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Review

Biomolecular Condensates and Gene Activation in Development and Disease

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SUMMARY

Activating the right gene at the right time and place is essential for development. Emerging evidence suggests that this process is regulated by the mesoscale compartmentalization of the gene-control machinery, RNA polymerase II and its cofactors, within biomolecular condensates. Coupling gene activity to the reversible and dynamic process of condensate formation is proposed to enable the robust and precise changes in gene-regulatory programs during signaling and development. The macromolecular features that enable condensates and the regulatory pathways that control them are dysregulated in disease, highlighting their importance for normal physiology. In this review, we will discuss the role of condensates in gene activation; the multivalent features of protein, RNA, and DNA that enable reversible condensate formation; and how these processes are utilized in normal and disease biology. Understanding the regulation of condensates promises to provide novel insights into how organization of the gene-control machinery regulates development and disease.

INTRODUCTION

The process of development is established by gene-regulatory networks, which determine body plan by controlling when and where specific genes are activated or repressed (Davidson, 2010). Through regulated readout of specific gene programs, a single genome can give rise to the large diversity of cellular phenotypes and functions found in multicellular organisms. Many processes play a role in gene regulation, and this review will focus on gene activation by recruitment of RNA Polymerase II (RNA Pol II) and its cofactors, which we will refer to collectively as the gene-control machinery. Hundreds of unique proteins and RNAs must work together at the correct genetic loci to ensure robust gene activation and must then be rapidly and precisely redistributed to activate new genes (Cramer, 2019; Roeder, 2019). In this context, activating new genes or gene programs during development requires the coordination of RNA Pol II and its hundreds of regulatory cofactors that must find one another at the right gene at the right time. This intricate choreography is performed within the highly crowded environment of the nucleus (Hancock and Jeon, 2014). Given the large number of independent events required, local retention of the gene-control machinery at specific genomic loci would mitigate stochasticity and enable or accelerate the process of gene activation (Hancock and Jeon, 2014; Lemon and Tjian, 2000; Matsuda et al., 2014). Indeed, components of the gene-control machinery are found in dynamic clusters where many copies of individual factors are at high local concentrations (Boija et al., 2018; Cho et al., 2016, 2018; Chong et al., 2018; Cissé et al., 2013; Iborra et al., 1996; Izeddin et al., 2014; Jackson et al., 1993; Liu et al., 2014a; Mir et al., 2017, 2018; Papantonis and Cook, 2013; Sabari et al., 2018; Tantale et al., 2016; Tsai et al., 2017). The exact nature of these clusters is not yet fully resolved, but it is generally agreed that constituents are concentrated because they engage in weak multivalent interactions with a dynamic nuclear substructure (Woringer and Darzacq, 2018). Emerging evidence supports a model where the gene-control machinery is locally concentrated by compartmentalization within dynamic biomolecular condensates.

Multivalent interactions among polymers produce networks that can yield phase separation once the attractive force of the interacting polymers becomes stronger than their interaction with the solvent (Flory, 1942; Semenov and Rubinstein, 1998). Phase transitions occur across sharp thresholds and are often reversible. These concepts were proposed to underlie cellular organization over a century ago (Wilson, 1899) and have recently been reinvigorated and expanded as a framework to study the numerous membraneless organelles, intracellular bodies, and other localized concentrations of functionally related macromolecules collectively referred to as biomolecular condensates (Banani et al., 2017; Brangwynne et al., 2009; Choi et al., 2020; Hyman et al., 2014; Shin and Brangwynne, 2017). The term biomolecular condensate is used to describe a concentration of biomolecules irrespective of the specific physical and chemical mechanism leading to its formation (Banani et al., 2017). While the phase separation of a single polymer in solution is well understood and has been shown to underlie the formation of macromolecular droplets *in vitro* and condensates in cells, the complex, heterogeneous, and nonequilibrium environment of the cell should be expected to cause deviations in the

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predictions of these formalisms (Choi et al., 2019; McSwiggen et al., 2019; Riback et al., 2020). Nevertheless, when applied to biological systems, these concepts have led to numerous advances that have helped in understanding cellular organization in various fields of biology (see other reviews in this issue).

Many nuclear processes are compartmentalized within condensates (Sabari et al., 2020; Strom and Brangwynne, 2019; Zhu and Brangwynne, 2015). Here, we focus on an emerging role for condensates in regulating the initiation and activation of gene transcription, how this process is used during development to switch between gene expression programs, and how is it dysregulated in disease. Formation of on-demand and reversible local concentrations of functionally related proteins at specific enhancers and promoters is an attractive model for how cells respond to signals and how cell-state transitions are accomplished. Coupling the activation of genes to the formation of condensates would allow for precise and nonlinear changes in gene-expression programs in response to signaling and during development. We will first discuss how condensates influence gene activation followed by a review of the multivalent features of the gene-control machinery and specific genomic loci, which together enable formation of condensates. We will then focus on how these multivalent interactions are regulated to reposition condensates to new targets during development and how this process is dysregulated in disease. We will close by highlighting a few of the many open areas for future work.

