Liquid-liquid phase separation in chromatin

Karsten Rippe

Division of Chromatin Networks, German Cancer Research Center (DKFZ) and Bioquant, 69120 Heidelberg, Germany

e-mail: Karsten.Rippe@dkfz.de

Running title: Phase separation in chromatin

Key words: Genome organization, nuclear subcompartments, liquid-liquid phase separation, intrinsically disordered protein region, nucleolus, transcription, heterochromatin, chromocenter

Citation: Rippe K (2021) Liquid-liquid phase separation in chromatin. *Cold Spring Harb Perspect Biol***,** published online 14 June 2021, https://doi.org/10.1101/cshperspect.a040683

Abstract

In eukaryotic cells, protein and RNA factors involved in genome activities like transcription, RNA processing, DNA replication and repair accumulate in self-organizing membrane-less chromatin subcompartments. These structures contribute to efficiently conduct chromatin mediated reactions and to establish specific cellular programs. However, the underlying mechanisms for their formation are only partly understood. Recent studies invoke liquid-liquid phase separation (LLPS) of proteins and RNAs in the establishment of chromatin activity patterns. At the same time, the folding of chromatin in the nucleus can drive genome partitioning into spatially distinct domains. Here, the interplay between chromatin organization, chromatin binding and LLPS is discussed by comparing and contrasting three prototypical chromatin subcompartments: the nucleolus, clusters of active RNA polymerase II and pericentric heterochromatin domains. It is discussed how the different ways of chromatin compartmentalization are linked to transcription regulation, the targeting of soluble factors to certain parts of the genome, and to disease-causing genetic aberrations.

Introduction

In a simplified and coarse-grained view, the interior of the eukaryotic cell nucleus can be separated into two main compartments: One is chromatin, consisting of the large supramolecular complex of genomic DNA wrapped around histone proteins and bound by a large number of chromosomal proteins as well as chromatin-associated RNAs. The other compartment is the soluble, liquid portion of the nucleoplasm, which is here simply referred to as nucleoplasm. It is a highly viscous fluid, rich in dissolved proteins and RNAs that surrounds the chromatin compartment. Inert proteins diffuse in a few seconds across the complete nucleus with the accessible space being dependent on their size (Baum et al. 2014). Thus, one would expect that proteins and RNA are homogeneously distributed in the nucleus unless locally excluded due to their size or bound to chromatin. Remarkably, the genome naturally self-organizes on the mesoscale by enriching protein and RNA factors into chromatin subcompartments (CSCs) that are around 0.1-1 µm in size (Misteli 2001; Cook 2002; Spector 2003; Misteli 2007; Wachsmuth et al. 2008; Caudron-Herger and Rippe 2012; Cremer et al. 2015; Cook and Marenduzzo 2018; Misteli 2020; Belmont 2021). CSCs are associated with a variety of activities and direct genome functions like transcription, DNA replication, recombination and repair. The exchange of marker proteins between a CSC and the nucleoplasm is surprisingly fast and frequently on the second scale, pointing to highly dynamic structures. This process can be observed in fluorescence recovery after photobleaching (FRAP) experiments as demonstrated in pioneering studies for nucleolar factors like fibrillarin (Phair and Misteli 2000) and RNA polymerase I (Pol I) (Dundr et al. 2002) in the nucleolus, the RNA polymerase II (Pol II) preinitiation complex (Kimura et al. 2002), linker histone H1 (Lever et al. 2000; Misteli et al. 2000) and

heterochromatin protein 1 (HP1) at transcriptionally silenced pericentromeric heterochromatin (Cheutin et al. 2003; Festenstein et al. 2003). It is noted that these studies also identified more immobile protein fractions that were bound to chromatin on the minute time scale. Thus, there appears to be a complex interplay of transient and more long-lived interactions that targets proteins to certain parts of the genome to assemble CSCs in a self-organizing manner reliably across the cell cycle as discussed previously (Wachsmuth et al. 2008).

In order to describe the process of CSC formation a definition of the relevant terms in the context of this review appears to be warranted. The general description of membrane-less cellular subcompartments as “biomolecular condensates” has been used rather broadly for the local accumulation of biological macromolecules independent of the formation mechanism (Banani et al. 2017; Sabari et al. 2020). On the other hand, in physics the term "condensation" and "condensate" is mostly used for a phase transition. Thus, we here suggest to apply “condensate” specifically for the assembly of subcompartments that are the product of a phase separation process. In contrast, the CSC designation makes no assumptions on the formation mechanism and only refers to the local enrichment of protein and/or RNA into a distinct chromatin domain on the mesoscopic scale of 0.1-1 µm. The term “liquid” is used here for a state in which biological macromolecules can independently change their location randomly in all dimensions like molecules in a fluid. Accordingly, the nucleosomes themselves by definition cannot be liquid as they are linked via the DNA into a polymeric chain, which constrains their individual translocations. This definition differs from other studies that refer to nucleosomes or chromatin as “liquid” or “fluid” if they are in a dynamic and disordered state where they retain some configurational flexibility relative to each other (Maeshima et al. 2016a; Sanulli et al. 2019; Maeshima et al. 2020). Here, this type of dynamic organization is referred to as “transient interactions” and the fast exchange of factors between the free and bound state in CSC as “transient binding” but not as “liquid”.

Mechanisms of chromatin subcompartment formation

Soluble protein and RNA factors are mostly homogeneously distributed in the nucleoplasm (**Fig. 1A**). Their local enrichment by binding to chromatin can be mapped along the linear DNA sequence. This sequencing-based analysis has been conducted for chromosomal proteins (Filion et al. 2010), histone modifications (Barski et al. 2007; Ernst et al. 2011) or associated RNAs (Li and Fu 2019). Thus, protein or RNA binding at clustered sites leads to the local enrichment of these factors (**Fig. 1B**). Furthermore, it is well established that the nucleosome chain folds into distinct 3D conformations via interactions between protein and RNA factors bound at distant parts of the nucleosome chain (**Fig. 1C**). This type of interaction drives the dynamic folding of the genome on multiple scales, which could additionally also involve associations via liquid droplets (Misteli 2020; Dekker 2021).

