Repetitive RNAs as Regulators of Chromatin-Associated Subcompartment Formation by Phase Separation

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Abstract

Repetitive RNA (repRNA) sequences emerge as important regulators of the dynamic organization of genomic loci into membrane-less subcompartments with distinct nuclear functions. These domains include sites of active transcription like the nucleolus as well as (peri)centromeric and telomeric satellite repeats. Recent studies point to an important role of repRNAs in complex with proteins to promote a phase separation-driven formation of chromatin domains. We review how key features of the phase separation process can be revealed by different experimental approaches and discuss the associated structure–function relationships for chromatin subcompartments that involve repRNA.

Genome Organization by Phase Separation

The eukaryotic nucleus has a typical diameter of 10–20 μm without internal membranes, and proteins can transverse through it by diffusion within seconds. Nevertheless, the nucleus is structured into subcompartments (also referred to asmembraneless organelles or nuclear bodies) that can dynamically partition the genome in a self-organizing manner [1–5]. RNA plays a crucial role as an architectural factor at ribosomal genes in the nucleolus, (peri)centromeres, telomeres or the inactive X-chromosome as described in a number of reviews [6–10]. Based on initial studies of the nucleolus, the model of a liquid droplet-like structure established by a liquid–liquid phase separation (LLPS) has been developed that rationalizes a highly dynamic but yet confined chromatin subcompartment organization [11–13]. LLPS as well as other phase separation (PS) mechanisms are described in detail in a number of excellent reviews [14–17], and distinctive features in the context of chromatin subcompartment formation have been discussed recently [18–20]. LLPS creates two liquid-like phases, a droplet-like cellular subcompartment and the surrounding nucleoplasm, similar to the demixing of oil drops in water. It is driven by multivalent interactions and frequently involves RNA interactions with low complexity intrinsically disordered regions (IDRs) of a protein that change the association properties of the complex [21–26]. IDRs are also commonly found in many chromatin-binding proteins [27] and are positively correlated with the propensity of a given protein to undergo an LLPS [28]. Here, we focus on the role of repetitive RNA (repRNA) sequences for regulating the formation of chromatin-associated subcompartments *via* different types of PS. These RNAs comprise tandem repeats that are present at satellite DNA elements like (peri)centromeres and telomeres. In addition, they can originate from interspersed repeat sequences of transposable element (TE) sequences that constitute up to ~40% of mammalian genomes [29]. Some TEs are transcribed into abundant RNA species with specific functions in nuclear compartmentalization as discussed in further detail below, while others have evolved to function as *cis*-regulatory elements for protein coding gene networks [30]. Given the high copy number, clustered distribution and partial self-complementarity, repRNAs bear the potential to function as platforms for locally concentrating protein factors and to

nucleate PS processes: (i) The repRNA sequences may provide a scaffold with locally enriched binding sites for interacting proteins, especially for those that contain tandem repeats. (ii) They may interact with themselves *via* self-complementary sequence motifs to provide additional interactions in RNA–protein assemblies. (iii) By interaction with IDRs, repRNAs might regulate their association into liquid droplets as mentioned above. (iv) They could be involved in cross-linking of regions of the nucleosome chain to induce the compaction of chromatin domains. In this review, we discuss the role of repRNA in PS-driven formation of chromatin subcompartments by different mechanisms.

The Different Flavors of Chromatin PS Processes

Chromatin subcompartments are frequently assigned based on the local enrichment of a given marker protein, e.g., nucleolin (NCL) or nucleophosmin (NPM) for the nucleolus [31], promyelocytic leukemia (PML) protein for PML nuclear body complexes at telomeres [32] or heterochromatin protein 1 (HP1) for pericentric heterochromatin compartments [33]. In the context of cellular PS, the term "biomolecular condensates" or simply "condensates" has been coined and implies assembly of macromolecules into a membrane-less supramolecular complex by PS [15,34]. A local enrichment of a factor with more or less sharp concentration boundaries to the surrounding nucleoplasm on fluorescence microscopy images into what traditionally has been termed nuclear "foci," "puncta," "bodies" or "speckles" is frequently taken as an initial finding that could point toward a PS process being involved. However, it is noted that a more formal definition would require it to demonstrate that indeed a given physical property, e.g., the protein concentration, is uniform throughout the subcompartment. For example, PML complexes might appear as homogeneous liquid-like droplets at diffraction-limited image resolution but rather organize into spherical shell-like structure [35]. Likewise, the PS boundary should display a steep concentration increase that can be distinguished from the enrichment due to "simple" binding to a cluster of sites on the nucleosome chain. Finally, the examination of a cellular subcompartment in steady state is not sufficient to conclude that it is indeed a PS product rather than being formed by coalescence of smaller structures, active transport processes or local macromolecule synthesis [36]. Accordingly, using the terms "phase separation" and "biomolecular condensates" needs to be justified beyond simply reporting a local enrichment of a specific factor in a chromatin associated domains. Finally, PS covers a broad range of different mechanisms that include liquid–liquid, liquid–gel and liquid–solid phase transitions, and it is not straightforward to distinguish between them in the native cellular environment [14,17,37]. With these caveats in mind, we here discuss three different prototypic PS scenarios, LLPS, liquid–gel phase separation (LGPS) and polymer–polymer phase separation (PPPS) that are informative for the mechanism of an RNA-driven chromatin subcompartment formation and are conceptually different from "simple" binding to a cluster of binding sites (Figure 1). The three different PS mechanisms share a common origin with respect to their targeting to certain genomic regions *via* specific binding sites. This chromatin binding step could involve RNA*via* transcription factors that bind certain RNA secondary motifs [10], RNA–DNA hybrids (R-loops) or RNA–DNA triplexes [38]. However, the relevance of these interactions in the context of PS, e.g., as nucleation sites for an LLPS/LGPS or as linkers between chromatin segments in PPPS processes, remains to be established.

For LLPS, the presence of liquid-like properties of the subcompartment is a defining feature. It emphasizes that macromolecules constantly rearrange their position with respect to each other in a random manner like molecules in a liquid, in a volume confined by the PS boundary. This behavior is to be distinguished from the fast exchange of a protein between a chromatin bound and unbound state, which would simply reflect transient binding with short residence times. Liquid droplets formed by association of oppositely charged molecules like negatively charged RNA interacting with positively charged proteins/peptides are referred to as "coacervates" [39]. The PS process can also involve a liquid–gel transition [14,17,22,40]. For gels that comprise a hydrophilic cross-linked polymer network, the term "hydrogel" is used to emphasize it has a high water content. An example for this state would be an agarose gel as it is used for gel electrophoresis of nucleic acids. Hydrogels can form in an RNA-dependent manner and might regulate transcription and chromatin organization as discussed in further detail below [40–43]. For LGPS, the mixing of gel constituting components within the PS compartment is largely reduced as compared to LLPS. Finally, a third type of PS can occur with respect to the conformation of the nucleosome chain and is referred to here as PPPS. It is characterized by a sharp transition of the chain from an open random coil conformation into an ordered and "collapsed" chromatin globule as it is induced by embedding the polymer in a bad solvent [44]. If this process involves only parts of a polymer, it is called a block copolymer microphase separation [45]. It is driven by an attractive interaction between segments. For the nucleosome chain, the latter can involve localized bridging interactions mediated by

