Biomolecular condensates at sites of DNA damage: More than just a phase⋆,⋆⋆

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Abstract

Protein recruitment to DNA break sites is an integral part of the DNA damage response (DDR). Elucidation of the hierarchy and temporal order with which DNA damage sensors as well as repair and signaling factors assemble around chromosome breaks has painted a complex picture of tightly regulated macromolecular interactions that build specialized compartments to facilitate repair and maintenance of genome integrity. While many of the underlying interactions, *e.g.* between repair factors and damage-induced histone marks, can be explained by lock-and-key or induced fit binding models assuming fixed stoichiometries, structurally less well defined interactions, such as the highly dynamic multivalent interactions implicated in phase separation, also participate in the formation of multi-protein assemblies in response to genotoxic stress. Although much remains to be learned about these types of cooperative and highly dynamic interactions and their functional roles, the rapidly growing interest in material properties of biomolecular condensates and in concepts from polymer chemistry and soft matter physics to understand biological processes at different scales holds great promises. Here, we discuss nuclear condensates in the context of genome integrity maintenance, highlighting the cooperative potential between clustered stoichiometric binding and phase separation. Rather than viewing them as opposing scenarios, their combined effects can balance structural specificity with favorable physicochemical properties relevant for the regulation and function of multilayered nuclear condensates.

Keywords

Biomolecular condensates; DNA damage response (DDR); DNA repair; Genome stability; Liquid-liquid phase separation (LLPS); Intrinsically disordered regions (IDR); Low complexity domains (LCD); Multivalent interactions; Higher-order assemblies

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1 Introduction

According to Richard Dawkins' 'The Blind Watchmaker: Why the Evidence of Evolution Reveals a Universe without Design', "biology is the study of complicated things that give the appearance of having been designed for a purpose", whereas "physics is the study of simple things that do not tempt us to invoke design" [1]. Oil droplets in water, from this perspective, could be considered simple things, with no sign of design. Living cells, on the other hand, with their intricate architecture and organizational complexity, are complicated things. As tiny high-precision machines, cells use energy to exert spatio-temporal control over the numerous biochemical reactions that take place every millisecond inside the intracellular space. Compartmentalization is key to such spatio-temporal control, and recent years have seen resurging interest in the biological, chemical and physical principles that govern cellular compartmentalization [2–5].

The cell nucleus is the biggest cellular compartment, a membrane-enclosed organelle and home of the chromosomes and the embedded genetic code. It comprises various layers of organizational complexity to maintain genome structure and function, including chromatin loops and higher-order chromatin architecture, but also a large number of chromatin-associated and non-associated nuclear proteins and protein complexes, which can form biomolecular condensates and thereby subdivide the nuclear space. Biomolecular condensates can be generally defined as intracellular compartments that lack surrounding membranes but function to concentrate biological molecules, and the term was chosen in part because it provides a link to concepts from condensed matter physics [5]. Such assemblies, despite the absence of a lipid membrane, can be considered physical entities, in which certain molecules are enriched, while others are excluded. Biomolecular condensates can form by different means and have different material properties, ranging from highly dynamic liquid droplets to dense and sometimes irreversible aggregates [6–8]. They can form through stoichiometric binding of molecules to one another, *e.g.* with the chromatin scaffold and histone modifications serving as binding site clusters for cooperative or non-cooperative protein binding, or, at the other end of the spectrum, by thermodynamically-driven liquid-liquid phase separation (LLPS). Lava lamps and vinaigrettes ('simple things') have served as popular examples for liquid demixing and to illustrate behavioral features of coexisting liquid phases. But of course, biomolecular condensates in living cells are in many aspects far more complex than oil droplets in water or than the 'lava' in lava lamps. They are composed of hundreds of molecules of different kind that make inter- and intra-molecular contacts, often in a highly dynamic manner and employing bonds of varying interaction strength. Their compositional control is energy dependent, and enzymatic reactions, including post-translational protein modifications, are involved in regulating their formation and functions [9]. Structured domains provide high affinity interaction surfaces for binding partners, and these interactions can be critical for the formation or maintenance of a compartment. Energy dependence does not refute a role for local thermodynamics, however, and lock-and-key type interactions involved in clustered binding can cooperate with dynamic multivalent weak interactions to form functional condensates [10]. Stable protein complexes with fixed stoichiometries represent a minority in protein interaction networks, which are instead dominated by weak, nonstoichiometric

interactions [11]. On the other extreme, intrinsically disordered proteins that form liquid droplets *in vitro* typically show more complicated behavior in their natural habitat inside cells, where homotypic interactions are dominated by various heterotypic interactions that involve multiple interaction partners [12]. A lot of biology ('complicated things') likely happens between the extremes of stable complexes with fixed stoichiometries and purely entropy-driven phase separation in idealized few component systems (Fig. 1).

Maintenance of genome integrity depends on local multi-protein assemblies, *e.g.* around sites of DNA damage, to mark genomic lesions and coordinate DNA repair reactions with other vital functions such as transcription and cell cycle progression. Denominating DNA repair foci as biomolecular condensates does not serve a purpose on its own, but focusing on material properties of these compartments and on the role of disordered protein sequences and their highly dynamic multivalent interactions complements classical biochemical and structural approaches and can open new areas of research. Here, we focus on and discuss connections between cellular mechanisms important for the maintenance of genome integrity and phase separation in the nucleus of eukaryotic cells, highlighting emerging examples of how protein properties involved in phase separation contribute to DNA damage sensing and compartmentalization of lesions, to cell cycle checkpoint activation, to the clustering and movement of DNA breaks, to replication initiation and replication stress signaling, and to telomere maintenance in cancer. We draw parallels to phase separated compartments outside the nucleus and discuss opportunities and future perspectives related to a better understanding, based on new conceptual frameworks and advancing technologies, of the specific interactions that form condensates and how such knowledge may be used to modulate condensate functions and to exploit condensate biology therapeutically.

2   Condensate formation by phase separation

Phase separation as principle implicated in the formation of biomolecular condensates has been discussed for a wide range of organisms [13] including bacteria [14], yeast [15], plants [16], animals [17], and also for viruses [18]. Example condensates include nucleoli [19–24], Cajal bodies [25–27], histone locus bodies [28], nuclear speckles and paraspeckles [29–34], PML nuclear bodies and APBs [35–38], SAM68 nuclear bodies [39], chromatin domains [40–51], transcription condensates [52–64], centromeres [65–67], DNA replication and repair condensates [68–79], stress granules and P-granules [80–82], and virus particles [83–92]. Several of these reside in the cell nucleus [93] and have genome-related functions (Table 1). Although attributing functional roles to phase separation has been difficult in some cases and requires further attention and dedicated research efforts, a rapidly growing list of cellular processes has been linked to phase separation properties and includes ribosome biogenesis and RNA metabolism, protein quality control and degradation, signal transduction, response to stress, cell division, endocytosis, autophagy, cytoskeleton regulation and cargo transport [94–111]. In the context of these processes, phase separation has been implicated in the enhancement or suppression of biochemical reactions and regulation of enzyme activities, signal amplification, storage, sequestration and molecular buffering, sensing of changes in the environment, control of viscoelasticity and viscoadaptation, and exertion of mechanical forces [112–119].

