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Functional organization of RNA polymerase II in nuclear subcompartments

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Abstract

Distinct clusters of RNA polymerase II are responsible for gene transcription inside eukaryotic cell nuclei. Despite the functional implications of such subnuclear organization, the attributes of these clusters and the mechanisms underlying their formation remain only partially understood. Recently, the concept of proteins and RNA phase-separating into liquid-like droplets was proposed to drive the formation of transcriptionally-active subcompartments. Here, we attempt to reconcile previous with more recent findings, and discuss how the different ways of assembling the active RNA polymerase II transcriptional machinery relate to nuclear compartmentalization.

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Nuclear organization, Transcription factory, Transcriptional activation, Transcription factor, Chromatin binding, Looping, Phase separation, Intrinsically disordered region, Binding kinetics.

Abbreviations

TF, transcription factor; Pol II, RNA polymerase II; CTD, C-terminal domain of the largest RNA polymerase II subunit; dense fibrillar component, DFC; fibrillar center, FC; LLPS, liquid–liquid phase separation; PIC, pre-initiation complex; 3D, three-dimensional; IDR, intrinsically disordered region in a protein.

Introduction

The mammalian cell nucleus is filled with DNA, RNA, and protein, which collectively take up ~30% of its volume [1]. Remarkably, this space segregates specific genome-associated activities into subcompartments without involving membranes [2–4]. In the early 1990s, it was experimentally demonstrated that active RNA polymerase II (Pol II) organizes into distinct 50–200 nm sized nuclear foci termed "transcription factories" with a mean density of at least one factory per μm^3 in mammalian cell nuclei [5]. Compared to the diffuse nucleoplasmic pool, these foci contained ~1000-fold higher levels of the relevant protein machinery, harbored the majority (> 90%) of nascent transcripts, and persisted inside of nuclei even after nucleolytic detachment of most chromatin [6]. However, the mechanisms governing the formation and dynamics of such factories are only partially understood [7].

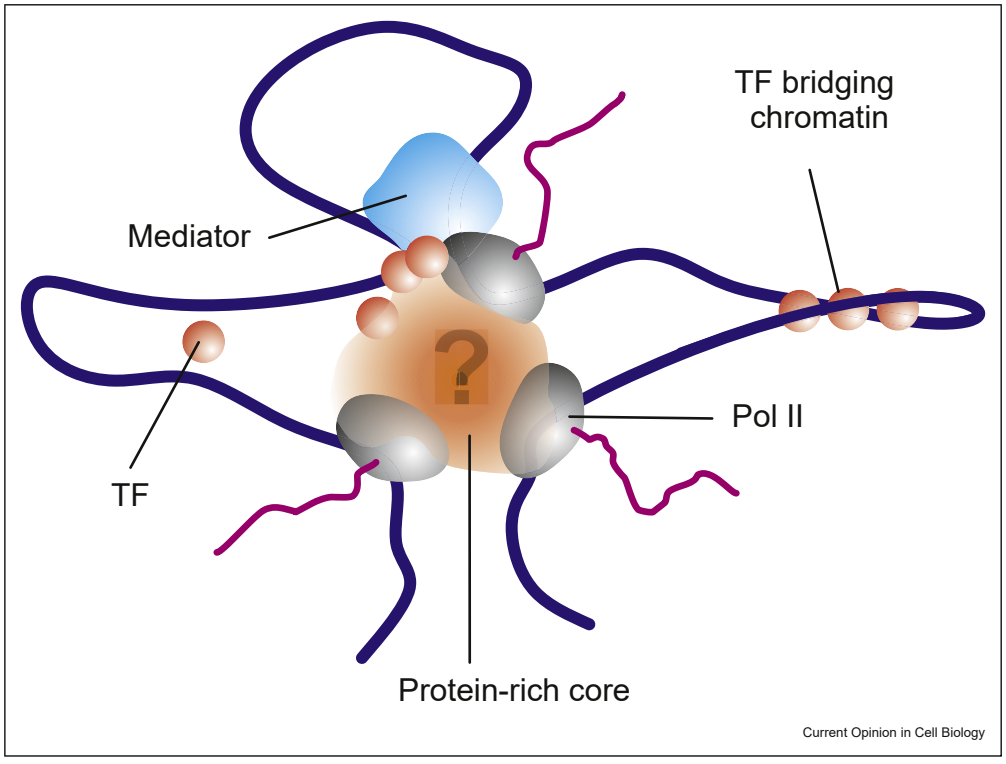
Recently, new insights into the organization of membraneless cellular subcompartments have emerged from soft matter physics, pointing to phase separation (PS) as a potential driver for the formation of Pol II transcriptional subcompartments [8–11]. One form of PS, liquid–liquid phase separation (LLPS), explains the formation of cytoplasmic protein–RNA bodies as demonstrated for P granules [12]. This allows these organelles to fuse, to maintain a border to their surroundings while exchanging with it, and to functionally compartmentalize the processes they harbor. Formation of a phase-separated droplet increases the local concentration of enzymatic activities and substrates and co-activators alike [13]. Thus, the idea of phase-separated Pol II subcompartments aligns with the cells' need for efficient transcriptional activation. However, whether PS is the mechanism that cells exploit to execute the different phases of the transcription cycle remains controversial [14,15].