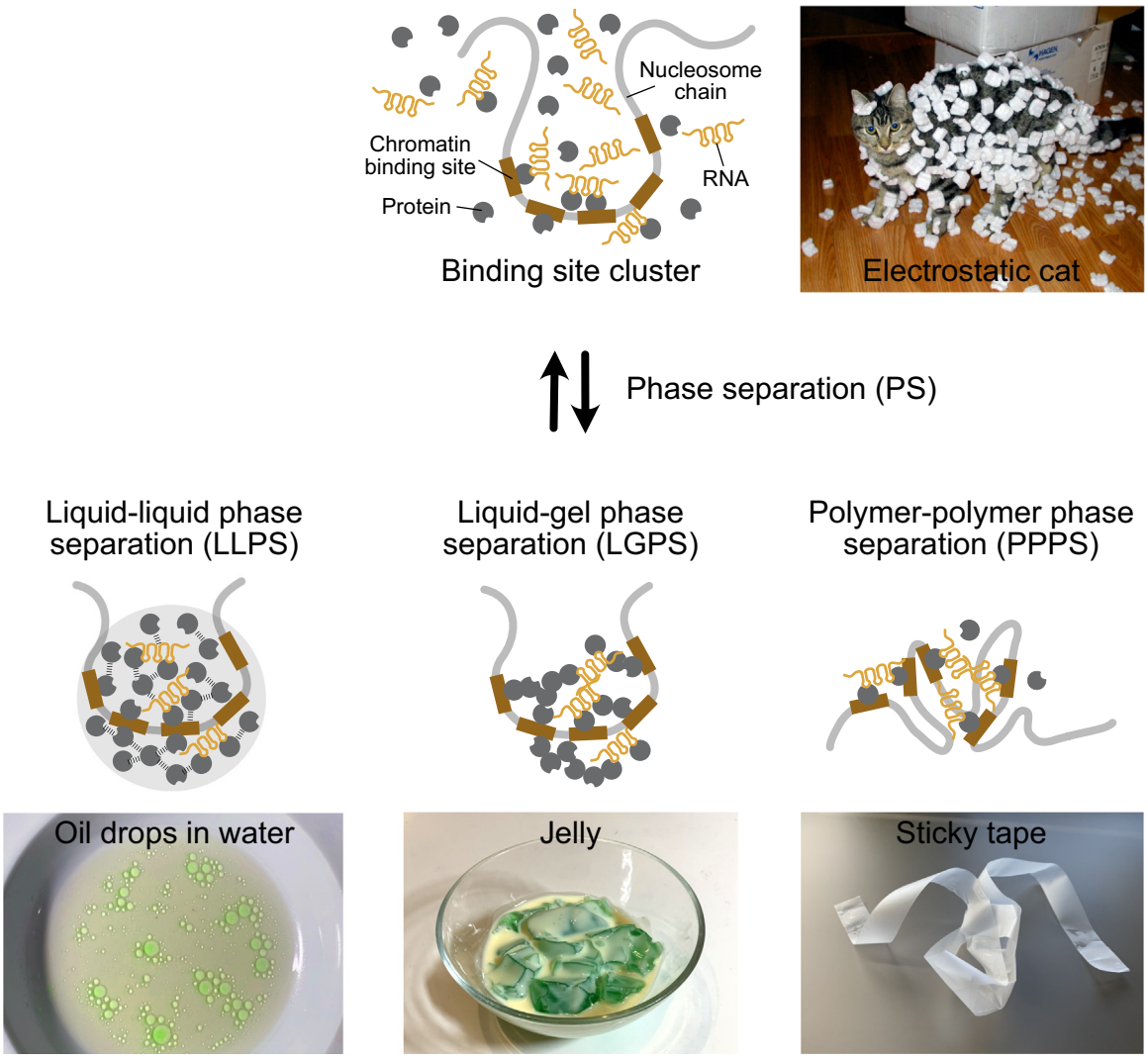

Figure 1. Different types of RNA-driven PS of chromatin domains. The clustered binding of proteins and RNA to genomic loci alone can compartmentalize activities by locally confining enrichment of certain factors on the nucleosome chain as shown at the top of the figure. This is illustrated by the "electrostatic cat" example. Due to the triboelectric effect, the electrically charged cat's fur acts as a "binding site cluster" for the styrofoam chips that are distributed in its environment (original image by Sean McGrath, NB, Canada). In the context of PS processes, clustered chromatin binding sites are essential to target a PS process to a certain chromatin locus. They can serve as nucleation sites for the further accumulation of protein and RNA by the LLPS or LGPS mechanisms depicted at the bottom. In LLPS, factors constantly re-arrange like solvent in a liquid, but clearly separate from the surroundings like in the "oil drops in water" model. An LGPS comprises organization into gel-like assemblies, which remain percolated by the solvent ("jelly" model). In a PPPS process, proteins and/or RNA form a linker that bridges two segments of the nucleosome chain. If these attractive interactions become sufficiently strong, they can induce a local collapse of the nucleosome chain into a "chromatin globule." This process is represented by the "sticky tape" model where parts of the adhesive tape stick together. Note that the three different mechanisms could also occur in combination. For further details and functional differences, see text and Table 1.

chromosomal proteins [46–49]. It is noted that LLPS,LGPS and PPPS are not mutually exclusive and that in reality frequently contributions from different mechanisms are present. For example, many low-complexity protein domains can form liquid-like droplets [50,51] but also have the propensity to polymerize into labile amyloid-like fibrils, e.g., *via* beta-sheet stacking [52]. This seemingly controversial observation indicates that self-assembly of intrinsically disordered proteins can occur through different PS mechanisms, potentially also with phase transitions that are mixed and/or

Table 1. Features of chromatin subcompartments formed by different PS mechanisms

	Binding site cluster	LLPS	LGPS	PPPS
Preferential internal mixing[a]	No	Yes	No	No
Chemical environment same as in nucleoplasm[b]	Yes	No	Mostly yes	Mostly yes
Coalescence[c]	No	Yes	Yes	Yes
Chromatin compaction[d]	No	Possible	Possible	Yes
Dependence of size on concentration increase[e]	None (except for unspecific binding)	Expansion	Expansion	None (for persistent bridging interactions)
Concentration fluctuation buffer capacity[f]	None	High	Low	Low
Exclusion dependence of other factors[g]	Fully accessible	Dominated by solubility in the phase	Dominated by particle size	Dominated by particle size
Exchange rate with other factors[h]	Fast	Slow	Fast	Fast

The table refers to the four different cases depicted in Figure 1 in their pure form. In reality, different combinations and intermediate states occur. For example, clusters of specific chromatin binding sites occur as part of the LLPS, LGPS and PPPS mechanisms.