In the context of equilibrium thermodynamics, a phase can be defined as form of matter, which within its boundaries is homogeneous in relevant properties such as chemical composition and physical state. As living cells are not at equilibrium, the definition of a phase broadens, yet without losing its general meaning or applicability. Accordingly, a polymer core is not per se incompatible with multi-layered condensate organization through percolation (an example for a cellular compartment with multiple co-existing phases being the nucleolus [20]), and local thermodynamic equilibria exist at mesoscopic scales despite living cells being out of equilibrium [120–122]. Phase separation and the regulation of intracellular condensates by active processes are therefore not mutually exclusive mechanisms, and a number of energy-consuming processes, including different post-translational modifications (PTMs), have been shown to participate in the spatio-temporal regulation of phase separation [9,117]. The relative abundance of the participating molecules determines composition and morphology of condensates [12,60,123], and PTMs not only change chemical properties of modified proteins, but are also central to the regulation of the abundance and local concentration of proteins and RNA. Although the exact nature, or molecular grammar, of interactions that drive phase separation and their regulation by PTMs is only starting to emerge, it is important to appreciate that, while they are often highly dynamic and may be considered 'fuzzy', they are not 'unspecific'. The multivalent interactions that participate in phase separation are sequence-encoded and often conserved [124,125], yet they differ from stoichiometric lock-and-key type interactions, for which structure-guided exchange of a single amino acid may be sufficient to abrogate binding (Fig. 1). This poses inherent difficulties to classic biochemical and cell biological approaches, such as the generation of separation-of-function mutants by single amino acid exchange, or structure-based design of small molecule inhibitors. However, these challenges should not only be seen as a disadvantage, but also as a chance for novel approaches in biochemistry and drug development. As the field moves towards a better understanding of the dynamic interplay between different types of interactions that contribute to condensate formation in different cellular contexts (Table 2), the easier it will become to modulate condensate behavior and tune condensate functions in a targeted manner.

A useful general framework for phase transitions driven by multivalent protein and nucleic acid molecules is provided by the 'stickers and spacers' model, adapted from the field of associative polymers [145,146]. In simplified terms, stickers provide intra- or intermolecular attraction between discrete sequence elements and are interspersed by spacers, which do not contribute significantly to the attractive interactions that drive condensate formation. Of note, stickers can be folded binding domains (*e.g.* SH2, SH3, SUMO, Chromo, Bromo, RRMs), labile structures (*e.g.* LARKS, low-complexity aromatic-rich kinked segments; RACs, reversible amyloid cores), or short linear motifs and even single residues that form attractive condensate-driving interactions [145–153]. Likewise, disordered sequence stretches can serve as neutral spacers between motifs that drive phase transitions. Intrinsically disordered sequences are thus neither necessary nor sufficient for phase separation [154]. Rather than sequence disorder per se, valence and patterning, *e.g.* of aromatic residues in prion-like domains, are important features associated with phase separation [129,140, 155]. In multi-component condensates, nodal scaffold molecules that are critical for condensate formation can be discriminated from low valency clients, although

the distinction, rather than being binary, might be more gradual and also vary depending on the cellular context [156]. Nevertheless, identifying driver molecules and driver interactions that form biomolecular condensates and elucidating their context-dependent regulation is among the key challenges in this area.

The three intrinsically disordered FET proteins, Fused in sarcoma (FUS), Ewing sarcoma breakpoint region 1 (EWSR1/EWS), and TATA box binding protein associated factor 15 (TAF15), with their aminoterminal prion-like domains and their carboxy-terminal RNA-binding motifs and RG/RGG-repeats, play a prominent role as drivers of phase separation. The FET proteins are abundant nucleic acid binding proteins, which bind to thousands of transcripts and affect multiple steps in mRNA biogenesis [157]. They can be seen as prototype intrinsically disordered proteins (IDPs), and their low complexity domains serve as translocation partners for DNA-binding domains of various transcription factors in several different cancers [2]. Point mutations, on the other hand, cause neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and frontotemporal lobar dementia (FTLD) [2]. As prototype IDPs with high medical relevance, their self-assembly features have attracted significant attention in recent years. Dissecting the molecular grammar of FET protein phase separation revealed that multivalent interactions among tyrosine residues from prion-like domains and arginine residues from RNA-binding domains and RG/RGG-repeats are critical [125]. Interestingly, when minimalist polymers of the positively charged amino acids arginine or lysine were investigated together with mono- or polynucleotides, poly-arginine condensates exhibited 100-fold higher viscosity as compared to poly-lysine droplets, and arginine outcompeted lysine for anionic binding, resulting in condensate immiscibility and inversion [135]. Consistently, lysine cannot substitute for arginine in the context of nuclear speckle condensation by intrinsically disordered mixed-charge domains, underpinning the specificity of interactions involved in condensate properties [30]. These results suggest that arginine motifs with their guanidium groups that enable simultaneous formation of charge-charge, π-π, and cation-π contacts play particularly important roles as stickers that drive condensate formation and govern their viscoelastic properties. On the other hand, such themes cannot be overly generalized, as sequence determinants of phase separation, both at the level of participating proteins and at the level of participating nucleic acids, are condensate type and context dependent. RNAs, for instance, not only act as molecular seeds that trigger protein phase separation, but based on their properties, such as abundance, sequence, length, secondary structures, and covalent modifications, also directly tune biophysical features of phase separated condensates, including size and shape, viscosity and surface tension, and molecular composition [31,60,81,158–161]. Such features can change over time and can result in what is typically referred to as 'aging' of phase-separated compartments towards increased condensate viscosity, enabling dynamic and sometimes adaptable transitions from liquid to gel and to solid [21,81,158,162–164]. While condensate solidification and protein aggregation may be more difficult (although not impossible) to reverse and are often associated with disease [165,166], a rather broad spectrum of (mixed) material property states is used in biological systems under physiological conditions and in response to cell stress. Within this broad spectrum, low complexity domains can even form labile, reversible cross-β polymer cores inside liquid droplets and reversible hydrogels

[167,168]. The formation of these labile amyloid-like fibers is regulated by PTMs and may thereby respond to changing cellular conditions, including cell stress.

A particularly dangerous type of stress occurs in the nucleus when cells experience replication stress or DNA damage, and emerging evidence supports that replication stress and DNA damage responses not only involve stoichiometric high affinity interactions at sites of DNA damage and among signaling molecules, but also make use of phase separation properties of proteins, nucleic acids, and of the DNA damage-induced polymer poly(ADP-ribose) (PAR) for spatio-temporal and physico-chemical control of genome integrity maintenance.