Here, we will focus on the Pol II holoenzyme as a candidate complex for coalescing into phase-separated condensates. Since Pol II alone is a very large structure that dwarfs its DNA template [16], the question that follows is: how is the transcriptional machinery organized in these apparent foci, and how do the consecutive steps in a gene's transcription cycle (i.e., initiation, elongation, co-transcriptional splicing, and termination) integrate or separate in space and time? Various mechanisms—that are not mutually exclusive—have been proposed to rationalize assembly and maintenance of active Pol II clusters [5,11]. Here, we discuss mechanisms for assembling transcriptionally-active subcompartments and their functional implications while attempting to reconcile previous with current research.

Chromatin binding and bridging interactions

Pol II transcription is initiated via sequence-specific interactions of transcription factors (TFs) with promoter and enhancers [7]. Subsequently, the general machinery assembles into preinitiation complexes (PIC; comprises TFIIA, -B, -D, -E, -F, -H, Pol II, and Mediator). The classical view of PIC assembly involves specific protein–DNA and protein–protein interactions that lead to a supramolecular entity the structure of which has been resolved at atomic resolution [17]. Long- and short-range bridging interactions via TFs can bring enhancers and promoters into spatial proximity and would suffice for the formation of Pol II factories with a spatial organization similar to that found in the cell [5,11,18,19] (Figure 1). As the residence times of active Pol II and TFs on chromatin are in the range of a few seconds (observed for most TFs), the resulting subcompartment is highly dynamic. However, at numerous gene loci, TF binding sites are often clustered such that, despite transient binding, chromatin remains on average continuously occupied to boost high local concentration of the transcription-related apparatus. There also exist chromatin-binding factors that bind to cognate sites for many minutes (e.g., CTCF and cohesin [20]), giving rise to conformations that persist for lengths of time comparable to longer-lived Pol II clusters. Moreover, despite formation of highly dynamic chromatin loops and clusters, polymer models of chromatin organization via transient bridging can recapitulate the formation of the transcriptionally-active subcompartment similar to that observed *in vivo*, as well as the experimentally-observed transitions between functional states [5,11,18,19]. In addition to these intrachromatin linkages, Pol II clusters might associate with the nuclear substructure, much like heterochromatin is physically linked to the nuclear lamina. The biochemical purification of Pol II factories from human cells required using caspases for their release from lysed nuclei. Their protein and RNA contents showed the expected selective enrichment for TFs and nascent RNA, but also numerous nucleoskeletal components that may potentially "anchor" the factory core [21,22]. Notably, Pol II clustering in response to signaling cues is enhanced by nuclear actin and its co-factors [23].

Figure 1



Pol II transcription factory organization. Chromatin is folded by specifically-bound proteins to promote enhancer–promoter interactions involving Pol II and the Mediator complex. Both promoters and enhancers can be Pol II bound, with enhancers producing short-lived eRNAs. Thus, they do not fundamentally differ from each other. Active transcription occurs at the periphery of a chromatin-depleted protein-rich core. This core could represent a phase-separated subcompartment stabilized by multivalent interactions between the IDRs of transcription factors (TFs) and co-activators.