[a] Macromolecules in LLPS subcompartments have per definition liquid-like properties so that their mixing within the compartment should occur fast. For gel- or chromatin globule-like states the constituting factors organize into more rigid structures. This does not exclude a simultaneously occurring exchange with molecules from the outside.

[b] In an LLPS, the accumulation of RNA and protein into a homogenous liquid-like droplet creates a chemical microenvironment that is different from that of the nucleoplasm, whereas in an LGPS and PPPS scenario, the compartment is still percolated by nucleoplasm.

[c] All three types of PS can exhibit coalescence.

[d] Chromatin compaction in a globule state is a defining feature of PPPS. LLPS or LGPS can occur without any change of the chromatin compaction state. However, they might induce a local environment that favors or initiates PPPS mediated compaction.

[e] LLPS and LGPS are not size buffered. Increasing concentration of the PS driving factor either expands liquid-like droplet volume in order to reach its steady-state concentration or extends the gel structure as additional binding partners associate to the existing assembly. The PPPS domain is size-buffered over the concentration regime that does not significantly affect bridging interactions.

[f] LLPS has the unique property that the liquid droplet state creates stable concentrations inside and outside the compartment.

[g] Passive access to the subcompartment from the surrounding nucleoplasm can be regulated by chemical properties (e.g, charge, LLPS) or size with compartment barriers acting as “molecular sieves” (LGPS, PPPS).

[h] LGPS and PPPS subcompartments are percolated with soluble factors from the surrounding nucleoplasm (unless restricted by their size) so that they can exchange fast.

change over time. Such a behavior was shown for both native and light-inducedFUS protein droplets, which can “mature” from a liquid-like state to exhibiting gel-like behavior (LGPS), up to even undergoing amyloid fibril-like aggregation, also termed liquid–solid PS [53,54].

Experimental Approaches to Study PS Processes

The mechanisms depicted in Figure 1 all segregate chromatin into distinct and mostly spherical structures, but the properties of the resulting assemblies are very different (Table 1). Molecules that form by LLPS tend to stay within the resulting liquid droplets with fast internal mixing, create a specific chemical microenvironment and insulate chromatin regions from each other. An LLPS compartment is concentration-buffered as it maintains a constant concentration of molecules in its interior against fluctuations from the outside. However, the droplet size follows concentration changes so that the chromatin content increases at higher concentrations. This feature could thus present a mechanism for spreading a given chromatin state. LGPS and PPPS subcompartments on the other hand are percolated with soluble factors from the surrounding nucleoplasm, but the size of macromolecules defines the accessible space [55]. Accordingly, factors within a LGPS and PPPS subcompartment can exchange fast with the surrounding space. The size of LGPS domains will also fluctuate with concentration as for LLPS. For the PPPS mechanism, however, domain size should be constant over the concentration range that leaves the driving attractive interactions between chromatin segments mostly unaffected. Since these and other key hallmarks of PS subcompartments are functionally relevant and need to be experimentally addressed, we summarize strategies to measure them in the following.

Concentration dependence and coalescence/dispersion

In vitroPS analysis

An essential part of any characterization of cellular subcompartments in the context of PS processes is identifying conditions for which a well-mixed solution undergoes a PS [56]. This issue can be directly addressed *in vitro* by varying relevant parameters (macromolecule composition, concentration, temperature, pH, ionic strength) to generate a phase diagram that defines the regime for which a PS is observed. These experiments typically comprise an analysis of liquid droplet formation or other types of

macromolecular associates by microscopy or other methods [56,57]. Examples for studies of RNA-dependent droplet formation are given in Refs. [25, 39, 58]. In order to quantitate the concentration dependence of the PS process, the turbidity of the solution can be determined by absorbance spectroscopy as described previously [57,59,60]. It involves measurements at wavelength where protein and nucleic acids do not absorb, e.g., 340 nm and above. The resulting signal reflects scattering of light in dependence of particle size and can be exploited to trace the formation of droplets or other particle types. In the context of the RNA-dependentchromatin-associatedPS discussed here, one challenge is it to adequately represent the chromatin part *in vitro*. Mono- and oligonucleosome particles can be reconstituted *in vitro* and have been shown to undergo an LLPS themselves [61–63]. However, it is difficult to envision how such a transition could occur in the context of a human chromosome that is connected by a continuous nucleosome chain that varies between 5 and 24 nm in diameter [64] and stably occupies a distinct territory in the nucleus [65]. In this environment, the nucleosome translocations on the second to minute time scale are confined to local movements of chain segments within a radius of about 70–80 nm [66–69]. Accordingly, chromatin appears in the cell as a mostly immobile scaffold as also reflected by the hour-long persistence of fluorescence bleach patterns of histones and lack of local positional changes [70–73]. How these pertinent cellular chromatin features can be adequately represented in *in vitro* experiments for studying the assembly of a chromatin subcompartment is currently an open question. A critical test whether the formation of chromatin droplets observed with mono- and oligonucleosomes occurs also with longer nucleosome chains would be to study the dependence of LLPS propensity on chain length. Technically, this can be accomplished by preparing native chromatin fragments up to 60–70 nucleosomes in size from human cell lines (e. g., [74]) or chromatin reconstituted *in vitro* with *Drosophila* extracts on DNAs that can exceed 40 kb (or 220 nucleosomes) in length [75].

Response to concentration changes

The response to concentration changes of protein and nucleic acids is a crucial parameter for PS diagrams that can be examined directly *in vitro* as described above. In the cell, over-expression or knockdown of phase-separating protein and RNAs in conjunction with quantitative microscopy readouts of local concentrations and subcompartment size is highly informative. For LLPS, the total cellular droplet volume scales with the cellular concentration of the phase-separating factors, while their concentration within the droplet is buffered [15]. For LGPS, the gel structure should also expand at increasing concentration as additional binding partners associate to the existing scaffold. A collapsed chromatin globule formed by PPPS, however, should behave differently and remain constant in size unless protein/RNA concentration affect the attractive properties of chromatin segments within the PS domain.

Coalescence and dispersion

The fusion/fission of liquid droplets is a crucial LLPS feature inherent to its fluid-like properties. It is not observed for domains that originate from (cooperative) binding to chromatin (Figure 1). The coalescence/dispersion process can be directly observed by microscopy, both *in vitro* and in living cells as done for the nucleolus [11,13,76] or for ectopically formed RNA–protein assemblies [25]. Interestingly, also collapsed polymer regions in PPPS that are separated by freely fluctuating chain parts can undergo coalescence after collision [77]. This process, however, requires that the attractive interactions between polymer segments are interchangeable between different globule regions and can dynamically rearrange on the time scale observed. For an LGPS, dynamic reorganization might be very slow (especially for the case of chemically cross-linked hydrogels), leading to a kinetically trapped state that would be incompatible with coalescence.