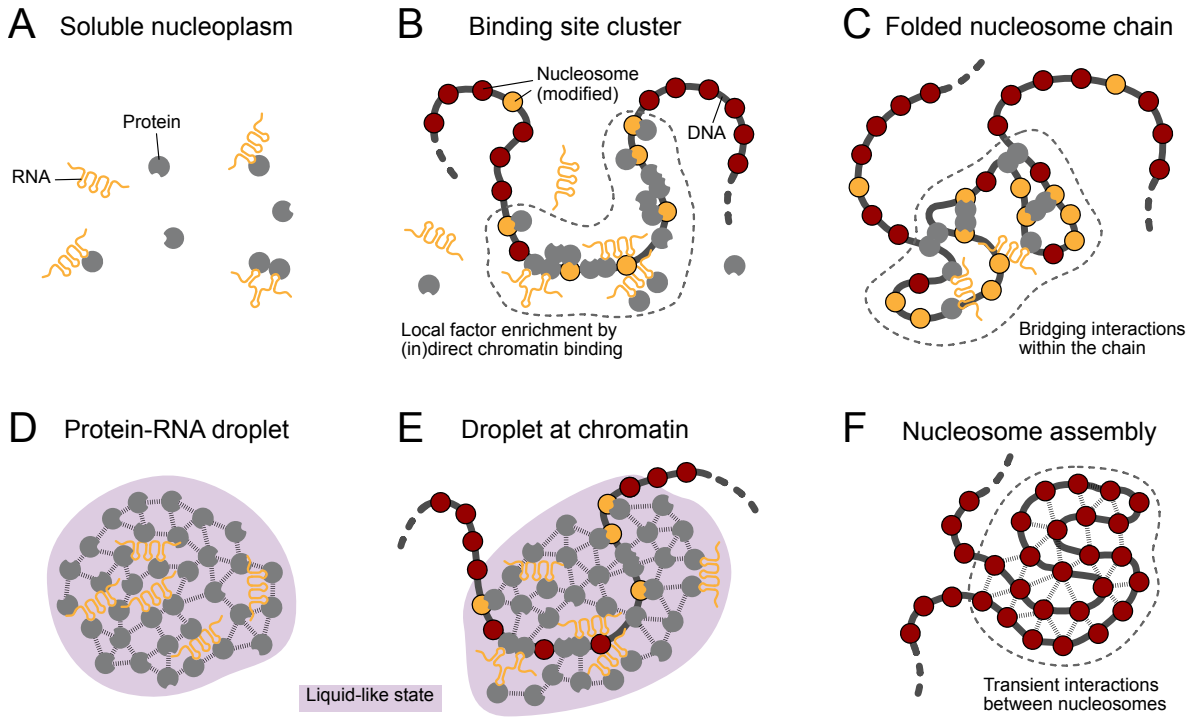

Fig. 1. Multiple mechanisms for formation of CSCs. (**A**) Macromolecules in the soluble nucleoplasm are homogeneously distributed as diffusion quickly equilibrates concentration gradients. (**B**) Direct or indirect binding to clustered sites on the nucleosome chain can locally enrich protein/RNA into a CSC indicated by the dashed line. (**C**) Bridging interactions induced by proteins and/or RNA fold the nucleosome chain into a spatially distinct domain. If a sufficient number of these attractive interactions between chain segments are present, they can induce a polymer-polymer phase transition into a condensed chromatin globule. (**D**) Protein and RNA can separate in the nucleoplasm or cytoplasm by undergoing LLPS into a liquid-like droplet that is mediated by multivalent interactions. (**E**) Chromatin-bound proteins and RNA could nucleate an LLPS event to accumulate additional protein and RNA factors into a liquid droplet. (**F**) Nucleosomes themselves assemble locally into a disordered state where they transiently interact with each other to form an irregular structure that excludes other macromolecules based on their size. It is noted that this state would not be called “liquid” here as the DNA connection between nucleosomes constrains their translocations relative to each other.

One well-established structure on the scale of 1 Mb are topologically associating domains (TADs) (Beagan and Phillips-Cremins 2020; Cavalheiro et al. 2021) and their substructures (Krietenstein et al. 2020; Szabo et al. 2020; Dekker 2021). The dynamic features of TADs observed in living cells are compatible with different polymer folding models (Wachsmuth et al. 2016). Transcriptionally active or inactive TADs segregate into distinct A-/B-compartments as inferred from chromosome conformation capture analysis, which measures the in situ cross-linking efficiency of genomic loci (Lieberman-Aiden et al. 2009). If the protein/RNA-mediated bridging between parts of the chain exceeds a certain threshold a sharp transition from an open random coil conformation into a collapsed chromatin globule can occur. This polymer-

polymer phase separation process is driven by attractive interaction between segments of the chain that induce the transition into a more densely folded chromatin domain (Leibler 1980; Williams et al. 1981; Bates 1991; Nicodemi and Pombo 2014; Michieletto et al. 2016; Jost et al. 2017; MacPherson et al. 2018) (**Fig. 1C**).

The CSCs depicted in **Fig. 1B** and **1C** arise predominantly from the direct chromatin binding of protein and RNA factors. Thus, the "null hypothesis" for forming a CSC against which a potential phase separation mechanism should be tested is the enrichment of protein and RNA factors by (cooperative) binding to a cluster of sites on the nucleosome chain (**Fig. 1B**). This process may also include additional indirect binding of proteins and RNA and can be described by well-established ligand binding models (Teif and Rippe 2010; Gutierrez et al. 2012; Phillips 2015). For example, the DNA sequence-dependent formation of heterochromatin nano-domains marked by the histone modification H3K9me2/me3 can be rationalized by this type of approach (Thorn et al. 2020). To explain how mesoscale proteins and RNA assemblies form with sharp boundaries against the surrounding regions, the mechanism of liquid-liquid phase separation (LLPS) has been applied (Hyman et al. 2014; Banani et al. 2017; Shin and Brangwynne 2017; Boeynaems et al. 2018). It describes the reversible demixing of an originally homogeneous solutions of proteins and RNA into two distinct fluid-like phases. This process can drive the formation of cellular subcompartments by sequestering certain proteins and RNAs into a liquid droplet-like state that segregates them from the surrounding solution in analogy to the demixing of oil drops and water. A molecular description of this process in the cell is given by the "stickers-and-spacers" model (Choi et al. 2020). It represents protein and RNA as flexible polymers were sequence motifs of one or more residues, the "stickers", mediate attractive interactions between different molecules while other parts of the chain act as mostly inert "spacers" between them. Above a critical concentration threshold, the stickers on the protein/RNA chain can induce a separation into a dense phase that coexists with a dilute phase in which the interacting macromolecules are depleted. If interactions in the dense phase are weak and transient it has liquid-like properties. However, the same framework can be used to also describe gel- or solid-like states with reduced protein/RNA mobility as their interaction strength increases (Choi et al. 2020). This LLPS description rationalizes the formation of cytoplasmic P granules, membraneless organelles formed by RNA and protein that are involved in RNA processing (Brangwynne et al. 2009) (**Fig. 1D**). LLPS arises via transient multivalent interactions and frequently involve RNA and intrinsically disordered protein regions (IDRs), creating an exclusionary local protein-RNA environment with distinct physico-chemical properties (Weber and Brangwynne 2012; Uversky et al. 2015; Banani et al. 2017; Drino and Schaefer 2018). It has been suggested to be also a crucial driver of genome organization (Erdel and Rippe 2018; McSwiggen et al. 2019b; Strom and Brangwynne 2019; Frank and Rippe 2020; Hildebrand and Dekker 2020; Narlikar 2020; Sabari et al. 2020). LLPS at chromatin involves directly chromatin-bound protein and RNA factors as nucleation sites so that a

liquid droplet assembles at a specific chromatin locus (**Fig. 1E**). Macromolecules not directly bound to chromatin can constantly rearrange and mix within the droplet and access to this type of CSC depends on the chemical nature of the CSC components. In contrast, access to a CSC formed by bridging interactions of the nucleosome chain (**Fig. 1C**) is controlled by particle size. Other properties like the response to concentration changes of constituting components in terms of size change or buffering also differ. Finally, reconstituted mono- and oligonucleosome particles have been shown to undergo LLPS *in vitro* and it has been proposed that this state exists also in the cell (Gibson et al. 2019; Sanulli et al. 2019; Wang et al. 2019) (**Fig. 1F**). However, within a chromosome, the DNA linkage between nucleosomes imposes a number of constraints with respect to their mobility relative to each other. Confined random translocations of the nucleosome chain can occur on the scale of 10-100 nm but on the mesoscopic CSC scale chromatin displays solid-like properties (Kimura and Cook 2001; Chubb et al. 2002; Gerlich et al. 2003; Walter et al. 2003; Levi et al. 2005; Jegou et al. 2009; Strickfaden et al. 2010; Chen et al. 2013; Wachsmuth et al. 2016; Maeshima et al. 2020; Strickfaden et al. 2020; Maeshima et al. 2021). Thus, liquid-like protein and RNA droplets could nucleate at certain points of a mostly immobile chromatin scaffold with confined motions of nucleosomes or parts of the chain within this droplet (**Fig. 1E**). It is noted that the mechanisms depicted in **Fig. 1** are not mutually exclusive. For example, the binding to clustered sites (**Fig. 1B**) would be part of both the chain folding (**Fig. 1C**) and LLPS (**Fig. 1E**) mechanism. In addition, liquid droplets as well as nucleosome-nucleosome interactions (**Fig. 1F**) could also act as bridging factors to promote folding of the chain into a compacted state.

