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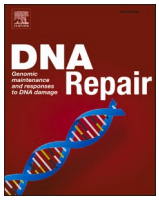

Compartmentalization of the DNA damage response: Mechanisms and functions

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ABSTRACT

Cells have evolved an arsenal of molecular mechanisms to respond to continuous alterations in the primary structure of DNA. At the cellular level, DNA damage response proteins accumulate at sites of DNA damage and organize into nuclear foci. As recounted by Errol Friedberg, pioneering work on DNA repair in the 1930 s was stimulated by collaborations between physicists and geneticists. In recent years, the introduction of ideas from physics on self-organizing compartments has taken the field of cell biology by storm. Percolation and phase separation theories are increasingly used to model the self-assembly of compartments, called biomolecular condensates, that selectively concentrate molecules without a surrounding membrane. In this review, we discuss these concepts in the context of the DNA damage response. We discuss how studies of DNA repair foci as condensates can link molecular mechanisms with cell physiological functions, provide new insights into regulatory mechanisms, and open new perspectives for targeting DNA damage responses for therapeutic purposes.

1. Introduction

Endogenous metabolites and exogenous agents such as UV light induce a variety of chemical alterations in the primary structure of DNA. These alterations, called DNA damage, are repaired by multiple mechanisms [1,2]. Briefly, excision repair mechanisms use the intact strand as a template to repair the damaged strand. Base excision repair corrects the lesion by excision of the damaged base and reinsertion of the missing base. Nucleotide excision repair corrects UV damage and bulky adducts by excision of an oligonucleotide containing the damage followed by DNA repair synthesis of the resulting single-stranded DNA gap. Mismatch repair corrects bases that are mis-incorporated during DNA replication. When both strands of DNA are broken, the double-stranded DNA break is repaired either by homologous recombination, using a homologous duplex as a repair template, or by nonhomologous end-joining, in which broken ends are simply spliced back together. The repair of covalent bonds between the two strands of DNA depends on the coordination of multiple repair pathways. Diverse repair pathways also ensure the repair of covalent bonds between proteins and DNA. The literature abounds with excellent books and reviews on DNA repair [1, 2]. The scope of the present review is to report on recent studies investigating the mechanisms and functions of protein compartmentalization during activation of the DNA damage response and repair of double-strand DNA breaks.

Induction of double-strand breaks with rare-cutting endonucleases, ionizing radiations or laser micro-irradiation have provided important insights into the spatiotemporal organization of DNA repair and checkpoint proteins in yeast and in human cells [3–5]. In response to DNA damage, hundreds to thousands copies of DNA repair proteins accumulate at DNA damaged sites and yield distinct foci visualized by conventional fluorescence microscopy [4,6]. For example, the local concentration of the recombination protein RAD52 in a repair focus is increased by more than 50 folds relative to the surrounding nucleoplasm [7]. The detection of focal structures induced by DNA damage has provided means to explore the orderly and interdependent recruitments of homologous recombination proteins at DNA breaks [2–5], and yielded important insights into the cell cycle regulated distribution of DNA repair and checkpoint proteins into distinct DNA repair compartments [4]. Interdependencies in protein recruitments at damaged sites indicate that the formation of nuclear foci is governed by a network of specific interactions [3]. Analyses of the precise kinetics of recruitment of 70 repair proteins to complex DNA lesions also corroborate the notion that different mechanisms orchestrate the recruitment of DNA repair factors to the lesions [5].

DDR foci concentrate molecules without a surrounding membrane and no fixed stoichiometry, a characteristic feature of biomolecular

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condensates [8–10]. The term condensate refers to membrane-less compartments and does not imply a specific assembly mechanism [9,11]. The spatiotemporal organization of DNA damage response proteins within nuclear foci may ensure the collective functioning of the different protein activities that compose the foci.