Structure, dynamics, and physicochemical properties

Subcompartment structure

Fluorescent microscopy-based approaches provide a wealth of information on RNA-dependentPS within the endogenous cellular environment. Candidate RNAs can be transcribed *in vitro* (and also fluorescently labeled if desired) and delivered into cells by microinjection or transfection to examine their coalescence/dispersion behavior or association with specific proteins over time [78]. The sequence specific tagging and fluorescent labeling of RNA with autofluorescent domains can be accomplished by engineering a high-affinity protein binding sequence in one or more copies into the RNA of interest ([79,80] and references therein). Examining the subcompartment's internal structure by fluorescence microscopy, preferably at super-resolution, provides crucial mechanistic information. A homogeneous distribution of components is indicative of LLPS, while the observation of distinct micro-domains would argue against a liquid-like state, at least at the level of the subcompartment.

Dynamics and accessibility of phase-separated factors in the nucleus

As discussed above, the kinetics of internal mixing and exchange with the nuclear environment distinguish between different PS mechanisms. This process can

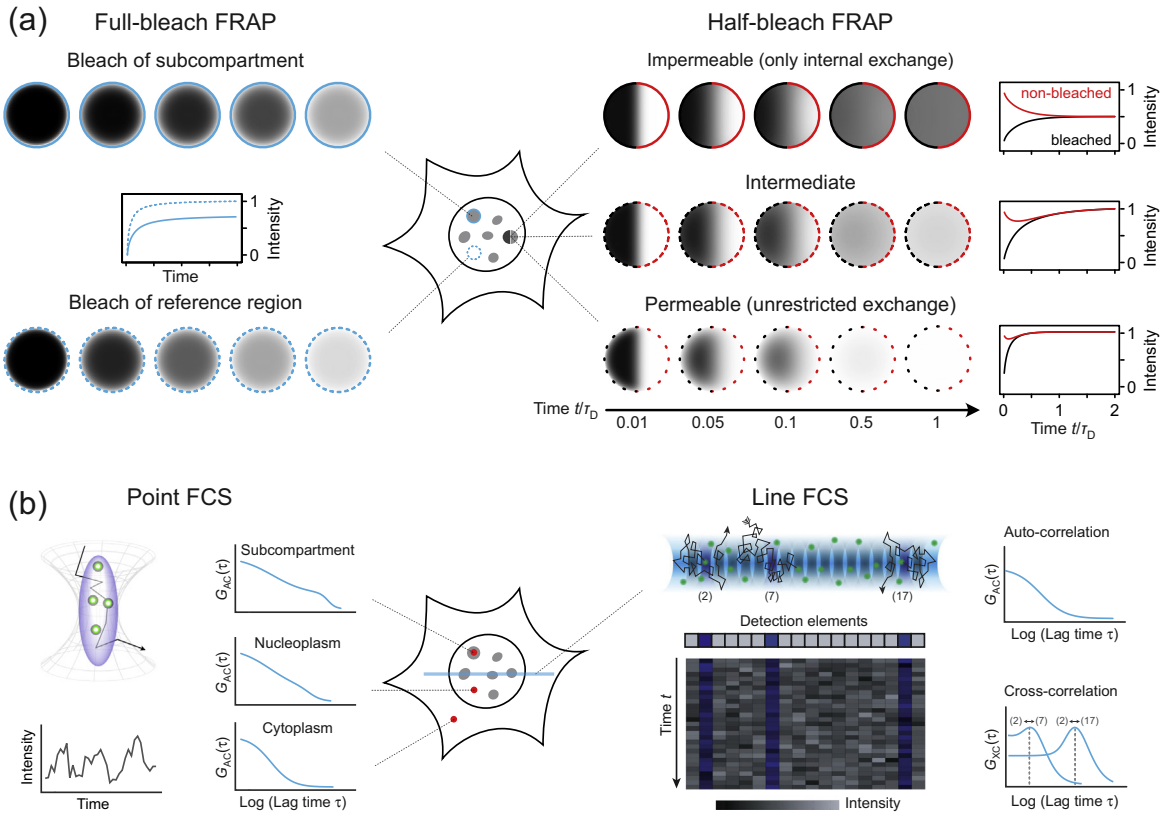

Figure 2. Experimental approaches to study protein mobility features informative about PS mechanisms by fluorescence fluctuation microscopy methods. (a) FRAP experiments that bleach the complete subcompartment ("full-bleach FRAP," left panel) in comparison to a reference region in the nucleoplasm reveal differences in protein–protein and protein-chromatin interaction. Right: Half-bleach FRAP. By bleaching only half of the subcompartment internal mixing and permeability of the boundary can be evaluated. The transport of molecules between the two parts reflects internal mixing, which can be then compared to the exchange with molecules from the surrounding nucleoplasm. 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. Further details on this approach are given by Erdel *et al.* [90] from which the figure panel was adapted. (b) In normal FCS fluorescent proteins enter and leave the excitation volume of a confocal microscopy by diffusion ("point FCS"). The resulting local fluctuations in the fluorescence signal are detected and used to compute auto-correlation (AC) functions that reveal spatially resolved protein mobility by subsequent measurements at different cellular loci, e.g., the subcompartment of interest, the nucleoplasm and the cytoplasm. Right: FCS with line-illumination ("Line FCS") allows it to compare protein mobility throughout the compartment and surrounding regions, either by repetitive fast scanning or, as depicted by parallelized multifocal fluorescence signal detection [55,81,82]. By applying correlation analysis to the fluorescence signal recorded at a given detector pixel, AC curves can be calculated at every pixel position. Correlation of signals from spatially separated detection volumes are evaluated by computing cross-correlation (XC) curves (e.g., signals of detection volumes 2 and 7 or detection volumes 2 and 17). In this manner, the transport in and out a compartment can be directly measured. The figure panel was adapted from Baum *et al.* [55].