Formation of transcriptionally active or silenced CSCs

In the following, we will not consider phase separation into mostly irreversible gel or aggregated states as it is a crucial feature of functional CSCs that they are dynamic and can form reversibly in a self-organizing manner across the cell cycle. Rather the focus is on three prototypical CSCs, the nucleolus, clusters of Pol II referred to as transcription factories as well as chromocenters. LLPS has been suggested to be operative for all three of them (**Table 1**) and several of their purified constituting marker proteins can undergo LLPS *in vitro* (**Table 2**). The review will use them as exemplary cases to discuss how their dynamic structure, material properties and biological activities are related to an LLPS process for their formation in comparison to alternative mechanisms. More general discussions of phase-separated processes that involve chromatin can be found elsewhere (Erdel and Rippe 2018; McSwiggen et al. 2019b; Strom and Brangwynne 2019; Frank and Rippe 2020; Hildebrand and Dekker 2020; Narlikar 2020; Sabari et al. 2020).

Nucleolus. The nucleolus is a prototypic CSC for an LLPS-driven formation mechanism (Brangwynne et al. 2011; Feric et al. 2016; Caragine et al. 2019; Lafontaine et al. 2021). Its

structure is characterized by the association of hundreds of nucleolar proteins around the nucleolar organizer regions containing the ribosomal DNA gene repeats (rDNA) from different chromosomes from which large amounts of rRNA are transcribed (Mangan et al. 2017; Nemeth and Grummt 2018; Lafontaine et al. 2021). In the nucleolus key marker proteins like Pol I, fibrillarin (FBL), nucleolin (NCL), and nucleophosmin (NPM1) are highly enriched together with the ribosomal RNA and form a sharp concentration boundary to the surrounding nucleoplasm.

Table 1. Features of exemplary CSCs for which formation by a phase separation mechanism has been proposed in relation to the surrounding nucleoplasm

CSC	Nucleolus[a]	Pol II transcription factories[b]	Chromocenters[c]
Organism	Human	Human, Mouse	Mouse, Drosophila
Marker proteins	Pol I, NPM1, NCL, FBL, UBF	Pol II, TAF15, BRD4, MED1/19, specific TFs	HP1α, MeCP2, H1
Structure	Heterogeneous	Diverse	Granular (HP1α, DNA)
Exchange with nucleoplasm	Seconds-minutes	Seconds-minutes	Seconds-minutes
Internal mixing	Yes	?	No
Fusion	Yes	?	Yes
Accessibility	Chemical properties	?	Size
Protein/DNA ratio	High	High	Average
RNA/DNA ratio	Very high	High	Average
Local viscosity	Increased	?	Average
Architectural RNA component	rRNA, aluRNA	Nascent RNAs, enhancer RNAs, LINE1, aluRNA	Major satellite RNA

[a] (Andersen et al. 2005; Nemeth et al. 2010; Brangwynne et al. 2011; Caudron-Herger et al. 2015b; Martin et al. 2015; Feric et al. 2016; Nemeth and Grummt 2018; Caragine et al. 2019; Frottin et al. 2019; Yao et al. 2019; Ide et al. 2020; Lafontaine et al. 2021; Lawrimore et al. 2021). [b] (Melnik et al. 2011; Ghamari et al. 2013; Papantonis and Cook 2013; Caudron-Herger et al. 2015a; Hnisz et al. 2017; Cho et al. 2018; Chong et al. 2018; Sabari et al. 2018; Guo et al. 2019; Nair et al. 2019; Quintero-Cadena et al. 2020; Sabari et al. 2020; Wei et al. 2020; Garcia et al. 2021b; Hilbert et al. 2021; Ma et al. 2021). [c] (Peters et al. 2001; Brero et al. 2005; Lu et al. 2009; Cao et al. 2013; Muller-Ott et al. 2014; Saksouk et al. 2014; Bosch-Presegue et al. 2017; Strom et al. 2017; Ostromyshenskii et al. 2018; Jagannathan et al. 2019; Erdel et al. 2020; Kochanova et al. 2020).

Pol II transcription factories. Transcriptionally active CSCs enriched with Pol II have been characterized as transcription factories (Jackson et al. 1993; Iborra et al. 1996; Osborne et al. 2004). They accumulate transcription factors, RNA and both promoter/enhancer DNA loci (Jackson et al. 1993; Iborra et al. 1996; Osborne et al. 2004). A number of previous studies have studied their features as well as their function as self-assembling organizers of the genome (Cook 2002; Chakalova et al. 2005; Papantonis and Cook 2013; Buckley and Lis 2014;

Cook and Marenduzzo 2018). In recent studies, the IDR mediated assembly of specific transcription factors (TFs) like SP1, OCT4, β-catenin, STAT3, estrogen receptor (ER) and SMAD3, the TBP associated general transcription factor TAF15 as well as transcriptional co-activators like MED1/19, GCN4 and BRD4 and the unstructured C-terminal domain (CTD) of Pol II into so-called transcriptional condensates has been described as a phase separation process (Hnisz et al. 2017; Frank and Rippe 2020; Peng et al. 2020; Sabari et al. 2020).

Chromocenters. Pericentric repeat sequences assemble into compact heterochromatin domains in mouse and Drosophila cells called chromocenters due to their strong fluorescence after DAPI staining (Probst and Almouzni 2008; Fodor et al. 2010). They contain mostly major satellite repeat sequences but also other types of repeats (Ostromyshenskii et al. 2018; Jagannathan et al. 2019). Recent work concluded that this type of CSC arises from HP1-driven LLPS that condenses chromatin (Larson et al. 2017; Strom et al. 2017; Fan et al. 2020; Li et al. 2020a; Wang et al. 2020) according to the scheme depicted in **Fig. 1E.** However, another study reported that chromocenters form independently of HP1 by polymer-polymer phase separation into a chromatin globule (**Fig. 1C**) (Erdel et al. 2020).

Table 2. CSC marker proteins that can undergo LLPS *in vitro*

Protein	Abbreviation	CSC	Reference
Nucleophosmin	NPM1	Nucleolus	(Feric et al. 2016; Mitrea et al. 2016; Mitrea et al. 2018)
Fibrillarin	FBL/FIB		(Berry et al. 2015; Feric et al. 2016)
Carboxyterminal domain of Pol II	CTD	Pol II transcription factories	(Kwon et al. 2013; Boehning et al. 2018; Lu et al. 2018)
TATA-Box binding protein associated factor 15	TAF15		(Chong et al. 2018)
p300/CREB-binding protein	p300/CBP		(Ma et al. 2021)
Bromodomain-containing protein 4	BRD4		(Sabari et al. 2018)
Mediator subunits 1/19	MED1, MED19		(Cho et al. 2018; Sabari et al. 2018; Guo et al. 2019; Zamudio et al. 2019)
Heterochromatin protein 1	HP1/α/β/γ, HP1a	Chromocenter (pericentric heterochromatin)	(Larson et al. 2017; Strom et al. 2017; Wang et al. 2019; Erdel et al. 2020; Qin et al. 2021)
Methyl CpG binding protein 2	MeCP2		(Fan et al. 2020; Li et al. 2020a; Wang et al. 2020)
Linker histone H1	H1		(Gibson et al. 2019; Shakya et al. 2020; Muzzopappa et al. 2021)

High-resolution structure

A CSC formed by LLPS would be expected to show a homogeneous distribution of a given marker protein within the droplet (**Fig. 1E**). However, other types of local protein enrichment (**Fig. 1B, C**) could also appear like a dense spherical structure at the limited resolution of light microscopy. This is illustrated by labeling endogenous intronic repeat sequence in the *MUC4* gene with dCas9-GFP, which results in punctuate structures with an apparent size of 0.5-0.8 µm (Chen et al. 2013). Thus, high-resolution CSC structures obtained with electron microscopy or fluorescence super-resolution microscopy methods are more informative to distinguish between protein-/RNA filled droplets as opposed to chromatin bound factors.