Pol II clustering around a protein-rich core

The aforementioned "classical" view of multiprotein complex assembly on chromatin as a sequential and temporally-ordered process clashes with the finding that supramolecular complex assembly occurs stochastically and in parallel in the cell (e.g., the studies by Wachsmuth et al., Stasevich et al., and Luijsterburg et al. [4,24,25]). The resulting complexes are highly dynamic, exchange their components within seconds, and involve unspecific multivalent interactions that lead to further accumulation of protein and RNA around sequence-specific TF nucleation sites. In this context, it seems particularly relevant that Pol II clusters contain significantly less chromatin than their surroundings and variable RNA amounts pending on gene expression amplitude and the phase of the transcription cycle (e.g., initiation versus elongation). Electron spectroscopic imaging of Pol II clusters reveals that active polymerases decorate the periphery of polymorphic protein-rich cores ranging from 50 to > 200 nm [5] (Figure 1). Similar topologies were described for transcriptional condensates forming upon ectopic overexpression of a transcription factor TAF15 construct: transcribing polymerases are at the periphery of these phase-separated clusters, the core of which was rich in TFs and (presumably) co-activators [26•]. Notably, in both these structures the engaged DNA and nascent RNA lie on the outer surface of a protein-dense core. This layered organization is supported by recent imaging studies [27•, 28] showing polymorphic Pol II clusters further varying according to the phosphorylation signature in their carboxyterminal domain (CTD) $(Y_1S_2P_3T_4S_5P_6S_7)_{n\ =\ 26\text{-}52}$ repeats. Ser5 versus Ser2 phosphorylation distinguishes initiating from elongating Pol II, and the post-translational modifications (PTMs) of its CTD can now reliably be mapped by mass spectrometry [29].

Studies of endogenously-tagged MED19 and Pol II in mouse embryonic stem cells identified two different assemblies [30]. One smaller than 100 nm in size and unstable (with average lifetimes in the order of tens of seconds), while larger (> 300 nm) clusters contained 200 to 400 molecules and persisted for several minutes. Another super-resolution microscopy study in living cells reported 200 nm-large Pol II clusters in human cells with dynamic size changes on the 10-sec scale [31]. In zebrafish, active Pol II clusters formed inside µm-sized subcompartments rich in RNA but lacking chromatin, with active transcription sites of 100–200 nm in diameter located at the RNA–chromatin interface [28]. Thus, on the one hand, hundreds of Pol II/TF clusters are detected at the ~100 nm scale in line with measurements previously of Pol II factories [5]. On the other hand, a much smaller number of larger condensates is detected at the ~1 µm scale, which could correspond to the active chromatin compartment defined previously [3]. How these different length scales can be bridged remains an open question. Furthermore, the spatial relation of Mediator and the Pol II-cluster core is not clear. Inspection of fluorescence images like those acquired by Cho et al. [30] only showed partial overlap of Mediator with the protein-rich core. This suggests that Pol II clustering might not rely on this large coactivator complex, in line with molecular evidence showing that its depletion does not affect 3D chromatin organization at active loci [32,33].

Phase separation as a mechanism for assembling Pol II around a protein-rich core

A prototypical example of a highly transcriptionally-active membraneless nuclear organelle for which PS has been proposed is the nucleolus [34]. The rDNA repeats on different human chromosomes fuse into a few large nucleoli containing high concentration of RNA, proteins including RNA Pol I, and specific TFs and extended intrinsically disordered regions (IDRs) are found in marker proteins like fibrillarin, nucleolin or nucleophosmin. The fibrillar center (FC) carries high concentrations of Pol I and UBF transcribing rDNA repeats and is surrounded by crescent-shaped dense fibrillar components (DFC), where nascent rRNA begins to assemble into ribosomes. Further rRNA processing takes place in the outer granular component (GC) marked by nucleophosmin. Nascent rRNAs are synthesized at the FC–DFC interface with actively-transcribed rDNA adopting a ring-shaped conformation of ~170 and ~240 nm in diameter in human and mouse fibroblasts, respectively [35]. Thus, active Pol I transcription zones within the nucleolus are comparable size-wise to Pol II factories and active Pol I also assembles around a protein-rich core provided by the FC. Features of this topology are reproduced *in vitro* with liquid droplets formed by nucleophosmin, fibrillarin, and a Pol I subunit [36].

PS could similarly drive the assembly of Pol II cluster protein-rich cores. Indeed, a microphase separation mechanism has been proposed, whereby Pol II and associated TFs locally nucleate transcriptional subcompartments upon zebrafish genome activation to create "transcription pockets" of 100–200 nm in size at the RNA–chromatin interface [28]. Pol II clusters could form via recruitment to such chromatin-associated LLPS condensates. In a next step, elongating polymerases are displaced relative to the core and result in the loosening up of the condensate [27•]. This model whereby Pol II is recruited to a core of a protein-dense surface-associated LLPS condensate is in line with experimental data showing that condensates by (endogenous or overexpressed) YAZ co-activators in human cells recruit Pol II several minutes after they initially form and also accumulate BRD4 and MED1 [37•,38]. The Pol II CTD could mediate interactions with this droplet-like protein core as inferred from *in vitro* experiments [39,40]. These observations imply that the PS-driven protein-rich core