3 Phase separation and maintenance of genome integrity

3.1 DNA damage sensing and PAR formation

Among the first cellular responses to DNA damage is the synthesis of PAR by enzymes of the PARP family [169,170]. PAR formation occurs locally at sites of DNA damage and is a transient response due to the antagonizing activity of PARG. Due to two phosphate groups per ADP-ribose unit, PAR is a highly negatively charged polymer, which during the peak of PAR production recruits a plethora of PAR-binding proteins by multivalent electrostatic interactions, including FUS, EWS and TAF15 [171]. While the prion-like N-termini of the FET proteins undergo concentration-dependent phase separation and form liquid droplets in cells, their positively charged RG/RGG-rich C-termini confer affinity to anionic PAR [68,172,173]. Interestingly, polyelectrolyte interactions can lead to ultrahigh affinity complexes in which the binding partners retain their structural disorder and highly dynamic character [174,175], implying that high affinity binding and structural disorder within complexes are not mutually exclusive. Hence, cooperative weak interactions can lead to very high binding affinities despite a high degree of conformational flexibility. PAR can reach chain lengths of several dozens to hundreds of units and can thereby generate a highly anionic environment at sites of PAR synthesis. FUS, on the other hand, with its multiple RG/RGG motifs is among the top 5% of proteins in terms of cellular abundance and has an estimated nuclear concentration between 4 and 8 μM [69]. The local induction of PAR chains nucleates the assembly of FUS both *in vitro* and *in vivo* [68,69]. Under physiologic conditions, intracellular FUS condensates stay liquid, but liquid to solid phase transition and aggregation into fibrous structures occurs for disease-associated mutations of FUS [68,69]. FUS-deficient or mutated cells have DSB repair defects and accumulate DNA damage, and they are sensitive to ionizing radiation and display chromosomal instability [176–180]. As FUS is involved in gene expression and transcription-associated processes, some of these effects might be due to altered transcription programs and transcription-associated DNA damage. Importantly, however, the PARP1-PAR-FUS system was recently reconstituted *in vitro* and analyzed at the single molecule level by atomic force microscopy [70]. This confirmed FUS recruitment to DNA damage through PAR-binding, and interestingly also revealed PAR- and FUS-dependent compartmentalization of damaged DNA [70]. Although direct *in vivo* evidence for PAR-dependent tethering of DNA ends by alteration of viscoelastic properties is currently missing, PARylation was shown to promote DNA repair by end-joining mechanisms that rely on close proximity of broken DNA ends

[181,182]. Moreover, phase separation of FUS at DNA damage sites was recently reported to be involved in the spatial organization and clustering of DNA damage-induced γH2AX nanofoci detected by 3D-SIM (structured illumination microscopy), further strengthening the link between DNA damage-induced PAR formation, protein condensation and damage clustering [183]. Due to the short-lived nature of PAR and the transient recruitment of PAR-binding proteins, the proposed tethering of broken DNA ends might primarily assist early events in the DDR (*e.g.* comparatively fast end-joining reactions), whereas more long-lived modifications and proteins recruitments have the potential to stabilize the damaged chromatin domain in a more sustained manner (see below).

The FUS-related proteins EWS and TAF15 show very similar transient recruitment behavior and co-assemble into PAR-seeded compartments [68,172,173], and several additional RNA- and PAR-binding proteins, including hnRNPs and hnRNP-like proteins, also get transiently enriched in PAR-seeded condensates [171,184]. While according to the scaffold and client model some of these RNA- and PAR-binding proteins likely play more important roles than others for the local demixing that occurs around DNA break sites, it seems reasonable to assume that collectively they shape the local environment and its viscoelastic properties. One example is the nuclear matrix protein SAFB, which through an R/G-rich region at its C-terminus is recruited to sites of DNA damage in a PAR-dependent manner and promotes γH2AX spreading and DNA damage signaling [185]. More recently, SAFB was shown to phase separate *in vitro* and its interaction with heterochromatin-associated repeat transcripts *via* its R/G-rich region is required for maintaining interchromosomal interactions adjacent to pericentromeric heterochromatin and heterochromatin condensation [186]. In response to DNA damage, PAR may thus compete with repeat transcripts such as major satellite RNAs for SAFB binding, and it will be interesting to address whether recruitment of SAFB (and potentially of other non-coding RNA-binding proteins) to sites of DNA damage is associated with compromised heterochromatin architecture.

The transient nature of PAR-seeded condensates may balance such risks, and the FET proteins, SAFB and other RNA- and PAR-binding proteins are later released and excluded from sites of DNA damage [68,185,187–191]. DNA damage-induced phosphorylation regulates this exclusion, and phosphorylation was shown to disrupt FUS phase separation by reducing self-interaction [167,192]. The bimodal assembly and exclusion dynamics that are observed for FET and FET-like proteins can help to remove nascent RNA and RNA-DNA hybrids from DNA break sites to facilitate repair [188,193]. In addition to exclusion from the damaged area, phosphorylation by the DDR kinase DNA-PK also promotes nuclear export and cytoplasmic accumulation of the FET proteins [194]. Interestingly, in ataxia telangiectasia (A-T) cells, which show deficiency in the DDR kinase ATM, neurotoxic protein aggregation occurs due to increased levels of reactive oxygen species (ROS) and hyperactivation of PARylation [195]. Elevated levels of PAR have also been reported for Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD) and for amyotrophic lateral sclerosis (ALS), and inhibition of PARylation was shown to be beneficial in PD, AD, HD, and ALS disease models [196–201]. Of note, while PAR formation is most strongly induced by DNA damage in the nucleus, it is also involved in cytoplasmic phase separation and protein condensation into stress granules, with important implications for health and disease [202–206]. As PARP inhibitors impact PAR-triggered

phase separation and can prevent toxic protein aggregation, their therapeutic value may go beyond modulating DNA repair. In the context of DNA damage, the dynamic nature and timely dissolution of PAR-seeded condensates facilitates the handover to downstream compartmentalization of the damaged area.