COMPARTMENTALIZED GENE ACTIVITY

Gene activation is a multicomponent and multistep biochemical process that must occur at specific genomic loci. This is analogous to the multicomponent and multistep biochemical assembly of signaling clusters at the plasma membrane, where diffusion away from the site of signaling is mitigated by local retention of reactants within a condensate, leading to increased specific activity of the pathway (Case et al., 2019b; Huang et al., 2019). Similarly, components of the gene-control machinery are locally retained at sites of gene activity (Cho et al., 2016; Li et al., 2019; Mir et al., 2018). These high local concentrations have functional outcomes for gene expression, as the number of RNA Pol II molecules locally concentrated is positively correlated with the number of transcribed RNA molecules (Cho et al., 2016) and preventing clustering of the gene-control machinery leads to reduced gene expression (Li et al., 2019). These high local concentrations can be attributed to RNA Pol II and its cofactors engaging in dynamic multivalent interactions with themselves and other components of condensates formed at specific genomic loci. Condensates composed of coactivator protein formed in cell-free nuclear extracts partition RNA Pol II and other cofactors (Sabari et al., 2018). The degree to which TAF15 hydrogels partition the low-complexity C-terminal domain (CTD) of RNA Pol II *in vitro* positively correlates with the ability of that protein to activate transcription of a reporter gene in cells (Kwon et al., 2013). RNA Pol II condensate formation *in vitro* and clustering in cell nuclei are dependent on the length of the CTD (Boehning et al., 2018), indicating that the enzyme's ability to cluster relies on the dynamic multivalent interactions modeled *in vitro*. In cells, synthetic condensates formed by light-induced clustering of defined protein domains compartmentalize RNA Pol II and locally enhance RNA synthesis (Wei et al., 2019). Intriguingly, while all protein domains tested formed light-induced condensates, only some were capable of enhancing transcription (Wei et al., 2019), highlighting the importance of condensate composition on function. These and other studies provide evidence that compartmentalization of the gene-control machinery within condensates promotes transcription of associated genes.

Based on these and other studies, a picture of how condensates can influence gene activation is taking shape, whereby macromolecules capable of engaging in multivalent interactions are clustered together at specific genomic loci where they concentrate RNA Pol II and its many cofactors (Figure 1). This model predicts that the extent and frequency with which a locus can concentrate RNA Pol II and cofactor molecules will influence gene-expression output. This regulation by organization does not supersede the regulatory mechanisms described for RNA Pol II recruitment and activity (Cramer, 2019; Roeder, 2019) but is a means to regulate whether involved factors do or do not find one another in the crowded environment of the nucleus. Additionally, the requirement for clustering of many factors together to ensure high transcriptional activity has the potential to reduce noise at sites of the genome less capable of promoting condensate formation, a feature that has also been proposed for signaling clusters (Case et al., 2019a). This ability to robustly activate specific genes while maintaining other sites unexpressed is particularly desirable during cell-state transitions and in maintaining cell identity.

This additional layer of regulation provided by condensates is mediated by dynamic multivalent interactions inherent to the gene-control machinery. As we will discuss in the next section, at least two tiers of multivalent interactions dictate where on the genome a condensate will form and what its composition will be, the interactions which include specific regions of chromatin and interactions among the locally concentrated components. The spatial organization of the gene-control machinery by highly dynamic and multivalent interactions has long been noted, but largely understudied by conventional paradigms of gene activation. In the subsequent sections, we will discuss these dynamic multivalent interactions are how they are central to condensate formation, composition, and function.

MULTIVALENT INTERACTIONS AMONG TRANSCRIPTIONAL MACHINERY AND THE GENOME

The combined effort of multivalent interactions among components of the gene-control machinery and their multivalent interactions with the chromatin scaffold underlie the formation of transcriptional condensates. DNA-binding transcription factors (TFs), transcriptional coactivators, regulatory enzymes, chromatin-associated cofactors, and RNA Pol II are capable of engaging in multivalent interactions with one another and with specific regions of the genome (Boehning et al., 2018; Boija et al., 2018; Kwon et al., 2013; Lu et al., 2018; Sabari et al., 2018; Shrinivas et al., 2019). Chromatin, the physiologic form of the genome, has many regulated inter- and intramolecular interactions leading to a variety of higher-order forms (Barbieri et al., 2012; Gibson et al., 2019; Lanctôt et al., 2007; Misteli, 2007; Sanulli et al., 2019; Woodcock and Ghosh, 2010). This

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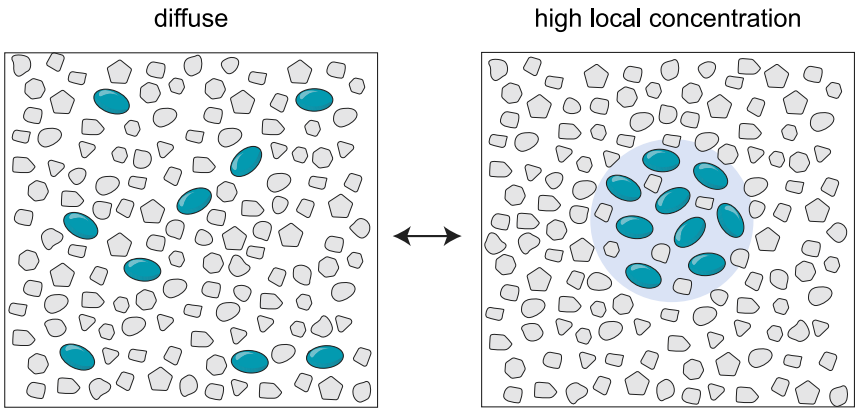

Figure 1. Condensates Compartmentalize RNA Pol II and Its Cofactors

RNA Pol II and its cofactors cluster dynamically in the cell. Their constrained diffusion and increased dwell time at specific genomic loci implicate weak multivalent interactions in their clustering. In this figure, a cartoon representation of RNA Pol II (blue oval) homogeneously dispersed among other nuclear proteins (gray shapes) (left) and locally concentrated by the formation of a condensate (right). This process is dynamic and reversible. This review covers the molecular features of the gene-control machinery that enable this type of dynamic compartmentalization, how they are utilized in development, and how they are co-opted in disease.

sets up at least two tiers of relevant interactions: those involving the chromatin scaffold and those involving components that are locally concentrated (Figures 2A and 2B). Together, these two layers can coordinate the establishment of condensates at specific genomic loci and determine the composition of concentrated components (Figure 2C). Modulating the interactions at either tier can change when and where condensates will form and with which components.