Many biomolecular condensates have been proposed to involve liquid-liquid phase separation (LLPS) [8–10,12–14]. However, the relevance of LLPS in the biogenesis and function of condensates formed in the physiological environment of the cell has been questioned because LLPS is a passive mechanism often used to model simple systems in equilibrium [14–19].

2. Liquid-liquid phase separation

LLPS is a thermodynamic process of de-mixing theorized by Flory and Huggins. This theory predicts that the free energy of mixing of a homogenous chain polymer with a single uniform solvent is determined by the difference of free energy between the polymer-solvent interactions and the sum of polymer-polymer and solvent-solvent interactions. LLPS occurs above a threshold concentration of the polymer called the saturation concentration (Csat). Above Csat, the interaction energy of polymer-solvent interactions is larger than the interaction energy of polymer-polymer plus solvent-solvent interactions. Consequently, the system phase separates spontaneously into a polymer enriched phase and a dilute phase, like oil and water. Thus, LLPS is a thermodynamic process that results from the sum of solubility-determining interactions in the system. Precise thermal and material parameters allow to predict the conditions for the system to be in a mixed or de-mixed state [20]. The existence of a saturation concentration is a *sine qua non* condition for phase separation [14]. A constant Csat and a dense phase volume that grows with increasing total polymer concentration are considered as strong evidence for LLPS [21]. Protein self-association processes without phase separation, such as oligomerization, are not expected to follow these rules [21]. A fixed Csat, however, cannot be determined for protein condensation in cells [22]. In the intracellular environment, the stabilization of condensates appears to be primarily governed by heterotypic interactions [22]. The cellular volume is occupied by a complex mixture of macromolecules, small molecule solutes, osmolytes and metabolites. This calls into question the generalizability of simple LLPS models based on two-component systems for understanding the principles of protein condensation in living cells. Yet, the conclusion that a particular protein undergoes liquid-liquid phase separation is often based on experiments performed in vitro with a purified protein in a simple buffer.

2.1. Sphericity

Membrane-less compartmentalization yields an interface with the surrounding milieu that is associated with an interfacial tension [23]. Since the minimum surface area for a given volume is a sphere, condensates formed by LLPS spontaneously adopt a spherical shape that minimizes surface tension. Whereas nuclear substructures often appear spherical by conventional fluorescent microscopy, super-resolution imaging may reveal more complex organizations and shapes.

2.2. Liquide-like properties

Cellular condensates exhibit liquid-like properties, fuse and divide, as revealed by time-lapse microscopy. Tagged proteins components of condensates typically show rapid recovery after photo bleaching. This liquid-like behavior is determined by the (short) time scales of the making and breaking of interactions between macromolecules, and the mobility of these macromolecules within the condensates and across the boundary of the condensates. Rapid recovery after photo bleaching is not a demonstration of LLPS. The liquid-like properties of physiological condensates are necessary for the function of enzymes that would be kinetically trapped in solid crystals [24]. A year of net measuring time on an Orbitrap mass spectrometer revealed that weak interactions dominate protein networks [25]. Stable protein complexes with a defined stoichiometry are rare [25]. Thus, the molecular interactions that underpin condensates are expected to span a wide range of dissociation constants, with a majority of high dissociation constants [24].