3.2 Chromatin topology and the chromatin response to DNA damage

The chromatin response to DNA double-strand breaks (DSBs) has been investigated in great detail [207–210]. Around the break sites, multiple DNA damage-induced chromatin modifications work together to remodel the chromatin and recruit repair factors. Many of the proteins that get recruited to damaged chromatin contain structurally defined chromatin reader domains with affinity for specific DNA damage-induced histone marks. An apical mark that is induced by DSBs and spreads at megabase scale is the phosphorylation of H2AX by ATM to form γH2AX compartments. The formation of these large domains depends on cohesin-mediated loop extrusion at DSBs, which results in bidirectional spreading of γH2AX within the boundaries of topologically associating domains (TADs) marked by CTCF [211]. In yeast, DNA-cohesin clustering resembles bridging-induced (polymer-polymer) phase separation, which may stabilize cohesin-CTCF complexes [212], and thereby potentially also stabilize the γH2AX domain. Downstream of γH2AX and its reader protein MDC1 the large scaffolding protein 53BP1 gets recruited. 53BP1 is a multivalent chromatin reader and its recruitment depends on γH2AX, on the abundant histone mark H4K20me2 (a mark that discriminates unreplicated from replicated chromatin and guides DSB repair pathway choice accordingly), and on DNA damage-induced de novo deposition of H2AK15ub [213–216]. 53BP1 binds modified chromatin as a dimer, but preformed 53BP1 dimers quickly self-assemble into dynamic higher order oligomers upon cooperative H4K20me2 and H2AK15ub binding [217,218]. Consistently, apart from H4K20me2 and H2AK15ub binding, also the oligomerization domain of 53BP1 is required for 53BP1 assembly into DNA damage-induced foci [219]. Stoichiometric one-to-one binding models can therefore explain the initial recruitment of 53BP1 dimers to modified nucleosomes, but simplified linear 'beads on a string' illustrations fall short of capturing the ensuing assembly and maturation of 53BP1 compartments. Of note, there is no dichotomy between initial, kinetically controlled, stoichiometric binding, *i. e.* one 53BP1 dimer per nucleosome, and the ensuing, thermodynamically driven nonstoichiometric assembly to generate the 53BP1 compartment (Fig. 2). Rather than viewing them as mutually exclusive, these mechanisms likely synergize to stabilize the domain and endow it with favorable properties.

Indeed, the 53BP1 compartment stabilizes chromatin topology at multiple levels: In the sub-micrometer range, 53BP1 forms dynamic nanodomains, which overlap with TADs, similar to γH2AX [220–222]. These nanodomains then cluster together to form circular microdomains, which provide an environment that favors DSB repair by non-homologous end-joining (NHEJ) and confines limited DNA end resection and homologous recombination (HR) factors to its center. 53BP1 compartments, which can also undergo fusions to form even larger repair condensates, are thus multi-layered, and multiple types of interactions, weak and strong, cooperate in their formation and functionality (Fig. 3).

This multi-layered organization is disrupted easily, in fact more easily than the upstream γH2AX and MDC1 foci, by changes in osmotic pressure, temperature, salt concentration, or inhibition of hydrophobic interactions [71]. This is important to keep in mind when detergents or methanol are used before or simultaneously with cell fixation, as such treatments may disrupt critical interactions. The self-assembly features of 53BP1, which are triggered by DNA damage, can be recapitulated in DNA damage-independent optoDroplet experiments using the *Arabi-dopsis thaliana* photoreceptor cryptochrome 2 (Cry2) as controllable and reversible optogenetic seed module [71]. On its own, this module does not result in light-induced protein clustering, but when fused to protein sequences that can phase separate by multivalent interactions, rapid formation of liquid droplets can be observed in living cells [71,223]. The C-terminus of 53BP1, which contains the oligomerization domain and is particularly rich in amino acids that have been implicated in phase separation, is sufficient to promote optoDroplet formation in this system, and the formed condensates are highly dynamic, frequently fuse, and once formed remain for several minutes in the absence of blue light [71]. When combined with a localized, chromatin-tethered seeding event such as endonuclease-mediated DSBs, blue light induction triggers rapid assembly of additional 53BP1 molecules specifically at these sites. 53BP1 also phase separates *in vitro* [71,72], and 53BP1 foci in irradiated cells display behavior consistent with nucleation, growth and coarsening, both when expressed ectopically and also in cells in which the endogenous gene locus had been edited to express fluorescently labeled 53BP1 [71,72]. Interestingly, DNA damage induced 53BP1 condensates also 'age' towards increased viscosity, and their formation and properties are modulated by additional macromolecules, including DNA damage-induced long non-coding RNAs (dilncRNA) synthesized by RNA Pol II from DSBs [72], and by the large 53BP1-interacting scaffolding protein AHNAK [73]. As more functions for RNA and RNA-binding proteins as regulators of genome stability are emerging [224–226], it will be interesting to elucidate exactly how different RNA species, their modifications and their abundance and dynamics around DNA break sites affect DDR condensate properties and repair outcomes.

53BP1 condensates also selectively attract clients, including the tumor suppressor p53 [71], which itself has phase separation properties and forms nuclear condensates to amplify target gene expression [29,227]. Consistently, p53 target gene expression and p21 induction are dependent on 53BP1 and can be tuned by 53BP1 regulators, which in turn determines cell fate decisions [71,73,228–231]. While structural and biochemical studies suggest that dimethylated p53 binds stoichiometrically to the 53BP1 tudor domain [228,232], cellular experiments have implicated the oligomerization domain and the BRCT domain of 53BP1 in p53 activation [71,73,229], raising the possibility that structural diversity and interaction multiplicity within a larger conformational space may contribute to full p53 activation.

DNA damage compartments not only form larger repair centers by fusion, but also relocalize within the nuclear space [233]. Nuclear actin polymerization, the actin-nucleating ARP2/3 complex, and nuclear myosins have been implicated in the directed motion of a subset of DSBs into discrete clusters to promote homology-directed repair [234,235]. Cell membrane-associated actin assembly by the ARP2/3 complex depends on phase separation and stoichiometry-dependent dwell times [236]. Potentially, relocalization of the subset of DNA breaks, which are difficult to repair, *e.g.* due to the chemical nature of the break

site itself, due to heterochromatic environment, or due to iterative cutting by an activated endonuclease, also depends on dwell times of the actin polymerizing machinery at the damaged domain. Thus, how long a DNA break remains unrepaired may work as a timer to eventually trigger mobilization of the domain and initiate a different type of repair, *e.g.* to switch from unsuccessful NHEJ attempts to relocalization and repair by HR.

In yeast, the meiotic DNA break machinery assembles by a DNA-driven condensation process that shares several features with phase separation [74]. Furthermore, the recombination protein Rad52, the functional homolog of the HR factor BRCA2 in mammalian cells, accumulates at induced DSBs to form liquid droplets with features reminiscent of phase separation [75,76]. Rad52 exhibits a slower diffusion coefficient and confined motion inside the condensate, and the diffusion coefficient changes sharply when molecules cross the phase boundary [76]. Furthermore, foci fusions result in condensates with twice the volume of individual foci, and Rad52 molecules inside fused domains explore the entire compartment. Together, these results support that rather than clustered binding, a nucleation core and phase separation cooperate to form the repair compartment [76].

Of note, Rad52 condensates nucleate DNA damage-inducible intranuclear microtubule filaments (DIMs), which are important for condensate fusions and, upon enrichment of tubulin in fused domains, also mediate the mobilization of damaged DNA to the nuclear envelope for repair [75]. Functional cooperation between membraneless liquid droplets, microtubule filaments, and lipid membranes is an exciting new concept, and the mechanical connections between membraneless and membrane-bound compartments, *e.g.* as can be observed for instance for phase separated RNA granules that 'hitchhike' on lysosomes for long-distance RNA transport in neurons [237], is largely unexplored in the context of mammalian DNA repair and maintenance of genome integrity.