be measured in living cells by single particle tracking and a variety of fluorescence bleaching and correlation spectroscopy approaches to compare the mobility inside/outside of the compartment of interest and to measure transport in and out of the compartment [4,81–83]. For structures in the 1-µm size range and above, fluorescence recovery after photobleaching (FRAP) can be applied. While it has been recently argued that FRAP would not be suited to test liquid-like properties [84], we do not share this view. Rather we consider FRAP measurements according to the different experiments depicted in Figure 2(a) as highly informative on protein mobility properties associated with different mechanistic features of nuclear subcompartments. By locally bleaching fluorescently tagged marker proteins of interest, its exchange can be determined and compared to other regions as, for example, done in our previous work for proteins that mark mouse pericentric heterochromatin [85,86]. Interactions can be identified *via* a reaction–diffusion analysis that are specific for a nuclear subcompartment of interest and absent in other regions of the nucleus [87]. In another type of FRAP experiment, only part of the subcompartment is bleached to directly measure the contribution of fluorescence recovery due to internal mixing of proteins [54,88–90] (Figure 2(a)). A recent application of this approach demonstrates how the preferential internal mixing (or low permeability at the

boundary) of a subcompartment can be quantitated and compared between different types of chromatin subcompartments [90]. A method providing complementary information to FRAP is fluorescence correlation spectroscopy (FCS) that can be conducted at different points in the cell (inside *versus* outside of the subcompartment of interest), along a line or at a light-sheet plane [81,82] (Figure 2(b)). The multi-pointFCS methods are particularly informative to directly measure protein transport boundaries for a given marker protein *via* a spatial cross-correlation analysis by simultaneous measurements at different cellular locations [55], although this approach has been hardly exploited in the context of PS processes. Finally, assessment of the local particle exclusion behavior with tracers, e.g., GFP monomers and oligomers, can reveal whether compartment accessibility is dominated by particle size *versus* chemical properties like charge or hydrophobic interactions [55,91,92]. For nucleoli, a prototypic example for a liquid-like subcompartment, it was demonstrated that nucleolar exclusion of wild-typeGFP could be completely reverted by addition of small arginine-rich peptides conferring a strong positive charge [93].

Chemical properties of phase-separated subcompartments

For an LLPS subcompartment that contains a fluid-like mixture of proteins and RNA, one would expect distinct changes of the chemical microenvironment that manifest themselves by differences in viscosity, pH, dielectric permittivity, hydrophobicity, macromolecular crowding etc. These parameters are highly relevant for the potential acceleration of the biochemical reactions due to special microenvironment created by a given subcompartment [94], and a number of molecular sensors that exhibit a change in their emission intensity or spectrum in response to the different features are available [95–97]. To probe the intracellular viscosity, the rotational diffusion coefficient can be measured by different methods that include (i) time-resolved anisotropy using fluorescence microscopy [98], (ii) extrapolation to short diffusion times coefficient of GFP tracers with measurements by multi-scale fluorescence cross-correlation spectroscopy [55,99] or (iii) polarization-sensitiveFCS to measure the local viscosity experienced by a given protein from its rotation diffusion time [90].

Ectopic induction of liquid droplets and RNA bodies in living cells

In order to investigate the link between RNA and nuclear bodies formed by PS within the cell, a number of approaches can be used. For a mechanistic analysis, it is also important to precisely control the PS process and follow it over time. This can be accomplished by fusion or tethering of a protein of interest to a domain that can be induced to promote association into liquid droplets. Subsequently, the stability and properties of these structure can be measured over time to quantify the capacity of candidate factors to phase separate (Figure 3(a)). An attractive system for performing such experiments are droplets formed by light-induced oligomerization of the CRY2-derivedPHR domain from plants [100]. Such “opto-droplet” systems have proven useful to induce the assembly of liquid-like RNA/protein body and to assess the PS propensity of proteins [53,90,101]. Another system to study the concentration-dependent RNA–protein PS in the cell has been induced recently and employs the formation of an ectopic multivalent scaffold of a protein of interest [25]. With this system, endogenous RNAs were found to locally enrich and to nucleate an LLPS.

To dissect the role of RNA for assembling of a chromatin-associated subcompartment, different strategies are depicted in Figure 3(b). Local enrichment of an RNA of interest on chromatin can be achieved by ectopic transcription [102] or tethering RNA to repetitive sequences [103] to nucleate the formation of “RNA bodies". This approach can also be applied to test for RNA-induced histone modification and compaction/decondensation of chromatin domains that would be associated with a PPPS transition [79]. Alternatively, RNAs can be tethered to endogenous loci by using the CRISPR-Display method, which involves dead-Cas9 (dCas9) targeting by fusion of target RNAs to a sgRNA [104]. These approaches can also be combined with opto-droplet systems (Figure 3(a)) and targeting of the droplet inducing factor to chromatin *via* dCas9 [101]. The resulting RNA-induced chromatin subcompartments can be examined by using the above-mentioned assays to gather information about a PS mechanism in the endogenous cellular environment.

RepRNAs Involved in Establishing Active/Silenced Chromatin Domains

The inherently multivalent repRNAs represent genome organizing factors that can interact simultaneously with multiple IDRs of chromosomal proteins and thus drive PS processes. Indeed, an increasing number of studies supports such a role for repRNAs and a number of specific cases are discussed in the following.

RNA-mediated nucleolus organization

The nucleolus represents a prototypic model for an LLPS induced droplet-like structure that becomes separated from the nucleoplasm and has NCL,NPM and fibrillarin as key marker proteins [105–107]. It contains hundreds of nucleolar proteins and large