Multivalency in the Chromatin Scaffold

The chromatin fiber has many layers of regulated interactions that can reversibly tune the type and number of interactions at specific regions (Figure 2A). The compaction of the chromatin fiber into various chromatin-rich condensates (Gibson et al., 2019; Larson et al., 2017; Plys et al., 2019; Sanulli et al., 2019; Strom et al., 2017) can occlude binding of activating factors. Chromatin-rich condensates can be dissolved by the regulation of nucleosome spacing and reversible acetylation of histone proteins (Gibson et al., 2019). The accessibility of DNA regulatory elements for TF binding is influenced by the action of chromatin remodelers recruited to specific enhancers and promoters (Clapier and Cairns, 2009). Acetylation negates the positive charge on the epsilon-amine of lysine and creates a binding site for acetyl-reader proteins. Many lysines on histones and nonhistone substrates are dynamically modified by what has been described as an "acetyl spray" (Weinert et al., 2018), leading to a dramatic change in electrostatic interactions and many new binding sites for acetyl-reader proteins. In addition to acetylation, chromatin is reversibly modified by an array of covalent modifications (Huang et al., 2014) that modulate chromatin accessibility and are bound by their own classes of reader proteins (Musselman et al., 2012; Taverna et al., 2007). Often noncoding RNAs are transcribed from enhancers that are capable of interacting with and influencing the activity of multiple components of the gene-control machinery (Sartorelli and Lauberth, 2020). The prevalence of chromatin-tethered RNAs (Sun et al., 2018; Werner and Ruthenburg, 2015) and RNA-binding proteins (Xiao et al., 2019) at active regulatory elements suggests an expanded role for RNA in assembling the transcriptional machinery. Each of these layers of regulation can modulate the "stickiness" of regions of chromatin for specific sets of multivalent interactors, thereby regulating where on the genome condensates form and with which components (Figure 2).

The combined interactions present at enhancers or promoters will likely dictate their ability to support condensate formation. Focusing explicitly on TF-binding sites, a threshold value of interactions was found to promote condensate formation and expression of a reporter gene in cells (Shrinivas et al., 2019). DNA with many TF-binding sites locally lowered the saturation concentration of TF and coactivator condensates. At low physiologic protein concentrations, condensate formation became dependent on the presence of DNA with the correct number and spacing of TF-binding sites (Shrinivas et al., 2019). These findings can be abstracted to any of the regulated interactions covered above, where a condensate can only form in the presence of the multiple points of interaction afforded by chromatin. Switching this "stickiness" on and off at specific sets of promoters and enhancers or moving it to other promoters or enhancers would change where condensates form on the genome, resulting in the reallocation of transcriptional resources.

Multivalency among Concentrated Components

Components of the gene-control machinery, signaling factors, and cell-type specifying factors can engage in multivalent interactions with one another. Much of what makes up the conventional paradigm of gene-regulation focuses on structured interactions that produce complexes stable in dilute cell-free lysates. Yet early protein-sequencing efforts indicated that stable interactions with fixed stoichiometries were insufficient to describe all aspects of gene regulation. The low complexity and intrinsic disorder of the CTD of RNA Pol II and the activation domains of TFs prompted Paul Sigler to write an essay calling for a reconceptualization of how gene activity would be organized (Sigler, 1988). Low complexity and intrinsically disordered regions (IDRs) have been recognized as a common feature of components involved in transcription (Fuxreiter et al., 2008; Liu et al., 2006; Xie et al., 2007), but there are many additional sources of multivalency in the gene-control machinery (Figure 2B). With the plethora of annotated protein-protein/RNA/DNA interaction domains (Finn et al., 2016), the prevalence of modification-regulated "reader" domains (Musselman et al., 2012; Ruthenburg et al., 2007), and the capacity of many factors to undergo reversible oligomerization (Gallego et al., 2020; Kwok et al., 2006; Minucci et al., 2000; Sun et al., 2013), most components of the gene-control machinery can engage in higher-order networks of multivalent interactions.

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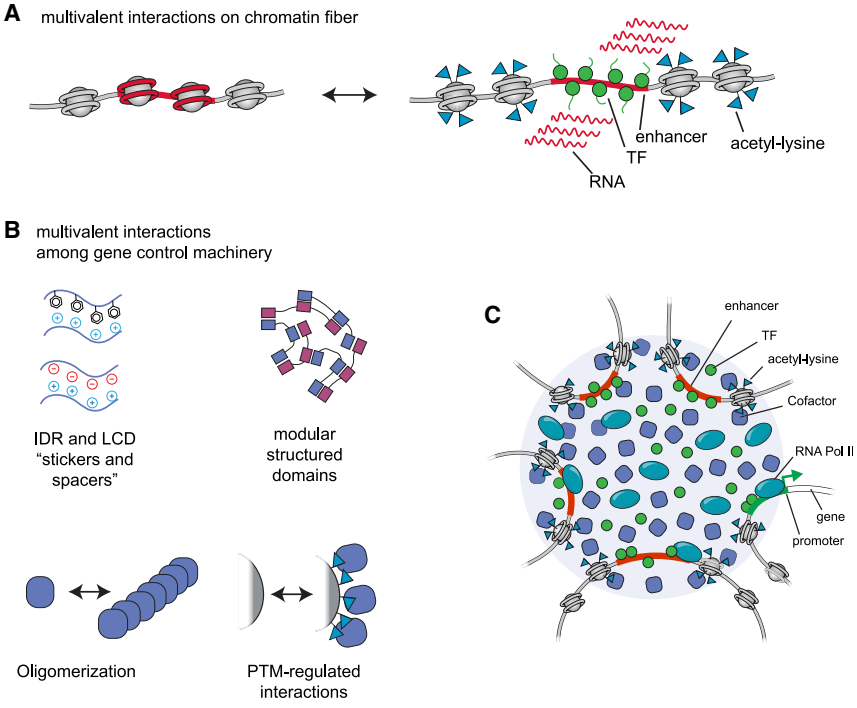