3.3 DNA replication and replication stress signaling

DNA replication and DNA replication stress are a major source of DNA damage and genome instability in cancer [238], and fine-tuned mechanisms have evolved to coordinate the firing of replication origins, replication fork speed, fork stability and repair with cell cycle progression and checkpoint signaling [239]. In mammalian cells, replication initiation involves licensing of replication origins and formation of the pre-replication complex (pre-RC), followed by origin firing in S-phase. While it is understood that replication initiation and origin firing are tightly regulated and well coordinated in space and time, the dynamic control of interactions involved in forming initiation clusters and replication factories in eukaryotic cells remains nebulous. Recent work on replication initiation in the fruit fly *Drosophila melanogaster* revealed that the origin recognition complex (ORC) and the initiation factors Cdc6 and Cdt1 contain intrinsically disordered regions that drive DNA-nucleated phase separation, which selectively promotes assembly and loading of the Mcm2-7 complex of the replicative helicase [77]. Cyclin-CDK-mediated phosphorylation disrupts phase separation of these condensates, likely by abolishing electrostatic interactions between charged disordered protein sequences and DNA [77]. In mammalian cells, a very similar mechanism seems to be in place, because short linear motifs (SLiMs) within intrinsically disordered regions of ORC1 and CDC6 drive phosphorylation state-dependent

interactions and liquid-liquid phase separation on DNA [78]. Also here, the process is neither untargeted nor independent of energy, but rather part of a multi-step initiator condensation process.

Downstream of origin licensing and formation of the pre-RC, origin firing during S-phase progression is controlled by the cell cycle checkpoint kinase ATR [240]. ATR activation in response to replication stress depends on the scaffolding protein TopBP1 and its intrinsically disordered ATR activation domain (AAD). Similar to the DSB repair factor 53BP1, TopBP1 was recently shown to form light-inducible optoDroplets with properties resembling phase separation [79]. Intriguingly, TopBP1 optoDroplets not only enriched the ATR kinase, but could also be used to reversibly switch ATR signaling on and off, resulting in blue light-controlled activation of the major downstream checkpoint kinase CHK1 and a slowdown of replication forks [79]. All these effects were mediated by the disordered AAD of TopBP1 and were abrogated by mutation of an essential aromatic tryptophan residue. TopBP1 is phosphorylated in response to replication stress, and mutation of a phosphorylation site in close proximity of the critical tryptophan to alanine reduced TopBP1 condensation and ATR signaling, suggesting a positive feedback loop between TopBP1 and ATR in TopBP1 condensates for signal amplification [79]. Consistent with the multi-step processes and multi-layered condensate organization discussed above, the authors propose the formation of a nucleation complex (based on stoichiometric interactions, *e.g.* between RPA and ssDNA and ATR-ATRIP, TopBP1 and the 9-1-1 complex), which stabilizes TopBP1 at stalled or damaged replication forks, and an ensuing condensation into dynamic higher-order assemblies by multivalent cooperative interactions to achieve robust ATR activation and signal amplification [79].

3.4 Alternative lengthening of telomeres (ALT)

Certain regions in the genome are particularly fragile, *e.g.* due to DNA secondary structures, repetitive sequence elements, heterochromatic environment, late replication timing, low density of replication origins, or a combination of these features [230]. Telomeres at chromosome ends fall into this category, and replication stress at telomeres is frequently seen in cancer cells. A subset of cancer cells uses alternative lengthening of telomeres (ALT) as telomerase-independent mechanism of telomere maintenance and show particularly high levels of telomere-associated replication stress [241]. ALT is characterized by telomere clustering in ALT-associated PML bodies (APBs) and by recombination-based telomere elongation involving break-induced replication (BIR), a noncanonical form of DNA synthesis [242]. APB formation is dependent on SUMOylation and on SUMO recognition by SUMO interaction motifs (SIMs), and recent work has implicated phase separation by SUMO-SIM binding modules in the formation of APBs [37, 38]. Poly(SUMO) and poly(SIM) scaffolds, or chemically induced tethering of SIMs at telomeres, drives liquid condensation and telomere clustering by coalescence, and induces ALT-like phenotypes such as mitotic DNA synthesis (MiDAS) at telomeres, BLM-dependent C-circle formation, and heterogeneous telomere length [37,38].

Interestingly, ALT-associated homology-directed repair also depends on carefully controlled PAR formation at broken telomeres [243]. PARylation at ALT telomeres assembles various

PAR-binding proteins, including the repair factors XRCC1 and LIG3 and the chromatin remodelers ALC1 and CHD7, but also the disordered FET proteins FUS, EWS and TAF15, several hnRNPs, SAFB1, SATB1, other RNA- and PAR-binding proteins such as RBMX and NONO, and the F-actin nucleation complex ARP2/3 [243]. Consistent with a role in PAR-triggered phase separation at ALT telomeres, loss of these proteins impairs APB formation and telomere clustering [243].

Finally, recent findings have suggested that telomeric BIR in APBs is a self-perpetuating process, due to BLM helicase-dependent BLM enrichment and BIR-dependent TRF2 SUMOylation to sustain SUMOSIM interactions and DNA repair factor recruitment [244]. These findings are consistent with the phase separation model of APB assembly, and self-perpetuation of biochemical processes might be a more general feature of biomolecular condensates during their respective lifetimes.

4 Opportunities and future perspectives

Investigating spatio-temporal aspects of genome integrity maintenance has been a long-standing endeavor, not least due to the clinical relevance of cellular replication stress and DNA damage responses. Resurgent interest in phase separation and its different flavors (*e.g.* liquid-liquid, liquid-gel, liquid-solid, polymer-polymer) has started to broaden the focus from stoichiometric binding between structurally defined binding partners towards studying multivalent interactions and structurally less well defined complexes. This has also spurred new interest in material properties of membraneless compartments and how these relate to function. Rather than being seen as mutually exclusive, stoichiometric binding between structured domains and multivalent interactions that can drive phase separation likely cooperate for condensate formation in the complex environment of the cell nucleus, and several intriguing examples related to different aspects of maintenance of genome integrity have recently emerged (Fig. 4).

Unlike phase separation in few component systems, the formation of biological condensates involved in the maintenance of genome integrity is more complex and typically follows a multi-step process, but in this process makes use of phase separation properties of some of the key components involved [245]. Studying these properties *in vitro*, using purified proteins, can be revealing (*e.g.* to identify relevant interactions and important sequence features, or to study mixing behavior in well controlled settings), and just like the biochemical characterization of an enzymatic activity in a test tube calls for an *in vivo* counterpart to provide context, cellular assays ideally performed at endogenous protein concentrations as was done in several cases, provide an important counterpart to phase separation experiments with purified components.