Nucleolus. In mammals, the nucleolus is structured into three domains clearly distinguishable in electron microscopy images (Thiry et al. 2011). Pol I is enriched in the fibrillar centers (FCs) and the actively transcribed rRNA genes (rDNA) are located at the interface between fibrillar centers (FCs) and dense fibrillar components (DFCs). The upstream binding factor (UBF), a key regulatory factor of rDNA transcription, is associated with both active and poised repeats at the DC/DFC border (Maiser et al. 2020). The resulting pre-rRNA is processed and assembled with ribosomal proteins in the DFC and in the granular component (GC), which is enriched in NPM1 and NCL. This internal compartmentalization can be rationalized as three coexisting, immiscible liquid-like phases (Feric et al. 2016; Lafontaine et al. 2021). Fluorescence microscopy super-resolution images are in line with this model as the distribution of marker proteins such as Pol I, FBL, NPM1 and NCL is quite homogeneous in the respective nucleolar subcompartments (Yao et al. 2019; Maiser et al. 2020; Lafontaine et al. 2021). However, it is also apparent that further fine structure exists for the organization of the actively described rDNA. These loci adopt a ring-shaped conformation of ~170 nm and ~240 nm in diameter in human and mouse fibroblasts, respectively (Maiser et al. 2020). Another study shows that FBL forms small clusters in the DFC of 50 nm in size spaced 100-200 nm apart (Yao et al. 2019).

Pol II transcription factories. Clusters of Pol II have been initially described as comprising 4-30 active polymerases that assemble around a protein-rich core with two or more transcription units with diameters of 50−180 nm in diploid human cell (Rieder et al. 2012; Papantonis and Cook 2013). The initial characterization of Pol II factories was conducted in fixed cells. Subsequent fluorescence microscopy analysis in living cells yielded similarly sized Pol II clusters of 220 nm (Cisse et al. 2013) as well as foci of CDK9, a kinase associated with active Pol II (Ghamari et al. 2013). Furthermore, active Pol II constrains chromatin movements, supporting the view that transcription factories link chromatin loci (Nagashima et al. 2019). Recent studies investigated the structure of active Pol II compartments in the context of a phase separation mechanism (Cho et al. 2018; Hilbert et al. 2021). The analysis of endogenously tagged MED1 and Pol II in mouse embryonic stem cells points to the existence of two different types of supramolecular complexes (Cho et al. 2018). One is relatively small (~100 nm) and instable with average lifetimes on the 10 second scale. The other population of larger clusters (>300 nm)

with ~200 to 400 molecules persists for several minutes. Another study characterized Pol II transcription compartments in zebrafish cells (Hilbert et al. 2021). Clusters of active Pol II were present in µm sized regions enriched in RNA but depleted of chromatin with the active transcription sites of 100-200 nm in size being located at the RNA-chromatin interface.

Chromocenters. The current high resolution structural data on chromocenters comprise electron and super-resolution fluorescence microscopy (Fussner et al. 2012; Erdel et al. 2020; Kochanova et al. 2020; Miron et al. 2020; Strickfaden et al. 2020; Xu et al. 2020). The results point to irregularly shaped domains with condensed chromatin in a granular structure in mouse cells with HP1 and H3K9me3 enrichment following the chromatin density (Erdel et al. 2020). Methyl-CpG-binding protein 2 (MeCP2) and linker histone H1 are also enriched in chromocenters but their fine structure is difficult to assess in the analysis conducted so far (Misteli et al. 2000; Cao et al. 2013; Muller-Ott et al. 2014; Linhoff et al. 2015). In Drosophila the chromocenter organization appears to be less granular and distinct with a multilayer organization of marker proteins (Jagannathan et al. 2019; Kochanova et al. 2020).

Internal mixing of marker proteins in CSCs and exchange with the nucleoplasm

The fast exchange of a large fraction of CSC marker proteins points to highly dynamic structures that nevertheless stably direct genome-associated activities to specific loci. LLPS could confine the translocations of protein and factors to the interior of the resulting liquid droplets so that they become segregated from the surrounding nucleoplasm (**Fig. 1E)**. In this environment they are concentration-buffered and maintain a steady concentration of molecules against external fluctuations that would only affect the droplet size (Banani et al. 2017) (**Fig. 2A**). The CSC types depicted in **Fig. 1B** and **Fig. 1C** on the other hand are permeated by soluble factors from the surrounding nucleoplasm. Access to the CSC is determined by the size of the macromolecule. For this type of CSC, factors can quickly exchange with the surrounding nucleoplasm and the domain size should be mostly unaffected by concentration changes (Erdel and Rippe 2018; Frank and Rippe 2020). However, at sufficiently high concentrations the bivalent attractive bridging interactions between chromatin segments could be competed out by monovalent chromatin interactions of the linking factors (Malhotra et al. 2021) (**Fig. 2A**). A fast exchange of bound proteins with the surrounding nucleoplasm that is measured in conventional FRAP can be explained simply by a short residence time in the chromatin bound state and does not represent evidence for the formation of a liquid droplet (McSwiggen et al. 2019b). The hallmark feature of LLPS that molecules can mix within the compartment like in a fluid can be evaluated by bleaching only half of the subcompartment and analyzing exchange of molecules with the unbleached half (**Fig. 2B**) (Brangwynne et al. 2009; Patel et al. 2015; Erdel et al. 2020). The resulting part of fluorescence recovery is then compared to the exchange with molecules from the surrounding nucleoplasm, which provides information on the permeability of the compartment boundary (Erdel et al. 2020).