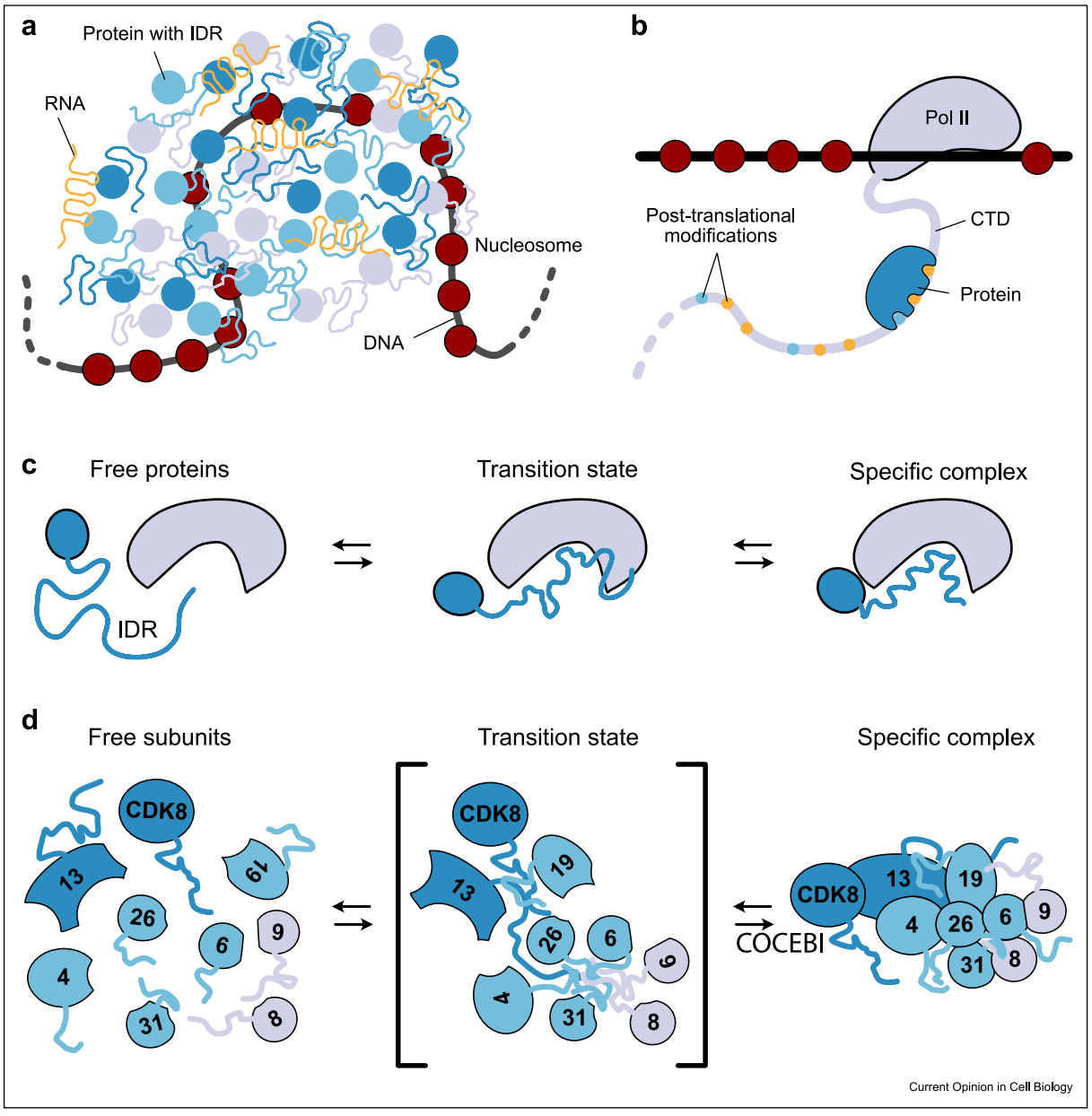
in the center of Pol II factories displaces inactive chromatin segments to give rise to the typical local organization pattern of euchromatin. Interestingly, these organizational aspects can be partly recapitulated via a dCas9-based optogenetic approach that exploits IDRs to form condensates. These preferentially form in euchromatic regions and could physically pull into proximity the genomic loci targeted, much like what is expected of factories [41] (Figure 1).