Existing technologies are being further developed and new ones are rapidly emerging, which can complement and extend classical biochemistry to inform about structure-function relationships in the context of biomolecular condensates. These include structure elucidation techniques such as solution and solid-state nuclear magnetic resonance spectroscopy (NMR, ssNMR), small-angle x-ray scattering (SAXS), cryo-electron microscopy (cryo-EM) and atomic force microscopy (AFM), as well as single molecule fluorescence

microscopy (SMFM) techniques including fluorescence fluctuation spectroscopy (FFS) and single molecule Förster resonance energy transfer (smFRET). Moreover, *in vitro* turbidity assays, microfluidics and optical tweezers, as well as light-inducible phase separation (optoDroplets) to study phase behavior under controlled conditions in living cells have emerged as informative techniques. Computational tools for sequence analysis and prediction of phase separation potential, for structural modeling, molecular dynamics simulations, and to calculate phase diagrams for multicomponent systems have become increasingly important, and intravital microscopy (IVM) may be used to examine condensates in their native environment [246–255]. As technologies advance and become more quantitative, a clearer picture of the relative contribution and functional relevance of phase separation properties in different biological contexts, also with regard to alternative mechanisms and interaction modes [256–259], is going to emerge. As with any single technology, limitations exist and have to be taken into account. For instance, as mentioned above, the choice of cell permeabilisation, pre-extraction and fixation has an impact on cellular architecture, and weak interactions involved in condensate formation may get easily lost. In live cell imaging experiments of endogenously labeled proteins (which, if the tag does not interfere with protein functions, is preferable over ectopic expression of fluorescently labeled proteins), signal intensity depends on target protein abundance, with limiting consequences for exposure times and phototoxicity. The development of particularly bright monomeric fluorescent proteins and advanced image segmentation tools using deep learning may attenuate some of these limitations in the future. While single cell and single molecule techniques provide insight into phenotypic and structural heterogeneity, this information is lost when population averages are analyzed. Moreover, microscopy-based approaches such as fluorescence *in situ* hybridization (FISH) and genomics-based approaches such as chromatin conformation capture (3C) techniques do not always yield congruent results, and it was suggested that 3C measurements may not always reflect spatial proximity [260]. Such limitations need to be taken into account when functions of biomolecular condensates are studied in the context of genome stability and organization.

Over the last couple of years the concept of phase separation has revived significant interest in and spurred new research on multivalent interactions, disordered protein sequences, dynamic protein and nucleic acid complexes and material properties of intracellular condensates. In multiple cellular contexts, including the processes involved in DNA repair and maintenance of genome integrity, there is mounting experimental support for intracellular phase boundaries, coarsening, and differential viscoelastic properties of condensates versus the surrounding milieu. At the same time there is experimental support for the notion that in nuclear multi-component compartments not all components are distributed uniformly and that their assembly resembles a tightly controlled and energy-dependent multi-step process, which often starts from a nucleation core. This process, however, once initiated, uses self-assembly properties of key components to foster percolation.

Working towards identifying and characterizing the specific interactions that are critical for the dynamics and for the material properties of intracellular biomolecular condensates may offer new opportunities to modulate their functions (Fig. 5). First examples of condensate-specific enrichment of cancer therapeutics have been reported [261,262], and the better

we understand the physico-chemical, viscoelastic properties of intracellular condensates and the molecular interactions that define them, the more specific the targeting of drugs to their relevant sites of action may work. This might be particularly relevant as derailed phase separation could well be a unifying theme for the development of a broad spectrum of conditions ranging from neurodegeneration to cancer and aging [263–266]. Finally, the development of bio-inspired materials such as designer organelles and nanocarriers can benefit from a deeper understanding of biomolecular condensates and their varied properties [267,268]. Although applying concepts from polymer chemistry and soft matter physics to cell biology and, vice versa, using knowledge from biological systems to engineer functional biomaterials comes with inherent challenges, the chances and new opportunities prevail.