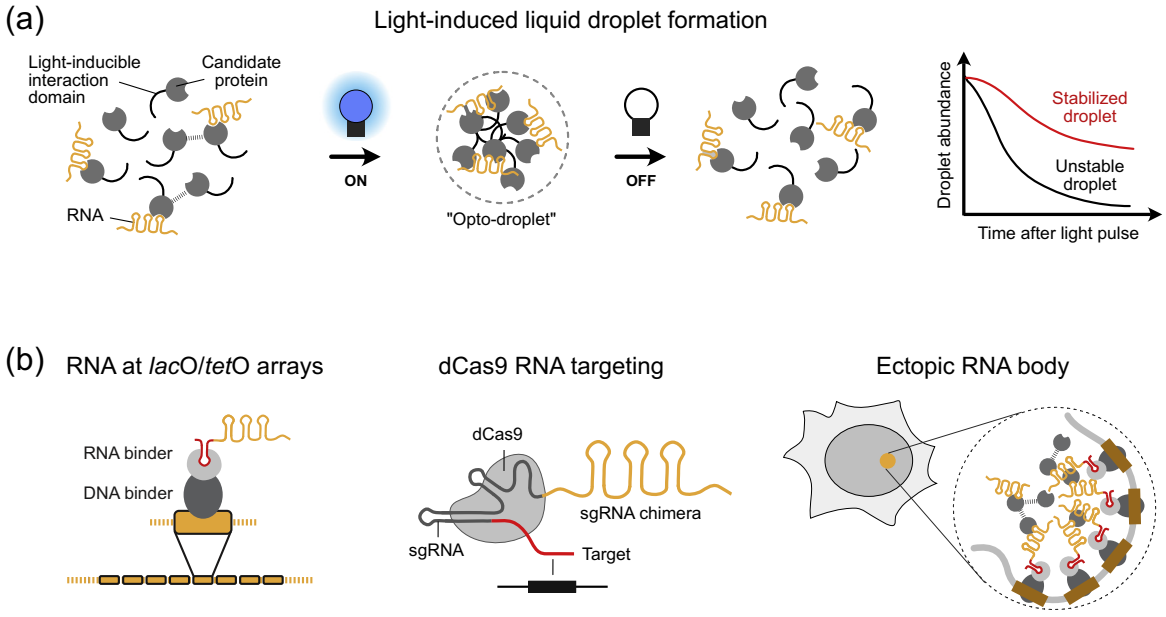

Figure 3. Strategies to induce ectopic RNA bodies to characterize their PS properties. (a) Light-induced formation of opto-droplets to assess the propensity of candidate proteins interacting with RNAs to form RNA–protein bodies [53]. The protein of interest is fused to the PHR domain, which promotes an LLPS upon illumination with blue light. The resulting RNA–protein bodies can be studied with respect to their stability in the absence of the light trigger. In a similar fashion, candidate RNAs with engineered high-affinity protein binding sites (e.g., MS2 loops) can be assembled into light-dependent droplets by fusion of PHR to the corresponding RNA binder (e.g., MS2 coat protein). (b) Formation of ectopic RNA bodies induced by tethering RNA to chromatin at *lac*O or *tet*O arrays with LacI/TetR fusion constructs at endogenous loci *via* the CRISPR-Display method [104]. The chromatin-tethered RNAs can nucleate a PS process to form a distinct chromatin subcompartment that can be studied with respect to its biophysical properties like, for example, internal protein mobility.

amounts of rRNA that are produced from long rDNA repeats within the nucleolar organizer regions [31,108] with typical volumes of 50 μm^3 (sum of all nucleoli) and 600 μm^3 (nucleus) measured for human cell lines [109]. Many of the rDNA genes are transcriptionally silenced *via* repressive chromatin modifications, but the active and silenced states are interspersed [110]. The average protein concentration is about 2-fold higher in the nucleolus as compared to the nucleoplasm with values of 0.20 g/ml and 0.11 g/ml, respectively [111]. The nucleolar RNA concentration is ~100-fold higher than the surrounding part of the nucleus [112], which would correspond to approximate concentrations of 10^8 and 10^6 nucleotides/μm^3 (or 50 and 0.5 mg/ml, respectively) for a value of 3 pg RNA in the nucleus [113]. In contrast, the estimated DNA content of the nucleolus is about 20 times lower than the nuclear average. The human rDNA repeats are 43–45 kb long and present in 300 copies [108]. This amounts to about 26 Mb of rDNA in a diploid human cell nucleus, which yields DNA concentrations of 0.5 Mb/μm^3 (or 0.6 mg/ml) *versus* an average of 12 Mb/μm^3 in other regions of the nucleus. Thus, the dominating contributions for the LLPS-driven structure of the nucleolus are likely to arise from multivalent protein interactions that are modulated by interactions with its RNA components, while the relatively low concentration of rDNA appears to be relevant for nucleating this process [114]. At the onset of mitosis, the nucleolus disassembles ([115] and references therein). Its reassembly requires RNA polymerase (Pol) I activity and the restart of rRNA production. In the absence of Pol I transcription, segregated mininucleoli with NCL, fibrillarin, and pre-rRNAs are formed at the nucleolar organizer regions of the genome [116]. For the nucleation of such droplets, rRNA plays a crucial role [107,117]. Inhibition of Pol I activity during interphase induces a decrease in the size of nucleoli and rDNA condenses into nucleolar caps [118]. Interestingly, a full dispersion of nucleoli throughout the nucleoplasm was only observed when Pol II instead of Pol I was inhibited [78], which also impaired post-mitotic assembly [115]. This process was linked to Alu element-containing RNAs (*alu*RNAs) originating from introns of Pol II transcripts that were enriched in nucleoli. It was suggested that *alu*RNAs can shift the equilibrium between the coalescent and dispersed state of nucleoli *via* an LLPS transition by interacting with IDRs of NCL and NPM [78,115]. The relatively small prenucleolar bodies containing NCL,NPM and rRNA

appear to require the association with Pol II*alu*RNA transcripts as the "glue" to assemble them into complete nucleoli. In addition to having a role in nucleolus formation, multi-phase separation was suggested as a model that functionally compartmentalizes rDNA transcription, rRNA processing and rRNA-ribosomal protein assembly within the fully assembled nucleolus [13].

RNA polymerase II subcompartments

Transcriptionally active subcompartments of Pol II have been termed "transcription factories" based on the initial observation that multiple Pol II complexes organize into distinct nuclear foci [119,120]. Transcription factories comprise Pol II, transcription factors, promoter/enhancer DNA and RNA, and numerous studies have characterized their features as well as their role as self-assembling organizers of the genome [2,5,121–123]. Recently, IDR containing components of transcription factories were reported to undergo an LLPS transition *in vitro* to form "transcriptional condensates" in different mixtures and nucleated by DNA in some instances. This process was described for the carboxy-terminal domain (CTD) of Pol II in dependence of its phosphorylation [124–126]. An LGPS transition of the CTD to form a phosphorylation-dependent hydrogel nucleated by RNA was observed for the CTD interacting with TAF15 and FUS IDRs [40–42]. In addition to the CTD, also transcriptional activators like MED1, BRD4, OCT4, GCN4, β-catenin, STAT3 and SMAD3 displayed an LLPS *in vitro* [127–130]. LLPS features were also observed in the cell upon binding of transcription factors TAF15 and SP1 to ectopic arrays of *lac*O repeats in dependence of an over-expression of these proteins [131]. Interestingly, also endogenously tagged MED1 and BRD4 [127] as well MED19 and the RBP1 Pol II core unit RBP1 [132] colocalized into nuclear foci that were associated with enhancers and transcriptionally active chromatin. Thus, a number of studies point to the formation of Pol II transcription factories by PS. Interestingly, an analysis of the RNA content of Pol II factories found a substantial enrichment of Alu and LINE1 (L1) RNA [133]. In the light of the finding that RNA can nucleate CTD interactions [42], this raises the possibility that these repRNA sequences are involved in establishing the Pol II subcompartment structure. Thus, it will be important to further characterize interactions between RNA, the CTD and transcription factor IDRs in Pol II transcription factories and evaluate them with respect to functionally relevant PS features within the endogenous cellular context. In addition, alternative chromatin binding mechanisms need to be considered. For example, it was shown that enrichment of Pol II and transcription factors in replication compartments of the herpes simplex virus appeared to be mostly driven by the creation of nucleosome free unspecific DNA binding sites and not by LLPS [134].