2.3. 1,6 hexanediol

The aliphatic alcohol 1,6 hexanediol disrupts weak hydrophobic interactions and is often used to dissolve condensates in cells [26]. While 1,6 hexanediol treatment can provide insights into the nature of the interactions involved in protein condensation, it does not allow distinguishing between LLPS and other condensation mechanisms [27]. It was first discovered as an agent that increases the permeability of nuclear pores [28]. Indeed, the phase separation of the FG-repeat of nucleoporin reproduces the permeability of nuclear pore complexes [29]. More recently, several aliphatic alcohols have been tested for their ability to melt labile cross-linked polymers and membrane-less cellular bodies. Of the four aliphatic alcohols 1,6-hexanediol (1,6-HD), 2,5-hexanediol (2,5-HD), 1,5-pentanediol (1,5-PD), and 1,4-butanediol, only 1,6-HD and 1,5-PD were able to melt liquid-like droplets in 5 min in vitro at concentrations ranging from 0.5% to 4%, and intracellular structures at 6–8% [30]. Because 2,5-HD has much lower melting activity, it has been suggested as a negative control for assaying dissolution of condensates by 1,6-HD. [31–33]. However, other reports show that 2,5-HD also dissolves condensates in living cells when used at 5% for 15 min [34]. Furthermore, and like 1,6-hexanediol, 2,5 HD suppresses chromatin motion at various concentrations (2,5–10%) after only 5 min [35]. In summary, 1,6 HD changes the permeability of membranes [28], interferes with the activities of kinases and phosphatases [36], and compromises chromatin organization [34,35]. Because the treatment of cells with 1,6 HD can lead to a variety of artefacts, we do not recommend 1,6 HD as a tool to probe the nature of DDR condensates. A recent study indicates that measurement of the interfacial properties of condensates through partial photo bleaching (FRAP), when possible, may help distinguish LLPS from other mechanisms [27].

3. A variety of condensation processes

In addition to LLPS, alternative and non-mutually exclusive processes can explain the local clustering of protein (Fig. 1).

3.1. Non-specific DNA binding

Replication compartments formed upon Herpes Simplex Virus infection are based on the increased accessibility of the viral DNA, which remains largely nucleosome free and facilitates non-specific protein-DNA interactions [37] (Fig. 1). Single particle tracking experiments revealed that molecules have the same diffusion constants inside and outside virus replication compartments, and that there is no restriction to the diffusion of molecules through the interface between the nucleoplasm and the compartment [37]. Yet, at the macroscopic level, HSV replication compartments exhibit liquid-like properties, fuse, are spherical, and have a refractive index different from that of the nucleoplasm.

3.2. Structural interaction with DNA

The simple binding of molecules to DNA such as the RPA complex and RAD51 that yield nucleoprotein filaments may be sufficient to yield microscopically visible foci (Fig. 1). Another example is mono-ubiquitinated FANCD2 that forms a closed ring with FANCI around double-stranded DNA and can produce filamentous arrays [38,39].

