal dynamic regulation of membraneless organelles by chaperone networks. *Trends Cell Biol.* **32**, 1–3 (2022).
210. Banani, S. F. et al. Compositional control of phase-separated cellular bodies. *Cell* **166**, 651–663 (2016).
211. Ditlev, J. A., Case, L. B. & Rosen, M. K. Who's in and who's out—compositional control of biomolecular condensates. *J. Mol. Biol.* **430**, 4666–4684 (2018).
212. Sanders, D. W. et al. Competing protein–RNA interaction networks control multiphase intracellular organization. *Cell* **181**, 306–324.e28 (2020).
213. Lyons, H. et al. Functional partitioning of transcriptional regulators by patterned charge blocks. *Cell* **186**, 327–345.e28 (2023).
214. Mir, M., Bickmore, W., Furlong, E. E. M. & Narlikar, G. Chromatin topology, condensates and gene regulation: shifting paradigms or just a phase? *Development* **146**, dev182766 (2019).

Acknowledgements

The work by H.J. on AKAP95 condensates was supported by the Department of Defense Breast Cancer Research Program (Breakthrough Award BC190343). J.G. is supported by The University of North Carolina at Chapel Hill (start-up funds and Jefferson Pilot Award), the National Institutes of Health (NIH) (R01-GM130866) and the National Science Foundation (NSF) (CAREER award 2239056).

Author contributions

The authors contributed equally to all aspects of the article.

Competing interests

The authors declare no competing interests.

Additional information

Peer review information *Nature Reviews Molecular Cell Biology* thanks the anonymous reviewer(s) for their contribution to the peer review of this work.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.

© Springer Nature Limited 2024