RNA from TEs

Repetitive DNA sequences have been implicated in PS processes as reviewed recently [135]. With respect to the corresponding repRNA counterparts, L1 and Alu sequences are among the most abundantly transcribed TE species. Interestingly, proteins encoded by TEs preferentially associate in *cis* with TE-derived RNAs (e.g., Gag, ORF1 proteins) and show a high degree of structural disorder [136]. The L1 and Alu RNA sequences arising from TEs accumulate in distinct nuclear foci that mostly do not colocalize with chromatin [137–139]. However, a euchromatin-associatedRNA fraction in human cells was characterized that comprised predominantly L1 sequences [140]. The loss of these sequences from euchromatin induces an aberrant chromatin distribution and PPPS-like behavior. This finding is in line with the observation that also a class of RNA from coding transcripts is needed to maintain an "open" and decondensed chromatin state [141]. The L1 and Alu RNA-dependent global euchromatin organization as reported [140] was dependent on the nuclear matrix scaffold attachment factor-A (SAF-A, also called HNRPU), which has been shown to regulate chromosome structure through interaction with nuclear RNA [142]. Notably, overexpression of a dominant-negativeSAF-A mutant protein induced the dissociation of RNA and chromatin compaction [140]. Thus, SAF-A and repRNAs may counteract a PPPS leading to a collapsed chromatin state. SAF-A has a RNA-specific binding domain, an IDR and, based on additional functional properties, an LGPS of SAF-A and interacting RNAs to form a hydrogel has been proposed to rationalize its function as an euchromatin organizer [43].

Satellite repeat transcription at pericentric heterochromatin

Pericentric repeat sequences assemble into compacted heterochromatin domains in mouse and *Drosophila* cells and are termed chromocenters due to their intense fluorescence observed after DAPI staining. In recent work, it was concluded that HP1 drives an LLPS *via* multivalent interactions at chromocenters [59,143]. However, alternative mechanisms exist [18,86] and recent experimental evidence argues against an HP1-dependentLLPS for mouse pericentric heterochromatin [90]. Rather, the results point to a PPPS of the nucleosome chain from a decondensed state into a collapsed chromatin globule that is independent of HP1. Another component of chromocenters, the lysine methyltransferase KMT5C (SUV4–20H2) that sets the H4K20me3 modification, is much more tightly

bound than HP1 in these subcompartments with an immobile fraction >90% on the minute time scale [86]. Interestingly, SUV4–20H2 was reported to display a preferential internal recovery at pericentric heterochromatin loci in half-bleachFRAP experiments indicative of a mechanism that confines translocations of SUV4–20 to this region [89].

RNAs derived from the major satellite repeats (mSat) that constitute mouse pericentric heterochromatin are involved in stabilizing these domains [144–146]. The mSat RNAs contribute to the retention of SUV39H1 and SUV39H2 methyltransferases that sets the histone H3 trimethylation at lysine 9 by RNA-nucleosome association and RNA–DNA hybrid formation. Thus, mSat transcription at chromocenters could provide an intrinsic mechanism to re-establish silencing if spurious transcription of this large constitutive heterochromatin region should occur. Such an activity could be achieved by simply binding SUV39H enzyme in an RNA-dependent manner to chromatin in the absence of a PS transition (Figure 1, top). Another role for mSat RNAs at chromocenters involves the SAFB nuclear matrix protein. According to a recent study, SAFB modulates chromatin condensation and stabilizes heterochromatin foci in mouse cells *via* the interactions of its R/G-rich region with mSat RNAs as well as other heterochromatin-associated repeat transcripts [147]. SAFB-GFP formed droplets *in vitro* that associated into larger clusters upon addition of mSat RNA. Based on this and other findings, the authors proposed that some kind of SAFB PS would occur at chromocenters and support the accumulation of more loosely bound HP1. However, given the controversy with regard to intracellular LLPS of HP1 [59,90,143], the mechanism by which mSat RNA-driven interactions with SAFB contribute to chromocenter stability and/or structure in the cell remain to be dissected in further detail.

Xist and L1 RNA-driven X chromosome inactivation

The inactive X chromosome (Xi) in female mammalian cells represents a large transcriptionally repressed chromatin subcompartment. It displays a distinct morphology upon DNA staining on microscopy images as a so-called Barr body that localizes to the nuclear or nucleolar periphery ([148] and references therein). The establishment of this silenced heterochromatic state is mediated by the *Xist* long non-codingRNA (17 kb in length in human) that contains the conserved repeat A (repA) region with up to nine repeated elements. *Xist* and its repA region can associate with components of the Polycomb repressive complex 1 and 2 (PRC1/2), which are involved in Xi heterochromatinization in a complex manner [149]. Furthermore, ectopic tethering of repA induces H3K27me3 [79]. *Xist* shows a spatial association with PRC2 in the nucleus [150] and also drives the formation of an Xi-specific chromatin subcompartment structure *via* recruitment of PRC1 [151]. In addition, L1 RNA has been shown to conspire with *Xist* in the assembly and spreading of the Xi nuclear compartment [152]. It involves a dual role of L1 loci as nucleation sites for X chromosome inactivation and of nascent L1 transcripts expressed from Xi to promote silencing of certain genes.

Recently, it was hypothesized that *Xist* induces an LLPS process that leads to X-chromosome inactivation [153]. In this context, a number of points are noted: (i) *Xist* assembles into distinct nuclear foci [148,153]. (ii) The complex interactome of *Xist* and its repA repeat point to its capability to promote multivalent interactions [149]. (iii) IDR-containing proteins are enriched in the *Xist* interactome [153]. (iv) The PRC1 protein chromobox 2 (CBX2), a member of the CBX protein family, has been shown to form liquid-like droplets *in vitro* and nuclear foci in the cell that overlap with H3K27me3 [154,155] and could be linked to Polycomb bodies [156]. (v) *Xist* foci are frequently located adjacent or partially overlapping to SAF-A foci [148], which has been proposed to undergo an LGPS [43] and interacts also directly with *Xist* [149,157]. Thus, a number of links to potential LLPS/LGPS features of the *Xist*RNA subcompartment exist, which await further experimental examination. It is also noted that the Xi chromosome displays a condensed chromatin structure that segregates it from the surrounding autosomal chromatin [148]. This compaction is indicative of a PPPS in which the Xi chromatin chain over the length of the complete chromosome displays attractive intra-chromosomal interactions that could be mediated by *Xist* and L1 RNA to induce a collapsed globule state.

Telomeres and TERRA

Telomere repeats are maintained in some cancers by an alternative lengthening of telomeres (ALT) pathway in the absence of telomerase. One ALT hallmark is the transcription of telomere repeats into a long non-codingRNA termed TERRA [158,159]. Another well-established cytological marker of ALT is the association of the PML protein with some telomeres to form distinct telomeric subcompartments referred to as an ALT-associatedPML nuclear bodies (APB) [32] that have TERRA enriched [160]. APBs have been shown to mediate telomere clustering in ALT [161–163] and the coalescence/dispersion of telomere-telomere [162,164], PML-telomere [68,164] and APB-APB [165] subcompartments has been demonstrated in living ALT cells. In line with these findings, a recent study reported LLPS-like features of artificially engineered APB-like subcompartments that