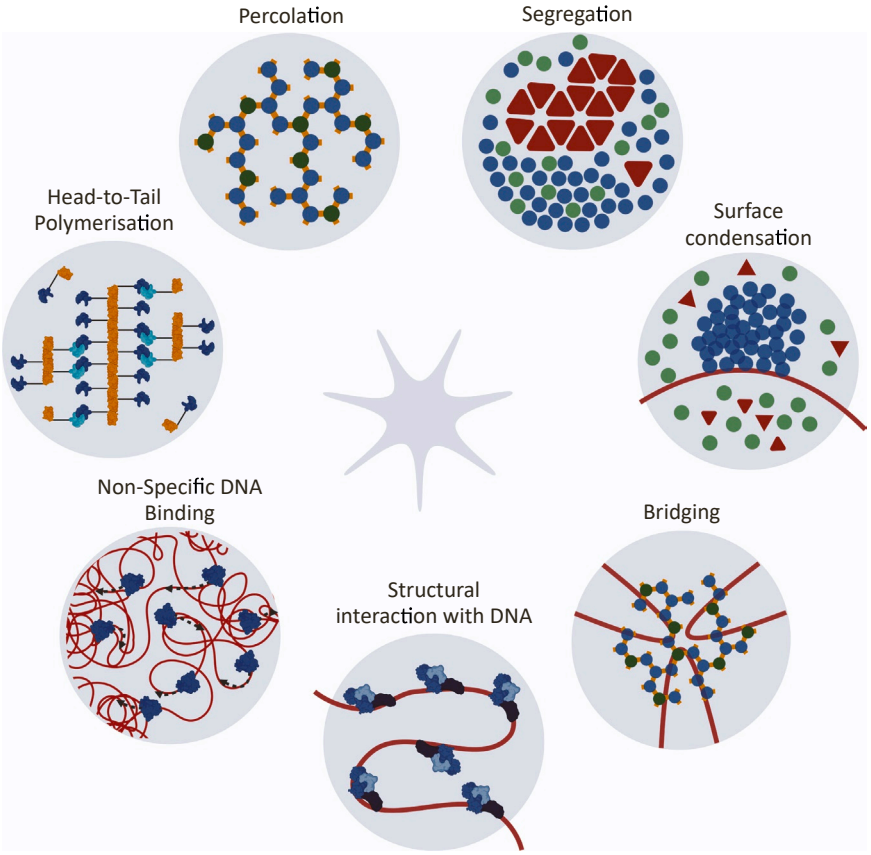

Fig. 1. A diversity of condensation processes that may combine with each other. Schematic representation of protein condensation processes. These mechanisms are not mutually exclusive. Some of these processes are likely to combine to yield nuclear condensates. The schematized molecular clusters are contained within a filled circle that represent a "focus", "nuclear body" or "puncta" visualized by conventional microscopy. This schematized circle makes no assumption about the biophysical properties of these structures. Schematized macromolecular arrangements within the foci emphasize on specific aspects of the condensation process. Nuclear foci can result from non-specific DNA binding based on increased protein accessibility to DNA, or by repeated structural interactions of proteins with DNA. The polymer-polymer phase separation model emphasizes on proteins that can bridge distant chromatin loci. Protein scaffolds that undergo head-to-tail polymerization yield filaments. Filament crosslinking by dimerization result in nuclear foci. Head-to-tail polymerization may combine with segregative transitions, as described below. Percolation is an associative transition driven by sequence-specific interactions that yields protein networks. Segregative transitions occur when macromolecules and macromolecular assemblies separate from other incompatible molecules. Biomolecular condensates have been proposed to form via coupled associative and segregative transitions. Surface condensation is a segregative transition that occurs at the surface of chromatin. Created with Biorender.com.

3.3. Polymer-polymer phase separation

In the Polymer-Polymer phase separation (PPPS) model, most simply known as the bridging model, condensates are formed by DNA-binding proteins that can bridge distant chromatin loci [15,40] (Fig. 1).

3.4. Surface condensation

Recent evidence suggests that chromatin provides a surface for the formation of growth-limited condensates at protein concentrations below the saturation concentration required for LLPS [41,42] (Fig. 1).

3.5. Head-to-tail polymerization

Alternatively, structurally - specific protein-protein interactions, like the DIX domain, can yield condensates (Fig. 1). In the Wnt signaling pathway, for example, the effector Dishevelled assembles reversible condensates via head-to-tail polymerization and crosslinking of the resulting filaments by dimerization [40]. A DIX like domain was identified in the N-terminus of TDP-43 [41]. It was found to be responsible for its head-to-tail oligomerization leading to the formation of functional dynamic condensates [42,43].

3.6. Percolation

The assembly of biomolecular condensates is typically driven by a few key multivalent associative scaffold proteins that are highly connected to other molecules [43,44]. Scaffold proteins often include a combination of well-folded domains and intrinsically disordered regions (IDRs) (Fig. 2). IDRs do not form well defined stable structures, yet are essential for protein functions. The functionalities of IDRs include molecular recognition, molecular assembly, protein modifications and entropic chains [45].

Non-redundant sequence-specific interactions play an essential role in the assembly of condensates. For example, the formation of cytoplasmic stress granules is dependent on site-specific interactions mediated by folded molecular interfaces [46–48]. Specifically, G3BP1 dimerization in combination with RNA binding domains drive the assembly of stress granules, whereas phosphorylation of the intrinsically disordered regions (IDRs) of G3BP1 exerts a regulatory role [46–48]. Multivalent molecular scaffolds necessary for the formation of condensates can be modeled using a sticker-and-spacer framework [14,49]. The valence of a macromolecule refers to the number of stickers. Spacers act as flexible linkers between stickers, whereas stickers include folded domains that enable specific protein-protein and protein-nucleic acids interactions, as well as intrinsically disordered motifs that provide highly dynamic, polyvalent and multivalent interaction surfaces [45] (Fig. 2). Individual amino - acids engaged into cation-π, π-π, electrostatic and other forms of attractive interactions can also contribute to the process of protein condensation (Fig. 2).