Figure 2. Two Tiers of Multivalent Interaction

Condensate formation for gene activation relies on at least two tiers of multivalent interactions. (A) Depiction of chromatin at a regulatory element before and after activation. Multiple points of multivalent interaction are dynamically regulated, including lysine acetylation (blue flags), accessibility of regulatory DNA (red) for DNA-binding TFs (green), and local synthesis of RNA (red squiggles). (B) The gene-control machinery has many sources of dynamic multivalency including IDRs, modular structured domains, homo- or hetero-oligomers, and PTM-mediated binding sites. (C) Combined, these two tiers of multivalent interactions lead to the formation of condensates localized to specific genomic regions. A cluster of three enhancers and their associated promoter concentrate RNA Pol II (blue ovals) and many cofactors (blue rounded squares). The TF and cofactors are depicted here as generic and are each meant to represent the respective class, each composed of hundreds of different members.

The combination of these two tiers of interactions promotes the localized formation of condensates (Figure 2C). If components are above their saturation concentration, they will form condensates spontaneously throughout the nucleoplasm and may eventually coalesce at the correct genomic locus, but if the associating factors are tuned low enough that the chromatin fiber becomes an essential component of the condensate, then condensates will form only at the correct locus when the right complement of factors is available. The formation of the nucleolus and other DNA-localized condensates are proposed to form by this type of seeded nucleation event (Strom and Brangwynne, 2019). This is consistent with recent models of localized induction regulating the formation and size of condensate (Söding et al., 2020) and diffusive capture (Bracha et al., 2018) used to explain the formation of condensates nucleated by engineered seeds of varying valences. These types of localized induction of condensates provide a means to rapidly, robustly, and precisely shift transcriptional resources to new locations in the genome and thereby activate new gene programs.

MODULATING MULTIVALENT INTERACTIONS DURING DEVELOPMENT AND IN RESPONSE TO SIGNALING

The type of reallocation of resources described above is accomplished during development and in response to signaling by modulating the multivalent interactions among transcriptional components and specific regions of chromatin (Figure 3). This is accomplished by carefully tuned regulatory elements, control of protein abundance, signal-induced nuclear localization, signal-induced posttranslational modifications (PTMs), or ligand-induced interactions. This modulation of multivalent interactions can create distinct transcriptional condensates within the same cell, in cells pre- and poststimulus, and across different cell types.

Developmentally regulated genes must be precisely activated to execute proper body-plan formation. This precision is accomplished in part by the accurate interpretation of information encoded in developmentally regulated enhancer elements. Curiously, some of these enhancers have evolved to be suboptimal for TF binding. Improving the affinity for TF binding within these enhancers increases the expression of the enhancer's target gene at the expense of accurate spatiotemporal regulation, leading to developmental catastrophe. In the developing *Drosophila* embryo, activation of similarly suboptimal enhancers for the *svb* gene is accomplished by the formation of a distinct microenvironment at the locus, consistent with the condensate model (Tsai et al., 2017). The TFs Zelda and Bicoid similarly create multifactor local concentrations that compartmentalize cofactors at low-affinity enhancers (Mir et al., 2017, 2018). This type of all-or-none response for sets of key developmental genes is essential for proper development, and the tight thresholds afforded by condensate formation and dissolution may underlie many of these events.

RNA is a major constituent and regulator of many cellular condensates (Roden and Gladfelter, 2020). In the nucleus, expression of RNA from a locus can itself nucleate the formation of a condensate. Tethering specific RNA molecules to chromatin leads to the formation of Cajal bodies, histone locus bodies (HLBs), and others (Shevtsov and Dundr, 2011), suggesting that chromatin-tethered nascent RNAs could have the same effect. A striking example of induced RNA synthesis during development occurs during zygotic genome activation, and it is concurrent with the biogenesis of many nuclear condensates (Arias Escayola and Neugebauer, 2018). In zebrafish embryos, the formation of HLBs and nucleoli coincides with the transcription of histone genes and rRNA, respectively. Once formed, inhibition of transcription leads to the dissolution of the HLB, and its components become incorrectly localized to Cajal bodies. This misallocation of resources is reversed upon the resumption of transcription (Heyn et al., 2016). These results suggest that