promoted telomere clustering as well as other ALT-like phenotypes [166]. Thus, the TERRA enriched APB and telomere subcompartments in ALT cells display PS characteristics. Independent of APBs and the ALT pathway, TERRA itself has the capacity to segregate into distinct micrometer-sized nuclear foci as visualized by fluorescence *in situ* hybridization and live-cell imaging [167,168]. Given the repetitive nature of TERRA as well as its ability to form intra- and intermolecular G-quadruplex structures [169], it is not surprising that TERRA can act as a scaffold for interactions with a variety of factors. The diffusive properties of TERRA in living cells are confined in telomere-neighboring regions of the nucleus, which may represent functional compartmentalization of telomere factors [170]. In fact, TERRA was shown to be involved in nucleation of telomerase molecules into clusters prior to their recruitment at a short telomere in yeast [171]. TERRA is expressed and accumulates into a nuclear focus following critical telomere shortening. The TERRA body then nucleates the recruitment of telomerase components, which are subsequently loaded onto the shortened telomere in late S-phase. Similar functional principles apply to other factors requiring dynamic exchange at telomeres in response to the cellular state. The protein hnRNPA1, for instance, participates in the exchange of the single-strandDNA-binding protein RPA for POT1 at telomeres in late S-phase [172]. Localization and activity of hnRNPA1 at telomeres are counteracted by binding to TERRA during early S-phase. In line with the above mechanism, a recent study provides evidence for sequestering hnRNPA1 into telomere-proximalTERRA foci [170]. Intriguingly, hnRNPA1 was also identified as a low complexity-domainRNA-binding protein that can undergo LLPS*in vitro* and *in vivo* [173]. Accordingly, it is tempting to speculate that TERRA acts in manner conceptionally similar to the NEAT1 non-codingRNA, which can induce the formation of phase-separated paraspeckles *via* interactions with NONO and SFPQ proteins [174]. In fact, TERRA can associate with telomeric chromatin *via* stable RNA–DNA hybrids, which in turn are suppressed by NONO and SFPQ proteins [175].

Finally, it is noted that TERRA is linked to the trimethylation of histone H3 at lysine 9 (H3K9me3) as a characteristic heterochromatic mark in a complex manner [176–179]. In human cells, the H3K9me3 density is inversely related with telomeric TERRA levels [177,178]. At the same time, it has been reported that TERRA interacts with the histone methyltransferase SUV39H1 that sets this mark [179] as well as with the heterochromatin proteins HP1α and HP1β that preferentially bind to nucleosomes that have the H3K9me3 modification [176]. These features of TERRA are reminiscent to the above-mentionedmajor-satellite repeat transcripts at chromocenters that have been reported to recruit the SUV39H methylase to maintain silencing of these loci [144,145]. In addition, the HP1-H3K9me3-SUV39H epigenetic circuit is also involved in the silencing of centromeric repeat transcripts that were linked to telomerase activity regulation in embryonic stem cells [180]. Accordingly, telomeric, centromeric and pericentromeric repRNAs might share common interactions with proteins involved in H3K9me3-mediated heterochromatin formation that lead to the establishment of repressive chromatin subcompartments at these loci.

Regulation of RNA-dependent PS

The nuclear subcompartments described above dynamically respond to different cellular conditions. Chromocenters and nucleoli, for instance, disassemble during cell divisions and are re-established during interphase. Thus, the question arises how the assembly process is regulated. One mechanism to achieve this are cell cycle dependent posttranslational protein modifications that affect protein–RNA interactions. Protein phosphorylation, for example, was shown to control the ability of protein–RNA mixtures studied *in vitro* to undergo an LLPS [39,124,125] or LGPS [40–42]. Likewise, sumoylation was suggested to modulate telomere clustering by LLPS-driven segregation of chromosomal ends into PML-body like subcompartments *in vivo* [166]. Modification of proteins with the nucleic acid-like molecule poly(ADP-ribose) (parylation) was implicated in regulating the liquid PS potential of the stress granule protein hnRNP A1 [181]. In addition, liquid-like properties of some DNA damage foci components were recently described [182] and linked to parylation as a regulatory modification [183]. Multivalent interactions between RNA and proteins could be influenced also by RNA modifications. The presence of multiple N6-methyladenosines was shown to enhance the PS potential of cytosolic N6-methyladenosines-binding proteins, which then partition into specific subcompartments [184]. Finally, it has been demonstrated that highly structured RNAs can bind a large amount of proteins, which affects the distribution of their interactors into different phases [185]. In this scenario, the expression levels of such a protein sequestering RNA would be the factor that modulates the PS process.

Conclusion

repRNAs can interact with proteins and other RNA molecules (including self-interactions), associate with chromatin through multiple mechanisms and act as scaffolding sequences. These features make them prime candidates for playing a role in a PS-driven formation of chromatin subcompartments *via*

different mechanisms as discussed above. In line with this view, an increasing number of studies invoke different PS mechanisms to rationalize the assembly of protein and RNA components at certain chromatin loci. One aspect in this context that so far has received little attention is the link between repRNAs and viral RNAs as many cellular repeats are of viral origin. RNA viruses have evolved to exploit their genome's secondary structure for efficient control of packaging and replication by highly optimized interactions of RNAs and binding proteins. Recent studies in measles [186], influenza A [187] and vesicular stomatitis virus [188] suggest that PS processes could be a general mechanism exploited by viruses to survive and replicate within their hosts.

While *in vitro* experiments provide ample evidence that mixtures of protein, RNA and DNA can indeed undergo an LLPS or LGPS, it is next to impossible to reproduce the complex macromolecule mixtures and solution conditions that are present in the cell. Thus, it is crucial to complement *in vitro* experiments with direct measurements in living cells to test for the presence or absence of functionally relevant PS features along the lines discussed above. The specifics of the PS mechanism that is proposed or tested in a given study need to be clearly defined and compared to (cooperative) binding of proteins and RNA to a chromatin scaffold as the "null hypothesis." Thus, distinctive PS features need to be experimentally demonstrated that go beyond a local enrichment of a given factor into nuclear foci. Toward this goal, identifying and characterizing the functional relevant "material properties" of chromatin subcompartment are highly informative and provide valuable information on their own. Protein and RNA composition, concentration dependence, subcompartment access, viscosity, pH, hydrophobicity, charge density, macromolecular crowding, etc. are essential parameters to rationalize how the efficiency of certain chemical reactions can be locally increased [94]. Perturbing key features identified in this manner and evaluating the corresponding reaction changes could then serve to demonstrate that a given subcompartment indeed creates a specific environment that modulates a specific genome-associated activity. By comparing the properties of PS processes, outlining experimental strategies to assess them and discussing these aspects in the context of specific examples, we hope to stimulate further work in this exciting area of research.

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LLPS, liquid–liquid phase separation; PS, phase separation; IDR, intrinsically disordered region; repRNA, repetitive RNA; TE, transposable element; NCL, nucleolin; NPM, nucleophosmin; PML, promyelocytic leukemia; HP1, heterochromatin protein 1; LGPS, liquid–gel phase separation; PPPS, polymer–polymer phase separation; FRAP, fluorescence recovery after photobleaching; FCS, fluorescence correlation spectroscopy; CTD, carboxy-terminal domain; ALT, alternative lengthening of telomere; APB, ALT-associated PML nuclear bodies.

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