Acknowledgements

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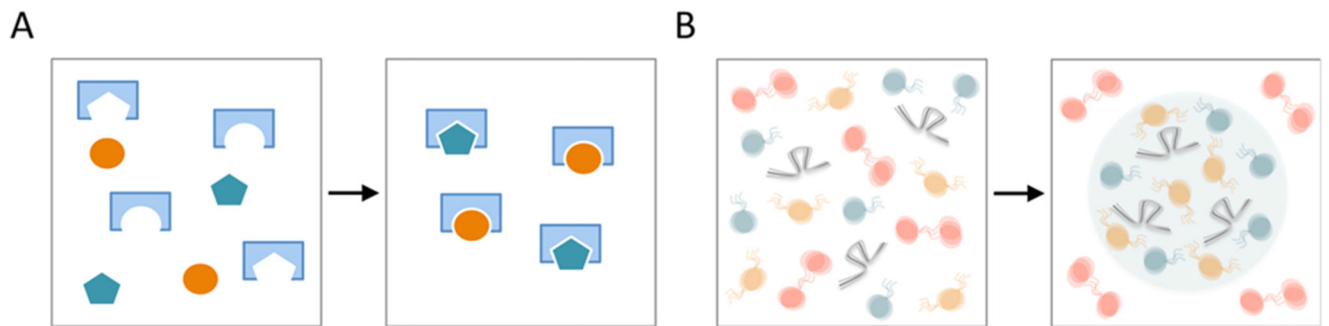
Fig. 1. Binding modes.
(A) Stoichiometric, in this case monovalent one-to-one binding, *e.g.* by lock-and-key or induced fit association. **(B)** Highly dynamic multivalent interactions involved in phase separation. Note that in both cases dynamic association and dissociation can occur and, importantly, high binding affinities can be reached.
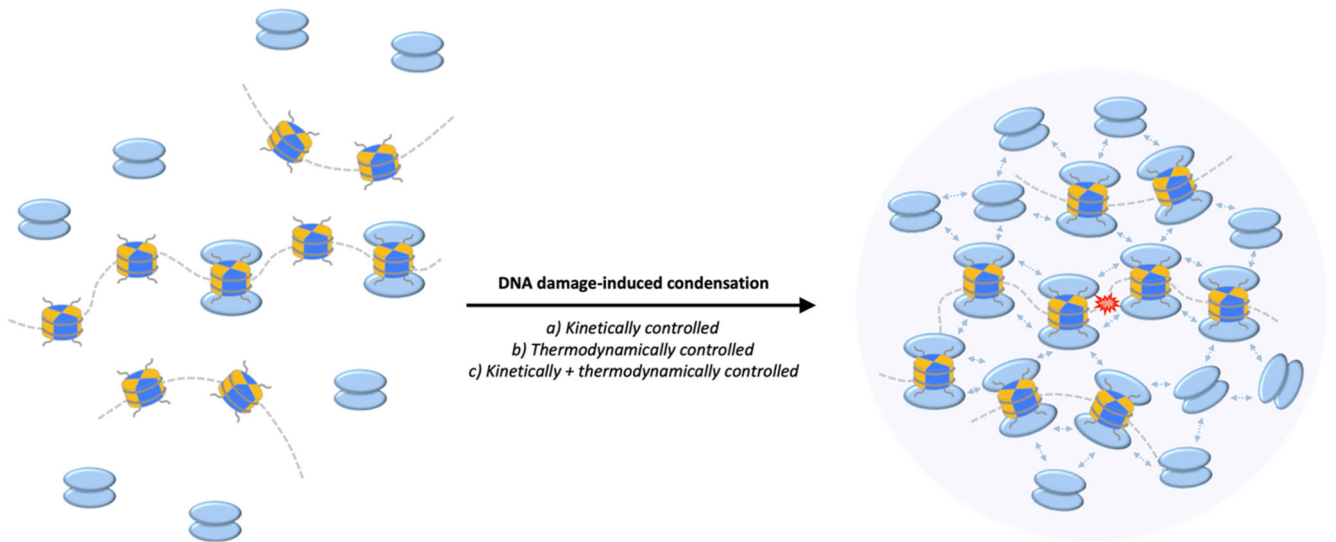
Fig. 2. Model of 53BP1 condensation at sites of DNA damage.
At the spatial and temporal scales relevant for the DNA damage response, 53BP1 condensation may be favored both kinetically, through enzymatically-controlled deposition of histone marks, and thermodynamically, through self-assembly of 53BP1 into mature oligomers to form a denser 53BP1 compartment.
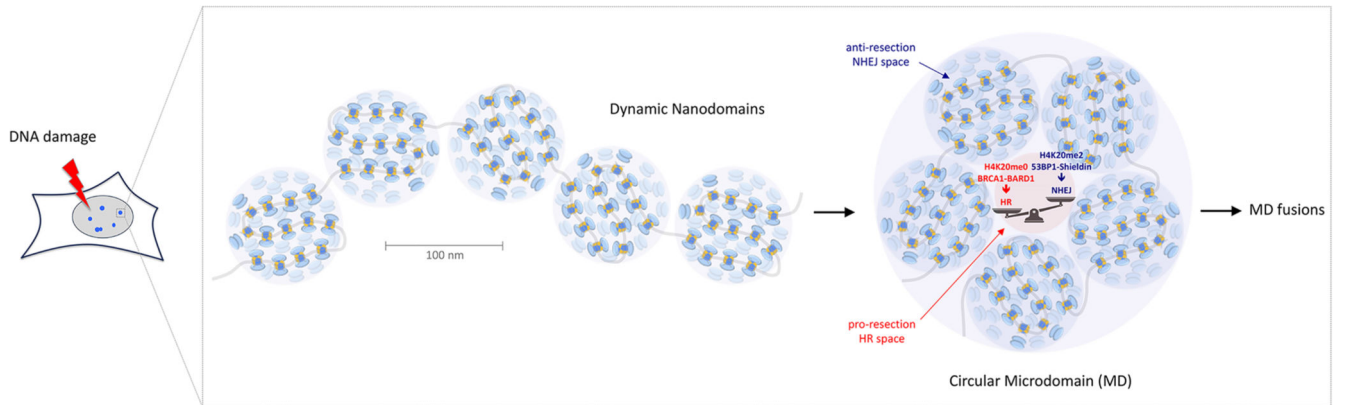
Fig. 3. Model of 53BP1 nano- and microdomains.
The propensity of 53BP1 to self-interact may play a role for the formation of 53BP1 nanodomains, for the assembly of multiple nanodomains into bigger microdomains, and for the fusion of microdomains into large DNA repair centers. The multi-layered organization of these domains enables tuning of DNA end resection and repair pathway choice according to replication and chromatin status, cell cycle position, and lesion complexity.
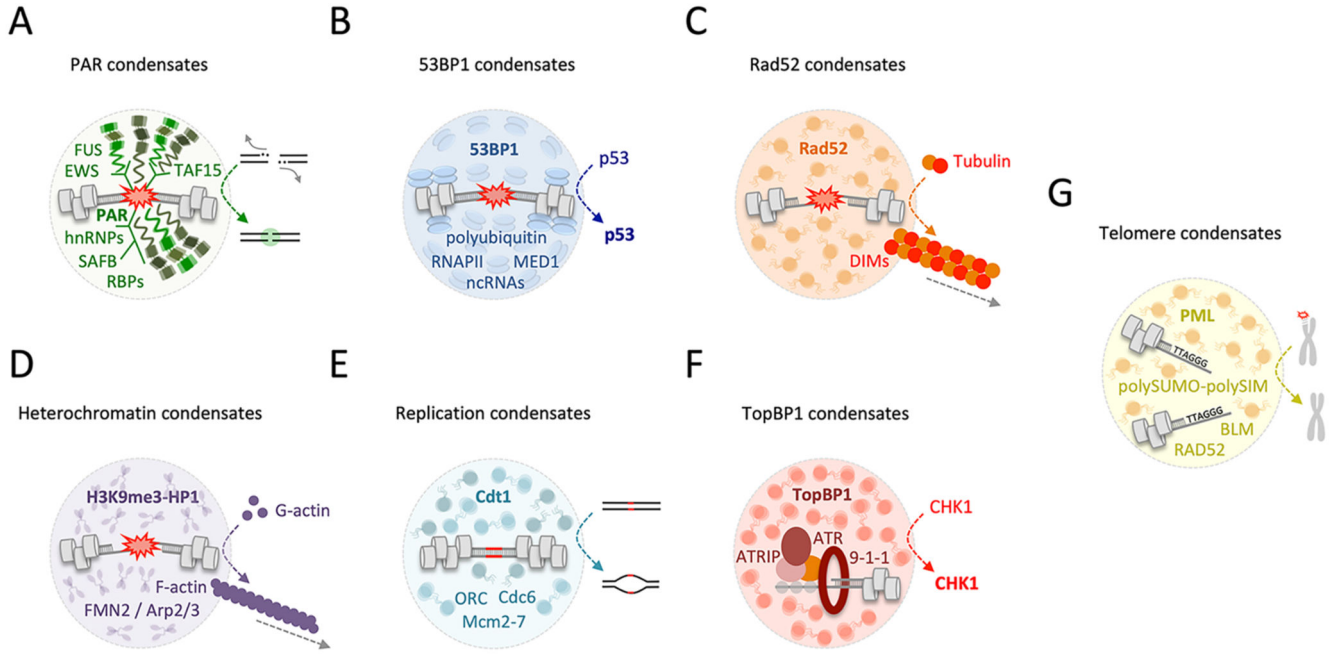
Fig. 4. Emerging examples of nuclear condensates with phase separation properties and roles in maintenance of genome integrity.