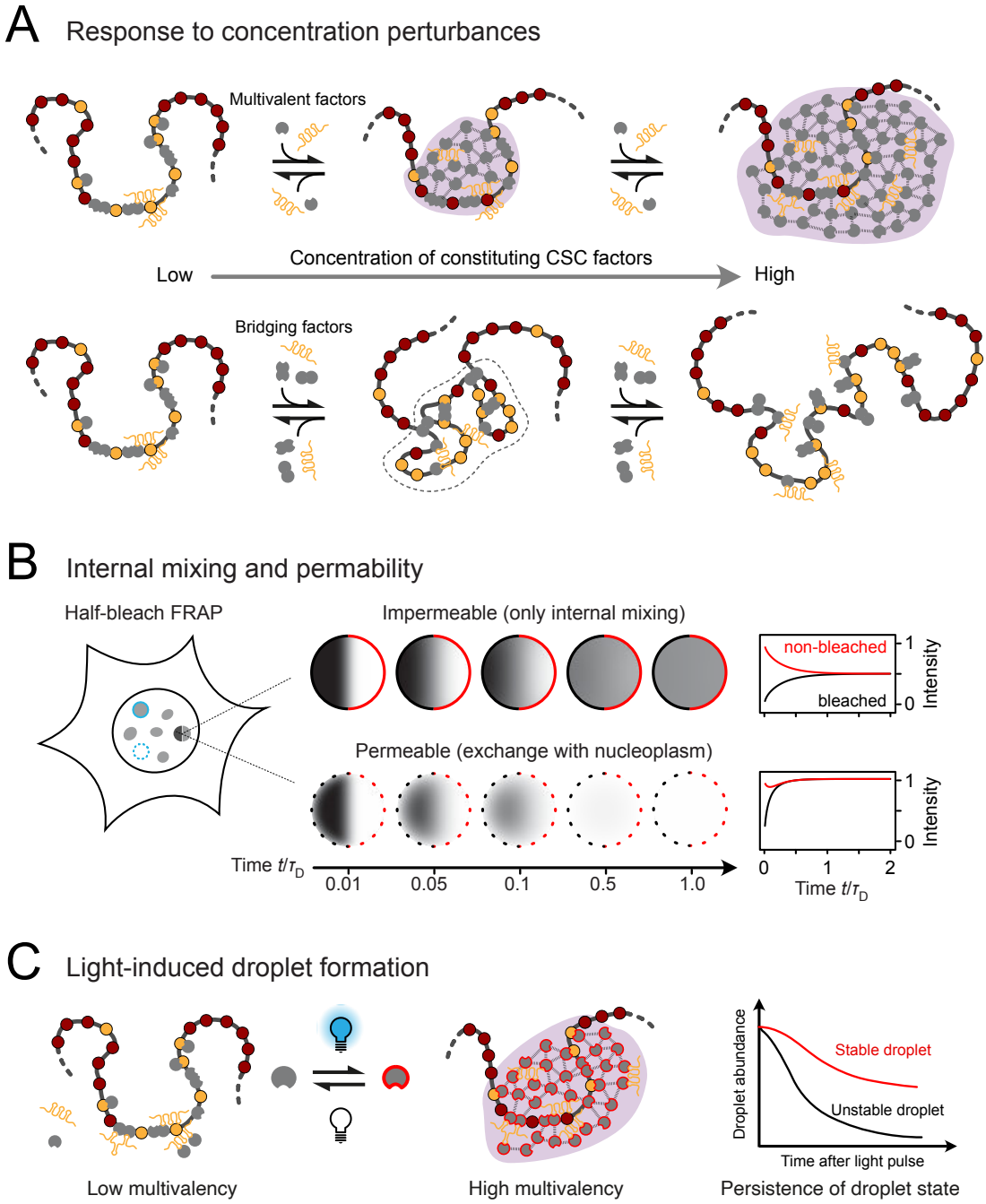

Fig. 2. Experimental approaches to analyze CSC assembly in the cell nucleus. (**A**) Response of CSCs to concentration changes. Top: Increasing the concentration of constituting proteins/RNAs is expected to expand liquid droplets while maintaining their internal distribution (Banani et al. 2017). Bottom: Bivalent chromatin cross-linking could be disrupted at high concentration of bridging factors (Malhotra et al. 2021). (**B**) Half-bleach FRAP evaluates internal mixing and permeability of the boundary (Erdel et al. 2020). Simulated temporal intensity traces for low, intermediate and high permeability are depicted, for a time axis normalized for differences in the diffusion coefficient by division to the diffusion time τ_D. (**C**) Light-induced formation of liquid droplets (Shin et al. 2017). In this assay the protein of interest is fused to the PHR domain, which promotes multivalent interactions and droplet formation upon illumination with blue light. This allows it to evaluate the effect of an artificially induced LLPS on the activity of a chromatin locus of interest, e.g., to study transcription activation. Furthermore, the stability of the resulting droplets can be assessed from their persistence in the absence of the light trigger.

An alternative approach to FRAP is the tracking of single fluorescently labeled particles as has been done for transcription factors (Chen et al. 2014; Kent et al. 2020; Garcia et al. 2021a; Garcia et al. 2021b). It provides direct information on the confinement of particle mobility but is typically limited to observation periods in the ~20 sec range due to loss of the fluorescence signal over time.

Nucleolus. Pol I and UBF have residence times on the 10-seconds to minute scale in the nucleolus, with prolonged retention at rDNA promoters upon activation (Chen and Huang 2001; Dundr et al. 2002; Gorski et al. 2008). Likewise, FBL, NPM1 and NCL show complete recovery in FRAP experiments on the 10-20 second time scale (Phair and Misteli 2000; Chen and Huang 2001; Dundr et al. 2002; Gorski et al. 2008; Frottin et al. 2019; Erdel et al. 2020). Interestingly, NPM1 displays preferred internal mixing within the nucleolus, a feature indicative of liquid droplet formation, which was less pronounced for NCL (Erdel et al. 2020) (**Fig. 2B**). Nucleolar access or exclusion is dependent on the chemical nature of a protein and less so on its size as expected for a LLPS compartment. In particular, certain peptides can carry a nucleolar localization signal that lacks defined sequence motifs and the exclusion of wild-type GFP was reverted by fusion of a small arginine-rich and positively charged peptide (Martin et al. 2015). Finally, it is noted that nucleoli are remarkably stable during their purification, which includes dilution/washing through multiple steps and allows for characterization of their protein, DNA and RNA content (Andersen et al. 2005; Nemeth et al. 2010; Caudron-Herger et al. 2015b). This property is difficult to reconcile with a reversible liquid droplet state, which would disassemble upon removing its constituting components from the surrounding solution.

RNA polymerase II transcription factories. Several FRAP studies evaluated the dynamic properties of Pol II complexes at chromatin (Becker et al. 2002; Kimura et al. 2002; Hieda et al. 2005; Darzacq et al. 2007). In these experiments, the Pol II fraction recovering over 10–20 minutes was assigned to the elongating state. In contrast the putative preinitiation complex was very dynamic and recovered within seconds after bleaching. These findings are in line with studies that report Pol II residence times in clusters of 5-10 seconds (Cisse et al. 2013; Cho et al. 2018) and that foci of the CDK9 kinase, which associates with active Pol II, exchange within seconds (Ghamari et al. 2013). In addition, these studies also report the existence of more long-lived complexes stable on the minute time scale or even for hours. In general, transcription factors show highly dynamic and stochastic binding with typical residence times of seconds (Mueller et al. 2013; Lionnet and Wu 2021; Lu and Lionnet 2021). In many instances, the residence times at their target promoter sites appear to be in the range of less than a minute although longer times have also been reported. Likewise, estrogen receptor α (ERα) (Nair et al. 2019) as well as SOX2 (Chen et al. 2014) were specifically bound for 10-20 s at their enhancers together with other TFs. However, the view of the chromatin residence time is bimodal and reflects essentially either specifically or non-specifically bound complexes might be too simplistic. A recent study concluded that several TFs (including ERα, FOXA1 and

CTCF) follow a power-law distribution of residence times and may involve longer binding events in the right-skewed tail of the distribution than previously derived from bi-exponential models (Garcia et al. 2021a).

Chromocenters. HP1, a marker protein enriched at transcriptionally silenced pericentromeric heterochromatin domains, exchanges within seconds with the nucleoplasm (Cheutin et al. 2003; Festenstein et al. 2003). However, this exchange arises predominantly by diffusion of factors from the nucleoplasm surrounding chromocenters in mouse fibroblast cell lines (Erdel et al. 2020). Neither HP1 nor MeCP2 displayed preferential internal mixing within chromocenters in half-bleach FRAP experiments (**Fig. 2A**) as expected from a liquid-like droplet. MeCP2 is relatively stably bound in chromocenters with 65% of the protein displaying a residence time of 25 s and around 20% of protein binding for more than four minutes (Ghosh et al. 2010; Agarwal et al. 2011; Muller-Ott et al. 2014). In Drosophila, the mobility of HP1a in chromocenters as measured by FRAP was highest at the early embryo stage (Strom et al. 2017). Subsequently, the fraction of immobile HP1a increased from 0% (nuclear division cycle 10) to 30% (cycle 14), pointing to a change of chromocenter organization during differentiation. Another interesting observation in the context of chromocenter protein mobility is it that KMT5C (SUV4-20H2) that trimethylates histone H4 at lysine 20 shows preferential mixing within mouse chromocenters (Strickfaden et al. 2020). Furthermore, its FRAP dynamics are dependent on the three different HP1 isoforms (Bosch-Presegue et al. 2017). The formation of a liquid droplet state of SUV4-20H2, however, is difficult to reconcile with its very tight binding (immobile fraction >90% on the minute time scale) and low abundance of 200 nM concentration in chromocenters (Muller-Ott et al. 2014). It will be therefore important to further characterize the origin of the confined mobility of SUV4-20H2. Another important factor for the dynamic structure of chromocenters is linker histone H1 that displays complex isoform specific interactions with chromatin and is involved in its compaction (Prendergast and Reinberg 2021). In the initial characterization of linker histone H1 binding by FRAP, the immobile fraction at chromocenters was increased by 10-25% (Misteli et al. 2000). Subsequent FRAP studies provided evidence for at least two different H1 chromatin-bound states established by simultaneous interactions of the H1 globular and C-terminal domain to different DNA regions (Brown et al. 2006; Stasevich et al. 2010; Wachsmuth et al. 2016). The longer-lived fraction shows a residence time of ~100 s and is likely to drive the linker histone-mediated packaging of nucleosomes (Maeshima et al. 2016b).