The contribution of IDRs to Pol II cluster formation

TF binding to cognate DNA sites represents a highly specific interaction targeting the transcriptional machinery to certain genomic loci. Subsequent accumulation of additional TFs and co-factors via transient and largely unspecific interactions could then occur more stochastically and in parallel for different factors. These factors would comprise the elusive protein-dense core described above, around which active Pol II clusters. The question that naturally follows is: which forces hold this core in place? Macromolecular crowding, entropic depletion attraction, and hydrophobic effects are all thought to mediate such assemblies [42,43]. Recently, IDRs have emerged as a potential key player of this process. They form a variety of molecular interactions and about a third of the eukaryotic proteome contains IDRs of $>$ 30 residues in length [44]. A large number of proteins involved in gene transcription carry long IDRs (e.g., the Pol II CTD, general and cell-type-specific TFs, co-activators like Mediator or BRD4, splicing factors) that are functionally important [45–47•]. For Pol II clusters specifically, this was exemplified in a well-controlled study in yeast, where gene expression and transcriptional bursting were compromised upon shortening of the Pol II CTD and loss of clustering. Remarkably, both effects were reverted by fusing the PS-prone FUS or TAF15 IDRs to the end of shortened CTDs [48••]. However, these cannot be considered completely unspecific since TAF15 is a known polymerase co-factor and FUS coalesces with Pol II in a transcription-dependent manner [49].

Different mechanisms that are not mutually exclusive involve IDRs and appear to be relevant for Pol II clustering. IDRs could provide the transient multivalent interactions that mediate PS of Pol II via its CTD and associated TFs and co-activators shown to form liquid droplets *in vitro* [10,50]. Thus, LLPS may rationalize formation of the protein-dense core of transcription factories [8,9, 26•, 51, 52] (Figure 2A). Such transient interactions would generate a phase of liquid-like properties so long as they are weak and transient (but will transition to gel- or solid-like states as strength increases) [52]. The resulting subcompartment would maintain high local concentrations of necessary factors with a sharp boundary to the nucleoplasm that could also keep promoters and enhancers in (transient) proximity to amplify transcriptional activation [8,9, 26•, 51, 52]. For PS to occur, sharp thresholds can be determined based on the affinity, number, and density of IDR–IDR and DNA-TF interactions [9]. The question of whether these are met at endogenous transcribed loci *in vivo* remains unaddressed, as most studies showing droplet production are either carried out *in vitro* or under non-physiological conditions. Moreover, multivalent interactions mediated by IDRs could enhance transcription below the critical concentration for droplet formation, suggesting that they activate transcription independently of LLPS [11,55•,56••]. One function of IDRs might simply be to mediate stoichiometric binding between TFs, co-activators and the Pol II CTD. Although the purified Pol II CTD adopts an unstructured random coil conformation, it may nevertheless establish interactions with sufficient strength in its unstructured state with other TFs via multiple contacts of certain amino acid residues in the CTD and dependent on its phosphorylation pattern [29,39,57]. This is apparent in experiments distinguishing initiating from elongating Pol II via differences in Ser5 versus Ser2 phosphorylation [29]. In addition, lysine acetylation occurring in CTD heptad non-canonical repeats also modulate protein binding to it [58•], suggesting that CTD modification patterns represent a "code" directing specific protein–protein interactions (Figure 2B) [53]. Binding to IDRs with certain patterns of PTMs could work like the recognition of epigenetic modifications of the unstructured histone H3/H4 tails by "reader" protein domains [59]. In addition, the induced (partial) folding of IDRs upon binding is a well-recognized mechanism for establishing specific interactions by these regions [44] (Figure 2C). A recent study dissected the induced folding of the disordered p53 activation domain during binding to the CREB binding protein (CBP) [54••]. The flexible IDR chain mediated binding via different pathways makes the reaction faster than it would occur for folded proteins and the diffusion-controlled reaction rate limit can be reached. Finally, IDRs could increase kinetic binding rates in a similar manner also without induced folding (Figure 2D). By stabilizing a transition state they could reduce the occurrence of unproductive encounters between macromolecules in the wrong steric orientation and, thus, increase the kinetic rate for establishing specific interactions during multi-subunit complex assembly [60]. Different IDRs may be interchangeable in this scenario as they would simply serve to sustain an intermediate state. A case in point would be PIC assembly as illustrated in the scheme of Figure 2D. Several high-resolution PIC structures show specific interactions between the different components leading to the assembly of a well-defined 4-MDa complex [17]. However, these structures contain IDRs that are not resolved. In addition, much like the Pol II CTD, many Mediator subunits (i.e., MED4, -6, -8, -9, -13, -15, -19,