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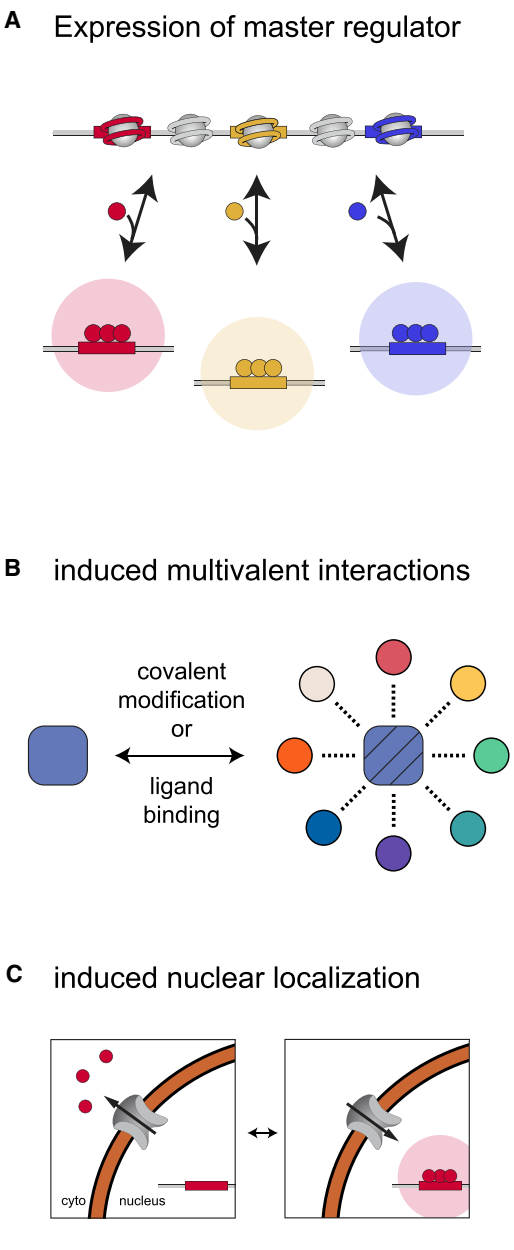

Figure 3. Regulation of Multivalent Interactions Due to Signaling
Both tiers of multivalent interactions are modulated during development in response to signaling.
(A) The expression of master regulatory factors leads to the activation of specific genomic loci. (B) Proteins can be reversibly induced to engage in multivalent interactions. Ligand binding for hormone receptors causes conformational changes that reveal their transactivation domains IDR-IDR interactions and interactions among modular structures domains are often regulated by PTMs. Reversible oligomerization can change the multivalency of interactions. Many proteins are reversibly modified at multiple sites with each site being a binding site for a specific class of reader proteins. (C) Control over the nuclear concentration of key regulators can lead to a rapid induction or dissolution of condensates.

both the formation and compositional integrity of nuclear condensates can be regulated by active processes such as RNA transcription and is a clear example of rapid reorganization of cellular resources that can occur during development.

Many cell types are defined by the expression of cell-type-specific transcriptional regulators. The overexpression of cell-type-specifying DNA-binding TFs can reprogram cell states and rewire gene-expression programs (Davis et al., 1987; Graf and Enver, 2009; Takahashi and Yamanaka, 2006) (Figure 3A). In addition to DNA-binding TFs, there are cell-type-specifying coactivators (Spiegelman and Heinrich, 2004), specific cofactor complex subunits (D'Alessio et al., 2009; Liu et al., 2011; Yoo et al., 2009), and enhancer-derived RNAs, which precede the expression of signal-induced gene expression (Arner et al., 2015). These factors can be considered initiators of "sticky" regions at new locations in the genome operating at both tiers of multivalent interactions.

Nuclear hormone receptors are involved in many aspects of development and physiology. Upon hormone binding, the combined effect of nuclear translocation and conformational change-induced interactions leads to the rapid activation of new gene targets (Figures 3B and 3C). Both the estrogen receptor (ER) and glucocorticoid receptor (GR) rapidly activate large clusters of enhancers where many additional TFs bind and cooperatively recruit coactivator complexes to activate target genes (Bojcsuk et al., 2017; Vockley et al., 2016). Recently, ER and GR have been shown to form nuclear condensates upon the respective hormone treatments at sites of gene activation (Nair et al., 2019; Stortz et al., 2020), suggesting that condensate formation may underly some features of their action. For ER, eRNA production and long-range regulatory element interactions were implicated in condensate formation (Nair et al., 2019). As condensates should self-assemble rapidly in response to small changes that cross a threshold, they may play a role in other rapid signal-induced chromatin occupancy and gene program changes.

Several signaling pathways engage with transcriptional condensates or create their own upon stimulation. Activation of the canonical wnt pathway leads to the nuclear localization of β-catenin, where it regulates gene expression (Figure 3C). β-catenin does not have a DNA-binding domain and engages with TF/coactivator condensates established at super-enhancers (Zamudio et al., 2019). Interestingly, the IDR portions of β-catenin are sufficient for correct genomic localization (Zamudio et al., 2019). This suggests that the amino-acid composition and any transient secondary structures that it forms specifically target the protein to the correct genomic locus, as has been observed for several other factors (Brodsky et al., 2020; Liu et al., 2014b; Lu et al., 2019). Activation of the Hippo pathway leads to the nuclear localization of YAP, a transcriptional coactivator. Immediately upon activation of the Hippo pathway by osmotic shock, the YAP protein forms condensates in the cytoplasm and nucleus (Cai et al., 2019). The nuclear YAP condensates rapidly create regions of highly accessible chromatin that partitions RNA Pol II over time, promoting RNA synthesis (Cai et al., 2019). These studies highlight the importance of composition to condensate function and different ways this can be modified due to signal activation.

Many aspects of the cell cycle are regulated by cyclin-dependent kinase (CDK)-mediated signaling. CDKs regulate the formation of the HLB, a condensate responsible for the expression of replication-dependent histone genes during early S phase. In all dividing eukaryotic cells, enough histone proteins must be made to package the newly synthesized genome. In metazoans, the