DNA, RNA and protein content and local viscosity

CSC formed by an LLPS mechanism (**Fig. 1E**) are expected to have a particularly high protein/DNA or RNA/DNA ratio as compared to the nuclear average. These parameters are compared for the nucleolus, Pol II factories and chromocenters in **Table 1**. The DNA (Nemeth et al. 2010), protein (Andersen et al. 2005) and RNA (Caudron-Herger et al. 2015b) content of

the nucleolus have been mapped and it is estimated that the DNA concentration in the nucleolus is about 20-fold lower while its protein content is 2-fold higher than in the surrounding parts of the nucleus. At the same time, the nucleolus is filled with ribosomal and other RNAs leading to a ~2,000-fold higher RNA/DNA ratio and ~40-fold higher protein/DNA ratio (Frank and Rippe 2020). Despite its low relative concentration, however, the rDNA sequences play an important role in nucleating the RNA-dependent assembly of the nucleolus (Grob et al. 2014; Berry et al. 2015; Falahati et al. 2016; Nemeth and Grummt 2018; Lafontaine et al. 2021). Thus, the composition of the nucleolus is quite similar to that of a cytoplasmic protein-RNA body (**Fig. 1D**) and fits well to a chromatin nucleated LLPS mechanism (**Fig. 1E**) (Lafontaine et al. 2021). Analysis of the protein (Melnik et al. 2011) and RNA content (Caudron-Herger et al. 2015a) of Pol II transcription show that for a relatively small factory size of 50−180 nm diameter the RNA/DNA ratio could be almost as high as that in the nucleolus (Jackson et al. 1998) and a high protein/DNA ratio is also estimated (Melnik et al. 2011). For mouse chromocenters, their DNA content has been determined after purification with major satellite repeats being the dominating component but also contain a 2 kb LINE element (Zatsepina et al. 2008; Ostromyshenskii et al. 2018). The total DNA concentration in chromocenters is about two-fold higher than the nuclear average (Muller-Ott et al. 2014). The proteins associated with the major satellite repeats have been mapped (Saksouk et al. 2014) and their chromocenter concentration is in general less than 5% of the nucleosome concentration (Muller-Ott et al. 2014). Thus, compared to the nuclear average, chromocenters display an average protein/DNA and low/average RNA/DNA ratio as their transcriptionally activity is silenced under normal conditions.

In summary, the high protein/DNA and RNA/DNA ratios of the nucleolus and Pol II transcription factories distinguish these CSCs from the surrounding nucleoplasm. The high local protein-protein and RNA that result from LLPS are expected to lead to an increased viscosity of the dense phase as shown previously for NPM1 and the nucleolus (Hyman et al. 2014; Feric et al. 2016). In contrast, the local intracellular viscosities in chromocenters as measured by polarization-dependent FCS is similar to that of the surrounding euchromatic regions (Erdel et al. 2020). Thus, it appears that high protein/DNA and RNA/DNA ratios will correlate with liquid-like CSC features and an increased local viscosity. Vice versa CSCs like mouse chromocenters that display average RNA/DNA and protein/DNA ratios and no significant viscosity differences may be less likely to be formed by LLPS.

Structure-function relationships

The different mechanisms that confine genome-associated activities by establishing CSCs (**Fig. 1**) lead to distinct structure-function relationships. In general, two main functional aspects

are apparent. One is to target macromolecules to certain parts of the genome, while the other is the formation of a specific local environment that enhances chromatin-mediated reactions.

Nucleolus. A number of findings show that the intact nucleolus structure and LLPS properties are directly linked to efficient ribosome biogenesis (Lafontaine et al. 2021). The tripartite nucleolar architecture of FC, DFC and GC is disrupted if Pol I or Pol II transcription is inhibited (Caudron-Herger et al. 2015b; Caudron-Herger et al. 2016). At the same time, dispersed pre-nucleolar bodies containing NCL, NPM and FBL that assemble post-mitotically at the nucleolar organizer regions to re-form the nucleolus only have a low rRNA content (Carron et al. 2012; Nemeth and Grummt 2018). Highly proliferating tumor cells, on the other hand, harbor larger and more active nucleoli for high rRNA and ribosome production (Derenzini et al. 2000; Montanaro et al. 2008; Weeks et al. 2019). In addition, cells from patients suffering from neurodegenerative diseases often present with less active nucleoli with structural aberrations (Parlato and Kreiner 2013). Such a correlation of size and activity would be expected for an LLPS-driven mechanism, in which a concentration increase of rRNA could increase the droplet size (**Fig. 2A**). Furthermore, it is well established that LLPS can create an environment with an increased local concentration of protein and RNA factors and enhance enzymatic (O'Flynn and Mittag 2021). Within the fully assembled nucleolus a multi-phase LLPS event could serve to compartmentalize rDNA transcription, rRNA processing and rRNA-ribosomal protein assembly (Feric et al. 2016). Interestingly, also repression of Pol I in the nucleolar cap has been reported by formation of a phase-separated subcompartment (Ide et al. 2020). The liquid-like properties of these distinct subcompartments within the nucleolus could also be important for quality control of misfolded proteins (Frottin et al. 2019). According to the latter study, the granular component of the nucleolus with its liquid-like phase prevents the irreversible aggregation of misfolding of proteins during heat shock.

Pol II transcription factories. For Pol II transcription factories providing specificity of gene regulation as well as promoting efficient transcription are important functional aspects (Papantonis and Cook 2013). It is, however, currently not clear what the driving mechanism of formation for this type of CSC is and how the formation mechanism would affect transcription. For example, the enrichment of Pol II and transcription factors in replication compartments of the herpes simplex virus appears to be mostly driven by locally enhanced chromatin binding (**Fig. 1B**) due to creating nucleosome free regions (McSwiggen et al. 2019a). Furthermore, modelling studies show that bridging interactions of TFs as depicted in **Fig. 1C** would suffice for the formation of Pol II transcription factories with a 3D organization similar to that found in the cell (Brackley et al. 2013).

The functional consequences of TF liquid droplet were studied with synthetic activator constructs using the approach of light-induced droplet formation depicted in **Fig. 2C** (Wei et al. 2020; Schneider et al. 2021). In these studies, it was concluded that droplets formed by TF fusion constructs increase gene expression or transcription activation, supporting the view that

LLPS of TFs and co-activators induces high transcription activity (Hnisz et al. 2017; Sabari et al. 2018; Sabari et al. 2020). However, corroborating these conclusions would require a comparison of TF activation capacity of the same factor in the presence/absence of LLPS under identical conditions (**Fig. 3**). It is noted that the propensity of a given TF or co-activator to undergo LLPS *in vitro* might simply reflect its ability to engage in multivalent interactions. These multivalent interactions could also promote interactions and enhance transcription activation in the absence of phase separation (Cho et al. 2018; Trojanowski et al. 2021). One alternative function would be that IDRs increase the kinetic rate for the formation of a specific complex between proteins and/or nucleic acids (Pontius 1993). In such a mechanism IDRs stabilize an intermediate state that allows the interacting factors to sample different orientations to each other, which increases the probability of specific complex formation during a diffusive encounter. Accordingly, it will be important to further dissect how IDRs modulate the interplay of interactions that differ in strength and specificity between TFs, co-activators and parts of the general transcription machinery in relation to the transcriptional output.