Figure 2



Mechanisms by which IDRs could be important for Pol II factories. (**a**) LLPS: Via relatively weak multivalent interactions, IDRs, frequently together with RNA, can drive phase separation of protein factors into liquid-like droplets above a critical concentration. (**b**) Readout of specific PTM patterns in an IDR: This mechanism has been reported for a number of proteins that interact with the Pol II CTD [53]. (**c**) Specific interactions via induced (partial) IDR folding: In the freely diffusing protein the IDR is in mostly random coil conformations with little helical structure. In an initial encounter, intermediate structures of different IDR conformation and the interacting protein are sampled. Subsequently, a structured complex forms wherein the IDR is stably folded at least in part [54••]. (**d**) IDR-mediated acceleration of complex formation: A hypothetical model is depicted to exemplify assembly of a part of the Mediator complex core. Large IDRs in MED4, -6, -8, -9, -13, -19, -26, -31 and CDK8 stabilize a transition state. In this state, the different subunits dynamically change their relative positions while remaining in contact. This enhances the probability of finding a configuration in which the specific interactions needed for complex formation are established, before dissociating again.

-25, -31) and CDK8 carry large IDRs conserved across metazoan, fungi and plants [45,46]. Hence, stabilization of intermediate states that accelerate PIC assembly could involve the interplay of these IDRs.

Structure–function relationships of Pol II clustering

Transcription can be carried out by an individual Pol II holoenzyme. Thus, any functional contributions from

the assembly of a larger Pol II cluster with multiple promoters/enhancers that involves more promiscuous multivalent interactions would be a modulation of this primary process. Several recent studies conclude that unspecific interactions of TF IDRs could reduce target search time and make Pol II regulation more efficient [61••–63•]. At the same time, high local concentration of TFs should increase binding site occupancy and enhance transcription activation capacity to reduce "noise" [64]. Whether phase-separating TFs and/or co-activators increase RNA production is currently not clear. While it has been frequently proposed that it can induce high transcription activity [8,26•,65–67], one has to demonstrate whether changes in transcriptional activity are indeed the result of PS. Recent work challenged the view that ectopic TF droplet formation at promoters would enhance transcription, but concluded that it is either neutral or even inhibits transcription [55•]. Interestingly, other studies report that the accumulation of regulatory factors in condensates inhibits Pol I [68] and Pol II transcription [69]. One could then also consider the scenario where the requirements for efficient transcription are met already at conditions below the critical concentration at which phase separated condensates form (but adequate for Pol II clustering), and that condensates arise from local crowding without enhancing functions [9]. Notably, endogenous Sox2 and Brd4 were recently shown to still form clusters after deletion of their IDRs, but not of their DNA-binding domains [70••]. Moreover, high throughput screens of promoter architecture found that even small changes to the location of TF binding sites relative to the core promoter and to each other profoundly affect activation amplitude [71,72]. Thus, it is important to separate the effect of the chromatin-bound TF fraction from TFs enriched, but mobile inside the subcompartment.

Conclusions and open questions

Invoking PS of transcriptional machinery components offers a mechanistic explanation for the formation and maintenance of Pol II clusters. It also helps rationalize the persistence of high local concentrations of polymerases, TFs, and co-activators at the active sites of transcription. A PS mechanism would not contradict the transcription factory concept, but rather comes to shed light on aspects of it that were obscure, for example, the formation of protein-rich factory cores. Despite this, multiple questions remain to be addressed. In the light of alternative mechanisms that center around classical stoichiometric protein–protein and protein–DNA/–RNA/–chromatin interactions, one needs to be clear about the experimental evidence that posit PS to govern Pol II transcription under endogenous conditions. For instance, how does the rather non-specific nature of condensates explain differences in bursting and transcriptional output? Similarly, if phase-separated condensates help explain how different TF mixtures can all recruit Pol II, how is spatiotemporal transcriptional specificity established? Also, how are the presumed transitions from an "initiating" to an "elongating" or "terminating" transcriptional condensate achieved (e.g., the studies by Cramer, Guo et al., and Cermakova et al. [16,73,74])? Finally, would phase-separated transcriptional condensates form at all transcriptionally-active loci or are they more of an exception that requires specific conditions that arise, for example, during stress response? Testing these questions at endogenous genomic loci should represent the next frontier in the field.

Conflict of interest statement

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

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