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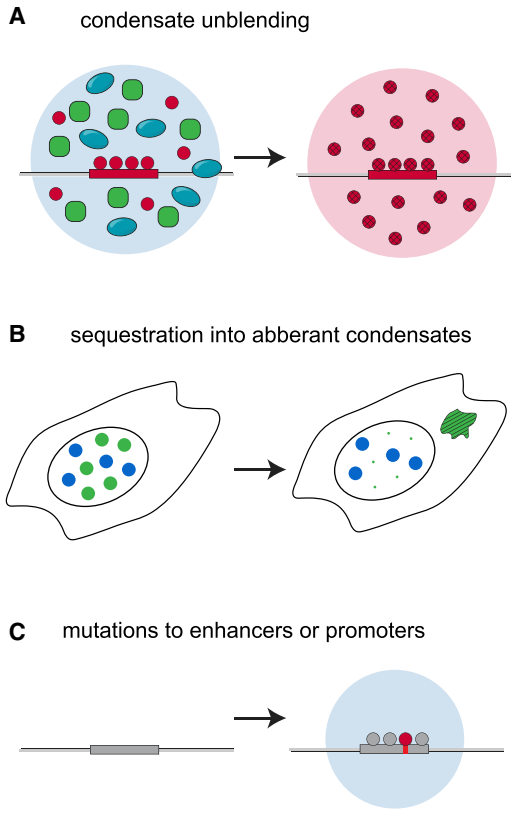

Figure 4. Dysregulation of Multivalent Interactions in Disease
The same multivalent interactions which are regulated for normal development and response to signals are dysregulated in disease. (A) Repeat expansion in the IDRs of DNA-binding TFs lead to increased homotypic interactions and reduced interactions with transcriptional cofactors. This leads to a condensate "unblending" and impairs gene activation, causing developmental defects. (B) Repeat expansion of glutamine in the Huntington's protein leads to intracellular inclusions where polyglutamine transcriptional components are sequestered away from the nucleus leading to reduced transcriptional activity. (C) Small insertions in regulatory DNA sequences introduce single binding sites for TFs that activate transcription of oncogenes leading to cellular transformation.

replication-dependent histone genes have been duplicated many times into arrays, and their coordinated expression is regulated by a nuclear condensate called the HLB (Duronio and Marzluff, 2017; Ma et al., 2000; Nizami et al., 2010; Zhao et al., 2000). The formation of the HLB is regulated by the nuclear localization and CDK-mediated phosphorylation of a key scaffold, NPAT/Mxc (Hur et al., 2020). Additionally, the number of histone genes present at the genomic locus is important for regulating the size and functionality of the condensate (Hur et al., 2020). The HLB highlights the key features of condensate regulation we have discussed: nuclear localization, multiple points of interaction on chromatin, and induced multivalent interactions among constituents. The HLB is a key example of how condensate formation can be coupled to gene activation to yield precise and robust regulation of transcription.

The combination of specific regulatory-element architecture, the abundance of interacting factors by either expression or nuclear localization, and ligand- or PTM-induced interactions (Figure 3) lead to the regulated formation of condensates during development and in response to signals.

IMPLICATIONS FOR GENE PROGRAM CHANGES DURING DISEASE

The same multivalent interactions that provide the regulation and specificity for activating specific sets of genes and developmental gene programs can be co-opted and dysregulated in disease. Disrupting, enhancing, or creating new multivalent interactions involving the gene-control machinery has been implicated in neurodegeneration, developmental disorders, and cancer (Figure 4).

Repeat expansions leading to aberrant condensates are found in several neurodegenerative diseases (Nedelsky and Taylor, 2019). Insoluble cellular inclusions or soluble oligomers of these mutant proteins either sequester or disrupt normal interaction networks of many proteins, including components of the gene-control machinery. Glutamine repeat expansions in the gene responsible for Huntington's disease (Htt) lead to intracellular inclusions that can sequester several components of the transcription machinery that contain large polyglutamine stretches, including CREB binding protein (CBP), a prominent coactivator (Figure 4B) (Nucifora et al., 2001). This sequestration leads to the depletion of CBP from sites in the nucleus and impaired transcription (Figure 4B) (Nucifora et al., 2001). In addition to sequestration within the insoluble inclusions, soluble oligomers of Htt mutant protein disrupts normal interactions among components of the gene-control machinery (Dunah et al., 2002; Kim et al., 2016). Similarly, expansions of dipeptide repeats within *C9orf72* found in amyotrophic lateral sclerosis (ALS) patients lead to disruption of interactions underlying cellular condensates, including several nuclear condensates (Boeynaems et al., 2017; Kwon et al., 2014; Lin et al., 2016; White et al., 2019; Zhang et al., 2019). In addition to repeat expansions yielding toxic proteins, the mRNA harboring the repeat expansion can also undergo aggregation to yield toxic RNA foci which can sequester RNA-binding proteins and disrupt other condensates (Jain and Vale, 2017). These findings highlight how condensate composition can be altered by transcriptional components' being mislocalized by aberrant condensates or by having normal multivalent interaction networks be disrupted by mutant protein or RNA.

Beyond neurodegeneration, repeat expansions are found in several developmental disorders. An alanine repeat expansion within the IDR of the TF HOXD13 causes synpolydactyly, a hereditary limb-malformation disorder (Muragaki et al., 1996). This alanine expansion promotes the homotypic phase separation of HOXD13 but prevents its ability to engage with cofactors. This inability to engage multiple cofactors, a proposed condensate "unblending," (Figure 4A) alters the transcriptional program in specific cell types during development coincident with limb malformation (Basu et al., 2020). These findings highlight that it is not condensate formation per se that is functional, but rather the capacity to compartmentalize specific cofactors at genomic loci within the correct developmental time window. Enhancing the capacity of TFs to self-associate promotes their condensate formation, but at the cost of intermolecular interactions with cofactors necessary for transcription, ultimately leading to developmental defects.