The sequence-specific interactions of multivalent associative scaffolds can yield a protein network maintained by reversible protein crosslinks (Fig. 1). In mathematics, a major change in the connectivity of a network is called a percolation transition. In theory, the process of percolation eventually results in a network that spans the entire volume of the system [14,44].

Concepts from graph theory proved useful in describing the organization of protein networks [47]. In this conceptual framework, a protein (a vertex) with a valence ≥ 3 is a node. A protein with only two interaction interfaces (V=2) would act as a bridge. If V= 1 then the protein would act as a cap [47], thereby limiting the growth of the network. Alternatively, the titration of an essential node may also limit the network to a localized cluster.

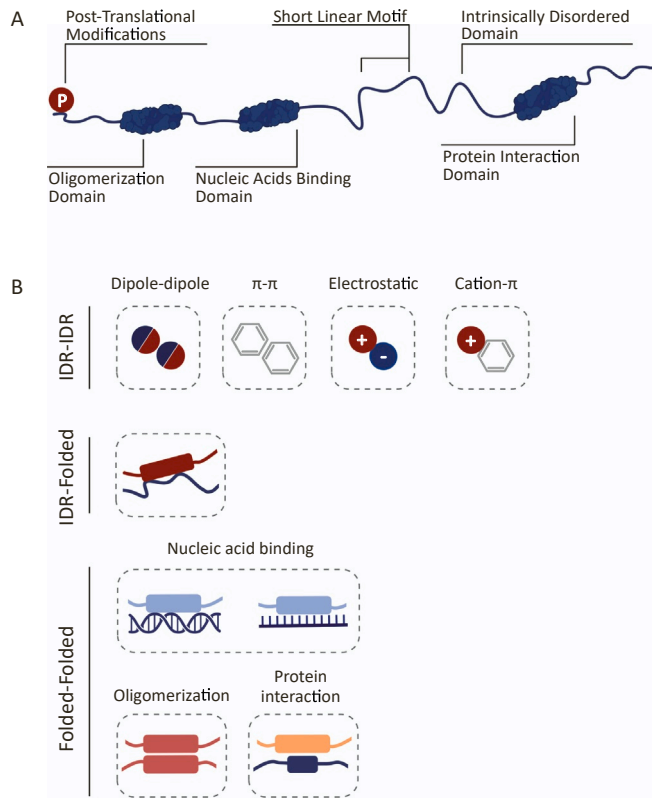

Fig. 2. Molecular driving forces for condensate assembly. A. Multivalent scaffolds that drive condensation often contain different types of sequence-specific interaction domains involved in protein oligomerization, nucleic acids binding and heterotypic protein interactions. The folded domains are often connected by intrinsically disordered regions (IDRs). IDRs are involved in molecular recognition, molecular assembly, are often modified post translationally, and can act as flexible linkers. B. Cooperation between different types of interactions is necessary for protein condensation. These interactions span a broad range of affinity constants. Transient interactions that break and reform rapidly determine the dynamics and liquid-like properties of the condensates. High affinity interactions contribute to the specific composition of the condensates and ensure precise molecular positioning. Created with Biorender.com.