(A) Poly(ADP-ribose) (PAR) triggers assembly of multiple intrinsically disordered and RNA-binding proteins (RBPs) including FUS/TLS, EWS/EWSR1, TAF15, hnRNPs, SAFB1/2 and SLTM and initiates their local demixing at sites of DNA damage, which may promote tethering and religation of broken chromosome ends. **(B)** 53BP1 condensates form around DSBs in a manner that requires multivalent chromatin binding and oligomerization and is promoted by DNA damage-induced transcription of long noncoding RNAs (ncRNAs). 53BP1 condensation is linked to activation of p53 signaling, and p53 dynamically co-assembles in 53BP1 condensates. **(C)** In yeast, the DNA repair protein Rad52 forms phase-separated condensates, which concentrate tubulin and project DNA damage-inducible intranuclear microtubule filaments (DIMs). DIMs mobilize the liquid Rad52 compartment to the nuclear periphery for repair. **(D)** In mouse and human cells, heterochromatin domains get mobilized when they experience DNA damage through FMN2/ARP2/3-mediated actin polymerization. Nuclear F-actin and myosin re-localize heterochromatic DSBs to the nuclear periphery for repair. **(E)** For initiation of DNA replication, DNA-dependent phase separation of the origin recognition complex (ORC), Cdt1 and Cdc6 facilitates Mcm2-7 loading. **(F)** TopBP1 condensation at DNA replication impediments switches on ATR/CHK1 kinase signaling to initiate a replication stress response. **(G)** Through multivalent polySUMO-polySIM interactions at chromosome ends in cells that use alternative lengthening of telomeres (ALT), ALT-associated PML bodies (APBs) form by phase separation for telomere clustering and telomere recombination.
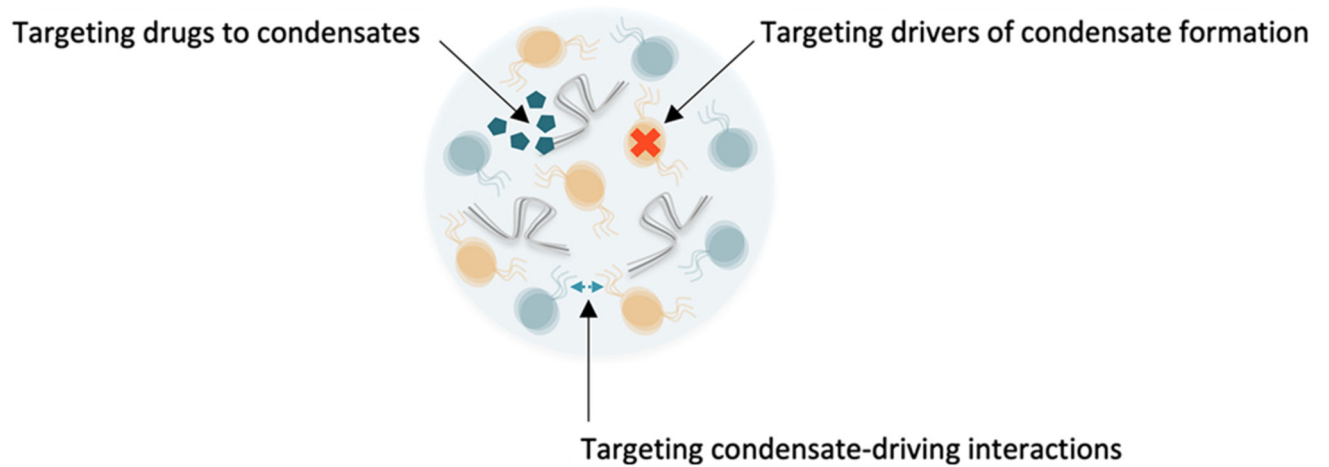
Fig. 5. Emerging opportunities for condensate therapeutics.
Condensate properties and functions may be modulated by targeting the molecular drivers of condensate formation, or by interfering with the intra- and intermolecular interactions that are important for condensate formation and maturation. Conversely, the physico-chemical properties of condensates may be exploited to facilitate partitioning of drugs into target compartments and enhance their efficacy.

Table 1

Biomolecular condensates in the nucleus and their main function(s).

Nuclear condensates	Main function(s)	References
Nucleoli	rRNA transcription and ribosome biogenesis	[19,20,21,22,23,24]
Cajal bodies	Biogenesis and maturation of snRNPs	[25,26,27]
Histone locus bodies	Processing of histone pre-mRNAs	[28]
Nuclear speckles	Regulation of pre-mRNA splicing	[29,30]
Paraspeckles	Protein and (lnc)RNA sequestration, gene regulation	[31,32,33,34]
PML nuclear bodies	Genome organization, transcriptional regulation, viral defense	[35,36]
APBs in ALT cells	Telomere clustering and telomere maintenance by ALT	[37,38]
SAM68 nuclear bodies	RNA metabolism	[39]
Chromatin domains	Genome organization, gene regulation, X-inactivation	[40,41,42,43,44,45,46,47,48,49,50,51]
Transcriptional condensates	Transcription initiation and elongation	[52,53,54,55,56,57,58,59,60,61,62,63,64]
Centromeres	Chromosome segregation during cell division	[65,66,67]
DNA repair compartments	DNA damage signaling and repair	[68,69,70,71,72,73,74,75,76]
Replication condensates	Replication initiation, coordination of origin firing	[77,78,79]

Listed are membraneless nuclear compartments, which have been linked to protein and nucleic acid phase separation, together with their main cellular function(s). Note that condensate formation does not necessarily rely on phase separation alone, and that for some condensates the causal relationship between phase separation properties of the involved molecules and cellular condensate function(s) is still debated.

Table 2

Interactions implicated in (liquid-liquid) phase separation.

Interaction	Residues involved	References
π-π	Phe-Phe; Tyr-Tyr; Phe-Tyr	[126,127,128,129,130,131]
Cation-π	Tyr-Lys; Phe-Lys; Tyr-Arg; Phe-Arg	[131,132]
Charge-charge	Asp/Glu-Arg/Lys; Phospho-Arg/Lys	[133,134,135,136,137]
Hydrophobic	Ala/Ile/Leu/M/Phe/Trp/Tyr/Val- or Pro-rich	[138,139,140,141,142,143]
Dipole-Dipole	Gly/Gln/Asn/Ser-Gly/Gln/Asn/Ser	[143,144]

Listed are interactions implicated in phase separation, together with residues involved. For example, attractive π-stacking between aromatic rings (aromatic stacking, π-π interactions), electrostatic (charge-charge) interactions, or dipoledipole associations including hydrogen bonding can promote protein assembly by phase separation. Note that multiple types of interactions can cooperate for phase separation, and that besides proteins and their indicated residues and sequence motifs also nucleic acids participate in phase separation and provide multivalent binding surfaces.