Small mutations within enhancers and promoters creating single TF-binding sites lead to the activation of oncogenes and

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cancer development (Figure 4C). A mutation within the promoter of the telomerase gene (*TERT*) introduces a single binding site for GABP resulting in the aberrant activation of *TERT* in multiple cancers (Bell et al., 2015). Similarly, an insertion within the enhancer for *TAL1* and oncogenic TF, introduces a single binding site for MYB nucleating a superenhancer that drives the expression of *TAL1* in T-ALL (Mansour et al., 2014). These small changes in TF-binding site number leading to dramatic changes in expression are in agreement with threshold events observed for phase separation of TF and coactivator pairs seeded by specific DNA sequences (Shrinivas et al., 2019). The degree to which population-wide polymorphisms in regulatory elements (Maurano et al., 2015) lead to similar changes warrants further investigation.

Enhancing or changing the multivalent interactions of TFs and cofactors have been implicated in cancer. Recurrent insertions within the YEATS domain of ENL, a chromatin reader factor, has been found in Wilms' tumor, a pediatric kidney cancer. These small insertions lead to enhanced self-association, condensate formation, and the increased recruitment of transcriptional machinery at key genes (Wan et al., 2020). Fusions involving developmental TFs and condensate-promoting domains of other proteins are drivers of two additional cancers, Ewing sarcoma (EWS) and acute promyelocytic leukemia. In Ewing sarcoma, fusion of the DNA-binding domain of FLI1 (an ETS family TF) and the IDR of EWS (an RNA-binding protein) leads to neomorphic condensates which activate the expression of target genes (Boulay et al., 2017). For acute promyelocytic leukemia, fusion of the DNA-binding domain of retinoic-acid receptor alpha (RARA), a nuclear hormone receptor, and the oligomerization domain of PML, the scaffold for PML nuclear bodies, prevents the differentiation activity of RARA, causing a differentiation block and uncontrolled proliferation of promyelocyte. Oligomerization of the PML fusion is required for its transforming activity (Kwok et al., 2006). As opposed to EWS-FLI, which leads to neomorphic activation of genes by recruitment of coactivators (Boulay et al., 2017), PML-RARA leads to neomorphic repression of genes by recruitment of corepressors (Martens et al., 2010; Wang et al., 2010). It is likely that additional examples of mutations leading to altered multivalent interaction networks are involved in cancer.

The diseases that have thus far been implicated in condensate dysregulation highlight the major nodes of regulation, which impact both tiers of multivalent interactions: the multivalent interactions among DNA-binding TFs and cofactors, the multivalency encoded in DNA regulatory elements, and their combined efforts in the effective recruitment of cofactors and RNA Pol II to target loci. Recent evidence suggests that different condensates will be more or less accessible to small-molecule therapeutics based on the composition and nature of their underlying dynamic multivalent interactions (Klein et al., 2020). Chemical screens have identified small molecules that disrupt interactions among mutant FUS proteins and reverse aberrant condensate formation and disease phenotypes in models of ALS (Wheeler et al., 2019). A deeper understanding of how these dynamic multivalent interactions contribute to specific disease states will provide new clues on how to therapeutically intervene to correct these aberrant condensates or design drugs that selectively partition into disease-associated condensates.

FUTURE DIRECTIONS

The rules for how organization by compartmentalization is accomplished are starting to be uncovered, but many open areas remain to be explored. These include, but are not limited to, the effects of the physical and material properties of the nucleus, the clustering of genes to share limited cellular resources, and functional dissection of condensate composition

Physical and Material Properties of Condensates and Chromatin

While this review has focused mainly on the diverse weak multivalent interactions among proteins, RNA, and DNA, collectively these interactions will engender physical and material properties, which also play a significant role in determining condensate formation and composition. Condensate formation is also influenced by the material properties of the local environment. Genome-templated condensates preferentially form in regions of low chromatin stiffness (Shin et al., 2018). The condensate does not mix with the local chromatin but pushes it away, consistent with reconstitution experiments where BRD4-chromatin condensates are immiscible with chromatin-alone condensate (Gibson et al., 2019). While condensates deform the local chromatin environment, they become constrained and unable to diffuse freely, bump into one another, and fuse (Lee et al., 2020). This expansion of local chromatin is in agreement with two recent studies investigating chromatin architecture during cell-state transitions. The first used live-cell locus tracking of a region before and after gene activation, showing constrained mobility, which increased upon activation (Gu et al., 2018). The second used two-color fluorescence *in situ* hybridization to investigate the distance between the *shh* enhancer and promoter before and after gene activation, showing that the loci were further apart when activated (Benabdallah et al., 2019). In both of these studies, gene activation led to increased distances being explored but still located within a confined region, perhaps due to condensate deforming the local chromatin.

The separation of loci by the rigid nature of chromatin suggests that changes to the material properties of chromatin would have a significant impact on where condensates can form and how they could interact with one another. Acute loss of cohesin, a major component in constraining chromatin conformation, increases long-range interactions between superenhancers (Rao et al., 2017), suggesting that the normal conformation of chromatin imparts a constraint which can be regulated. During development, global chromatin architecture undergoes dramatic changes. For example, during differentiation of embryonic stem cells, chromatin transitions from loosely packaged to highly condensed regions (Fussner et al., 2010; Ricci et al., 2015). These changes are often framed in a restriction of cell lineages due to DNA regions becoming inaccessible to TF binding, but often TFs are bound to regions without activating target genes. What consequence do the material properties of local chromatin have on the ability to form condensates? Early indication that these are linked comes from observations that large and stable RNA Pol II condensates observed in mouse embryonic stem cells become less prevalent upon differentiation (Cho et al., 2018). It will be interesting to understand how these local and global changes in the material properties of the nucleus impact

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where along the chromatin fiber condensates can form and the impact of this on activating gene expression programs.