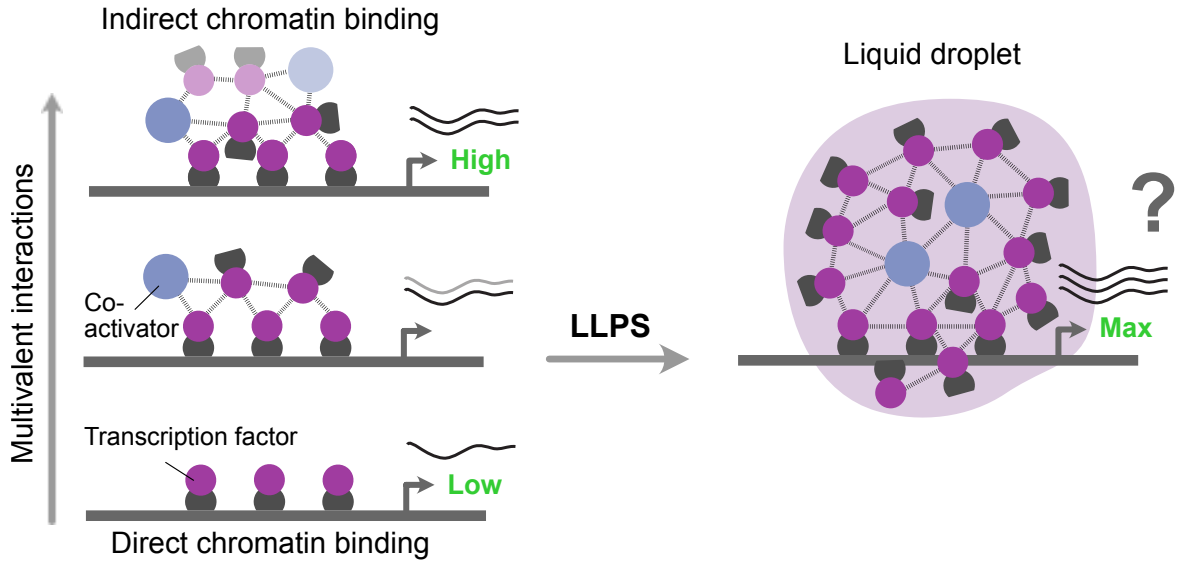

Fig. 3. Multivalent interactions, chromatin binding and LLPS. Direct chromatin binding of a TF is accompanied with indirect interactions of coactivators like histone acetylases, BRD4 or components of the mediator complex that enhance transcription. LLPS would largely increase the amount of indirectly bound factors. It would also lead to a sharp concentration boundary between the droplet and the nucleoplasm while indirectly chromatin-bound factors would be expected to show a concentration decrease as the distance from the directly chromatin-bound TFs becomes larger. Furthermore, it is currently not clear if the formation of a liquid droplet around a given promoter would indeed increase transcription as proposed in a number of studies as compared to the indirect binding of coactivators depicted on the left side of the scheme.

On the mesoscale, liquid droplet formation itself could also accelerate the binding reaction of TFs and/or co-activators to their target sites (Brodsky et al. 2020; Kent et al. 2020; Garcia et

al. 2021b). Confining a random search process to a chromatin-associated droplet and increasing the local concentration of a given factor could greatly increase its kinetic binding rate. Finally, several studies link the IDR-mediated formation of liquid droplets to the phenomenon of "transcriptional bursting" where the promoter enters a refractory state after being in a period of active transcription for several minutes (Rodriguez and Larson 2020). The propensity of transcription factor activation domains to form liquid droplets with the transcription factor p300 as well as the length of the Pol II CTD correlates with an increased frequency and longer duration of transcriptional bursts (Quintero-Cadena et al. 2020; Ma et al. 2021). Remarkably, Quintero-Cadena et al. also show in their study that the loss of Pol II activity due to shortening the CTD can be partially rescued by fusion with an IDR from FUS or TAF15. The stability of the putative LLPS-driven condensates formed via these IDR interactions could be dependent on their RNA content as shown for MED1-IDR droplets in vitro (Henninger et al. 2021). These findings raise the possibility that transcriptional bursting arises from the periodic formation and disruption of an activating liquid droplet state formed between IDRs of Pol II, TFs and co-activators and nascent RNA. However, the switching of a given gene between an active and silent state can also explained by the promoter proximal and distal binding and dissociation of regulators and their chromatin mediated interactions with the transcription machinery (Rodriguez and Larson 2020).

Chromocenters. The assembly of intact chromocenters is linked to chromosome segregation and silencing of repeat transcription (Probst and Almouzni 2008; Fodor et al. 2010; Janssen et al. 2018). How these functions might be affected by proposed LLPS events of relevant chromocenter proteins like HP1, MeCP2 or H1 is currently not clear. It is noted that a number of studies show that global compaction, accessibility and size of mouse chromocenters is largely independent of HP1 (Peters et al. 2001; Schotta et al. 2004; Mateos-Langerak et al. 2007; Bosch-Presegue et al. 2017; Erdel et al. 2020). Notably, the knock-out of HP1α, which has been proposed to be crucial for LLPS in mammalian heterochromatin (Larson et al. 2017; Wang et al. 2019), has no apparent phenotype in mice (Aucott et al. 2008; Singh 2010; Mattout et al. 2015). The chromocenter structure in HP1α$^{-/-}$, HP1β$^{-/-}$ and HP1γ$^{-/-}$ knockouts in mouse embryonic fibroblasts was mostly unaffected on the mesoscale in terms of DNA compaction as compared to wild type cells (Bosch-Presegue et al. 2017). MNase digestion experiments in the latter study point to a decrease in accessibility at nucleosome resolution of chromocenters if HP1α is lost. Likewise, structural phenotypes of chromocenters in differentiated Drosophila cells at the mesocale are not associated with HP1 but rather with two sequence-specific satellite DNA-binding proteins, D1 and Prod (Jagannathan et al. 2019). In embryonic cells, however, HP1a binding is required to establish the clustering of pericentromeric regions and the overall chromosome folding, while it is dispensable in differentiated cells for these functions (Zenk et al. 2021). Thus, multiple studies arrive at the conclusion that HP1 does not induce chromatin compaction in differentiated cells. Rather, chromocenter-specific interactions of

HP1, which can act as a transcription repressor (Hathaway et al. 2012), might prevent spurious induction of satellite repeat transcription (Erdel et al. 2020). In this manner, HP1 would stabilize the transcriptional silencing of a collapsed chromatin globule (**Fig. 1C**) rather than forming liquid droplets (**Fig. 1E**). On the other hand, MeCP2, linker histones and KMT5C are important for the structural integrity of chromocenters. MeCP2 induces clustering of pericentric heterochromatin upon overexpression in mouse myoblasts (Brero et al. 2005) and it could thus be involved in changes of chromocenter structure. In fact, mutations of MeCP2 that cause Rett syndrome, a severe neurological disorder, have recently been proposed to be detrimental because they prevent the formation of liquid droplets in vitro (Fan et al. 2020; Li et al. 2020a; Wang et al. 2020). It is noted, however, that the structural phenotype of these MeCP2 mutations has been rationalized previously as being the result of perturbed chromatin interactions that decrease the ability of MeCP2 to compact heterochromatin (Agarwal et al. 2011). In addition, in neurons, loss of MeCP2 is accompanied by redistribution of the H3K20me3 modification at chromocenters (Linhoff et al. 2015). Furthermore, linker histones are highly abundant in the nucleus at a stoichiometry of about 0.7 H1 per nucleosome (Fan et al. 2003) and enriched at mouse chromocenters (Cao et al. 2013). Their depletion leads to chromocenter clustering and de-repression of the major satellite repeat sequences in them (Cao et al. 2013; Healton et al. 2020). In Drosophila H1 is also required for the structural integrity of chromocenters (Lu et al. 2009). Since linker histones have been shown to undergo LLPS *in vitro* (Gibson et al. 2019; Shakya et al. 2020; Muzzopappa et al. 2021) it will be important to investigate if H1 at chromocenters in the cell displays material properties indicative of its accumulation via LLPS. It is noted, however, that the ability of H1 to form liquid droplets is lost with increasing DNA length, which promotes the formation of more solid-like aggregates (Muzzopappa et al. 2021). Finally, KMT5C is enriched at chromocenters and mediates changes of pericentric repeat organization and chromatin accessibility (Hahn et al. 2013). As discussed above its mobility appears to be confined to chromocenters (Strickfaden et al. 2020), which makes it an interesting protein for further investigation of LLPS at chromocenters. Apart from dissecting the contributions of factors beyond HP1 to the dynamic chromocenter organization it will be important to further investigate embryonic cells. In these cells in Drosophila, the chromocenter mobility of HP1a is increased (Strom et al. 2017) and the protein is required for the 3D organization of pericentromeric heterochromatin (Zenk et al. 2021).