Clustering of Genes and Regulatory Elements to Combine and Share Resources

In the same way that the material properties of chromatin might keep some regions apart, this organization allows condensates to bring regions together. Genome-templated condensates at two defined loci lead to locus tethering by condensate fusion (Shin et al., 2018). Nuclear condensates associated with multiple genomic loci can spatially organize these loci. The Cajal body, a nuclear condensate responsible for spliceosomal RNA synthesis, tethers regions of the genome together (Sawyer et al., 2016; Wang et al., 2016). The HLB, a nuclear condensate involved in the transcription and processing of replication-dependent histone genes, coordinates these loci (Carty et al., 2017; Hur et al., 2019). Unbiased investigation into genome organization observed enrichment of multi-wise and long-distance interactions among genomic loci associated with superenhancers, splicing speckles, and the nucleolus (Beagrie et al., 2017; Quinodoz et al., 2018), which have all been implicated in condensate formation. Arrays of interchromosomal enhancers in the olfactory regulatory region are organized in multi-interaction hubs (Clowney et al., 2012; Monahan et al., 2019) suggesting a condensate level organization in these highly specialized cell types. The clustering of genetic loci by a shared condensate may allow these genes to share limited cellular resources, potentially enabling more robust expression and coordinated regulation. This type of coordinated expression has been observed for two promoters which share an enhancer (Fukaya et al., 2016) and at a larger scale for the coordinated expression of replication-dependent histone gene array in early S phase. The extent to which condensate-mediated gene clustering coordinates coregulated gene-expression programs during development remains to be investigated.

Probing Interactions within Condensates

While we understand many of the biochemical activities and interaction interfaces involved in gene activation at an atomistic level, how these activities and interactions behave within condensates and the effect condensates have on their potential for interactions with partners and substrates is less understood. The application of recently developed tools to systematically identify components of transcriptional condensates will be an important advance. There have been exciting advances in unbiased identification of protein, RNA, and DNA components of other biomolecular condensates by proximity biotinylation strategies (Kurihara et al., 2020; Markmiller et al., 2018; Youn et al., 2018) and by fluorescence-activated particle sorting (Hubstenberger et al., 2017). These new techniques, together with more conventional biochemical methods (Andersen et al., 2002) followed by proteomics or high-throughput sequencing of RNA or DNA, have potential to uncover the compositional dynamics of condensates and their impact on function. Continued cataloging of condensate components and changes to their composition during stimulation or cell-state transitions will be important to deciphering how condensate composition is dynamically regulated.

Once components are identified dissecting the molecular features of their engagement with condensates is necessary to perform functional experiments. Remarkable progress has also been made in predicting the required functional patterning and valences of specific amino acids for phase separation (Martin et al., 2020; Wang et al., 2018). For TF-activation domains, the functional molecular features within these IDRs have been defined by high-throughput screening efforts where large libraries of synthetically designed protein sequences are screened for their ability to activate gene expression (Erijman et al., 2020; Ravarani et al., 2018). An evolutionary perspective to identify conserved features of IDR sequences is also showing promise (Zarin et al., 2019). Based on these pioneering studies it is becoming easier to selectively mutate domains predicted to be necessary for condensate formation allowing correlation of condensate disruption and functional outcomes. New tools for inducing synthetic intracellular condensates with defined protein domains, defined valences, and defined genomic locus (Bracha et al., 2018; Shin et al., 2018, 2017) now enable the *de novo* formation of cellular condensates. The application of this toolkit to dynamic transcriptional condensates will allow for the dissection of the molecular interactions enabled by condensates and further investigation into their molecular and cellular functions.

CONCLUDING REMARKS

The compartmentalization of gene activation within condensates creates a new layer of regulatory biology at the level of spatiotemporal organization. The multivalent interactions necessary to create condensates—the protein, RNA, and DNA features that enable these types of interactions—and the properties which emerge as a consequence represent a new arena of regulatory biology. Investigation into these pathways does not supersede the molecular mechanisms currently understood at atomistic detail but will consider how these biochemical processes operate at larger length scales; instead of, how does an enzyme-substrate pair yield activity?, the question is, how are enzymes and substrates organized so that they will or will not find one another? These weak multivalent interactions play important roles during cell-state transitions as new gene-expression programs are activated. Continued investigation of how condensates organize the process of activating new gene programs will require a great diversity of experimental techniques and likely the development of new tools. Similarly, progress will require a truly multidisciplinary approach combining biology, chemistry, and physics in a way that each field can learn and grow from the complementary insights that each provides.

GLOSSARY

Saturation Concentration

In the context of mixtures of macromolecules in the test tube or in the cell, the saturation concentration describes the concentration above which condensates begin to form.

Threshold

A point along a linear scale at which discontinuous change in a measured parameter is observed. For example, increasing the concentration of a protein past the threshold value of its saturation concentration will lead to the formation of condensates.

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Seeded Nucleation

A form of heterogeneous nucleation where the nucleation and growth of new phases does not form homogenously but is seeded at specific locations. In the context of the nucleus, individual genomic loci can act as seeds to ensure that condensate formation occurs at specific locations and not at random locations throughout the nucleoplasm.

Scaffold

In the context of condensates, scaffolds are the essential components which enable the local concentration of other molecules, often referred to as clients.

Weak Multivalent Interactions

Weak refers to the low-affinity or relatively high dissociation constant relative to interactions between subunits of a complex. Valence refers to the number of interactions a macromolecule can engage in simultaneously. Multivalent is defined as simultaneously engaging in 3 or more interactions thereby enabling interaction networks. The combination of weak and multivalent promote the formation of dynamic condensates by enabling conformationally dynamic networks of interactions among biomolecules.

Stable Interactions

As a foil to weak multivalent interactions which promote condensate formation, stable interactions are those which promote complex formation. These interactions typically adopt a low number of conformations and thereby occur with defined stoichiometries.

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