Assessing the contribution of LLPS to the structure of the nucleolus, Pol II transcription factories and chromocenters

With respect to the three CSCs compared here, the following tentative assignment is made: Evidence for a CSC formed in the cell by LLPS is currently strongest for the nucleolus, which has a number of features in support of this mechanism. These comprise liquid-like properties of constituting factors, transitions between coalescent and dispersed states and an increased

local viscosity as discussed above. These features are likely to be related to its unusual composition with respect to the high enrichment of RNA and proteins and very low DNA content. Thus, the overall properties of the nucleolus are dominated by multivalent interactions of protein and RNA. The direct association of these factors with the DNA of the nucleolar organizer regions makes a relatively small contribution albeit being important for nucleating and targeting the assembly. It is noted that the transcribed rDNA locus adopts a folded conformation (Maiser et al. 2020) and a recent study in budding yeast reports that it forms distinct condensates by a polymer-polymer phase separation (**Fig. 1C**) within an LLPS subcompartment of ribonucleoproteins (Lawrimore et al. 2021).

For Pol II transcription factories or transcriptional condensates, it is difficult to conclude at this stage by which mechanism they form. Several lines of evidence indicate that multivalent interactions mediated by IDRs are important to form the active transcription machinery. Thus, many of the factors involved in transcription activation have a high propensity to undergo LLPS as demonstrated with purified proteins *in vitro*. However, evidence that such a phase separation indeed occurs under endogenous conditions in the cell is scarce. Rather, multivalent interactions of IDRs might simply mediate protein-protein interactions between specific and general transcription factors as well as co-activators (**Fig. 3**) (Chong et al. 2018; Trojanowski et al. 2021). Furthermore, it is currently an open question, if the formation of a liquid droplet state induced by sufficiently high endogenous cellular protein concentration would indeed amplify gene expression or increase transcription activation.

For chromocenters a number of criteria and corresponding experimental tests to dissect how this type of CSC is formed in mouse fibroblasts have been presented (Erdel et al. 2020). The results argue against HP1-driven LLPS as a major driver of chromocenter formation in differentiated cells (**Fig. 1E**). A similar type of analysis appears to be generally warranted to make conclusions about LLPS at chromocenters in other organisms (e.g., Drosophila or Arabidopsis) or cell types such as embryonic stem cells. Furthermore, HP1 appears to be irrelevant for chromocenter structure as corresponding phenotypes are lacking as discussed above (Peters et al. 2001; Mateos-Langerak et al. 2007; Aucott et al. 2008; Singh 2010; Mattout et al. 2015; Bosch-Presegue et al. 2017; Erdel et al. 2020). These observations lead to the model that HP1 binds and bridges H3K9me3-modified nucleosomes without inducing chromatin compaction (**Fig. 1B, C**). In mouse cells the latter process is likely to be driven by linker histone H1 that mediates the interchromosomal packing of the nucleosome chain (Hansen 2020), and counteracts clustering of chromocenters from different chromosomes (Cao et al. 2013). This clustering could be mediated by DNA methylation-dependent chromatin binding of MeCP2 (Brero et al. 2005; Agarwal et al. 2011), which competes with H1 for binding sites (Ghosh et al. 2010). In Drosophila, which lacks DNA methylation, chromocenter clustering is dependent on D1 and Prod (Jagannathan et al. 2019). The resulting chromocenter conformation in mouse fibroblasts would be that of a collapsed chromatin globule induced by H1- and MeCP2-mediated

interactions between the nucleosome chain (**Fig. 1C**) and HP1 binding providing an additional safeguard against spurious transcription activation (Erdel et al. 2020).

Conclusions

The concept of LLPS-driven assembly of chromatin compartments provides a novel and inspiring perspective on how the cell organizes genome-associated activities. Such a mechanism could have far-reaching implications and has been associated with a variety of human pathologies like Rett syndrome (Fan et al. 2020; Li et al. 2020a; Wang et al. 2020), oncogenic RNA splicing (Li et al. 2020b) and various neurodegenerative diseases (Zbinden et al. 2020). The latter, together with developmental disorders, could involve deregulated LLPS due to the expansion of repeat sequences within TFs (Basu et al. 2020). Another study linked the formation of nuclear droplets to drug targeting and metabolism via the preferential enrichment of anti-cancer drugs in CSCs (Klein et al. 2020). However, as discussed here, a number of considerations and findings challenge the general application of the LLPS mechanism to chromatin that need further investigation: (i) The formation of a CSC is clearly different from the assembly of a complex that comprises protein and RNA, such as a cytoplasmic P body, which are devoid of chromatin. The binding of proteins and RNA to clustered sites on a mostly immobile chromatin scaffold could be fully sufficient to target genome-associated activities to specific loci in the nucleus. Thus, invoking LLPS to rationalize local chromatin enrichment might be a solution to a problem that does not exist for chromatin patterning in many instances. (ii) CSCs have very heterogeneous properties as illustrated here for three exemplary cases. Thus, a “one size fits all” approach does not seem appropriate to rationalize how CSCs are formed. Accordingly, a more systematic comparison of different mechanisms and cell types against each other is needed that considers the scenarios depicted in **Fig. 1**. (iii) Informative material properties like high-resolution structure, mixing within the CSC versus the exchange with the surrounding nucleoplasm, RNA/DNA/protein content and local viscosity need to be determined in a consistent and well-defined manner in living cells. In some instances, the currently available results argue in favor of an LLPS while in others against it. (iv) A general challenge in the field of chromatin organization is to derive structure-function relationships for a given CSC. This is exemplified by the well-established organization of the genome into TADs. Despite their ubiquitous presence across organisms, defining the specific functions of TADs has proven to be difficult (Beagan and Phillips-Cremins 2020; Cavalheiro et al. 2021). Likewise, for LLPS even for artificial systems with ectopic expression of factors, evidence is often lacking that the transition from direct and indirect chromatin binding to a phase-separated droplet state is associated with functional changes. (v) Perturbation experiments of proteins and RNA factors as well as chromatin states are highly informative to reveal underlying organization principles and could be integrated with structural features in high-content screening

approaches (Berchtold et al. 2018). In combination with appropriate readouts structure-function relationships can be revealed. Thus, perturbation analyses should be integrated more frequently into studies of phase separation in chromatin. In summary, an integrative approach that considers different mechanisms across a variety of CSCs is needed to elucidate the role of phase separation as a self-organizing principle of chromatin domains. Towards this goal the "infusion" of the field by biophysical experimental methods and quantitative mechanistic models in the context of phase separation studies creates a unique opportunity to take our understanding of chromatin patterning and its functional consequences to the next level.

Acknowledgments

I am grateful to Maïwen Caudron-Herger, Akis Papantonis, Jorge Trojanowski, Lukas Frank, and Robin Weinmann for discussion. Work from my laboratory is supported by DFG Priority Program 2191 "Molecular Mechanisms of Functional Phase Separation" via grant RI1283/16-1.

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