3.7. Coupled associative and segregative transitions

Macromolecules typically occupies 20–30% of the cellular volume [50]. These macromolecules do not interpenetrate, and this exclusion volume effect has an impact on the thermodynamics of the system. Solvent molecules that interact with the solute macromolecules also occupiy a volume called the solvation volume. Molecular crowding creates the conditions for entropic segregation [39], where entropy measures the degrees of freedom of the system [51]. So, segregative transition is a process in which the molecules of a system separate from each other due to their incompatibility. Phase separation is the segregation of macromolecules from other incompatible macromolecules or solvent molecules. Liquid-liquid phase separation refers to the formation of two co-existing immiscible liquid phases. Entropic forces likely play an important role in intracellular organization. For example, entropically - driven segregation has been proposed to be the main driving force that guide the action of proteins such as type II topoisomerases and promote the segregation of bacterial chromosomes [51]. By contrast, entropic forces cannot drive the partitioning of smaller macromolecules such as plasmids [51].

Therefore, a nuanced model that integrates the driving forces of protein organization in the crowded cellular environment suggests that the formation of cellular condensates results from the coupling of associative and segregative transitions [14,44] (Fig. 1). This model considers the importance of changes in the connectivity of macromolecules for the formation of biomolecular condensates. Hence, the formation of large protein network through associative transitions would promote entropically driven segregative transitions. The model is consistent with observations that membrane-less compartments such as Cajal bodies, nucleoli and splicing speckles have a low density and a sponge-like structure [52].

4. Functions of biomolecular condensates

One key question that must be addressed is the function(s) that emerge specifically from the assembly of DDR condensates. Compartmentalization enables the spatiotemporal organization of biochemical pathways via enzymes co-localization. The product of an upstream reaction becomes the substrate for the subsequent reaction catalyzed by a co-localizing enzyme [53] (Fig. 3A). High local concentration of binding sites favor rebinding events after dissociation, enhancing the overall binding avidities for enzymes and substrates, thereby enhancing the efficacy of biochemical reactions [54] (Fig. 3A). Furthermore, the high local concentration of repair factors may limit promiscuous reactions ensuring that DNA repair occurs at the right time and the right place, and/or fulfill architectural functions required for the faithful restoration of the genetic information (Fig. 3B). The simplest hypothesis is that the structural organization and the composition of DDR condensates, defined by the repertoire of protein binding sites, determine their physiological functions (Fig. 3C). Below, we discuss recent insights into the formation and functions of condensates assembled in response to DNA damage.

5. Double-strand break repair compartments: Focus on Rad52

Pioneering work from the Rothstein laboratory has provided important insights into the dynamics of homologous recombination proteins in live yeast cells [3,7,55–57]. Upon induction of a single DNA double-strand break (DSB) in haploid cell, an estimation of 600–2100 Rad52 molecules promptly relocate to the break and form a focus [55]. A fluorescent tag placed next to a specific DNA cut site revealed that Rad52 foci indeed co-localize with the DSB, and that a single Rad52 focus can include multiple DSBs, suggesting that Rad52 foci represent DNA repair centers [7]. Furthermore, the formation of DSBs increases the mobility of damaged chromatin, and thereby facilitates homology search during homologous recombination [58,59]. In Drosophila and mammalian cells, the nucleation of actin filaments by the Arp2/3 complex promotes the clustering of DSBs into discrete sub-nuclear domains [60,61]. Nuclear actin polymerization induces multiscale alterations in genome architecture that facilitates homology-directed repair but also increases the odds of chromosome translocation [62].

In the early stages of homologous recombination, the DNA ends are resected 5' to 3' and the resulting single-stranded overhangs are first bound by replication protein A. Rad52 is recruited to DNA repair foci through direct interaction with Rfa1, the large subunit of replication protein A [3,63]. Single particle tracking (SPT) Photo Activable Localisation Microscopy (PALM) experiments revealed that Rad52 molecules exhibit confined diffusion within the focus, and are subjected to an attraction potential towards the center of the focus [64]. The concentration of binding interfaces may increase Rad52 binding avidity within the condensate. For example, the SUMOylation of Rad52 reduces its mobility within the focus [64]. Yet, Rad52 diffuses approximately six times faster than Rfa1 bound to single-stranded DNA [64]. Hence, the majority of Rad52 molecules is not bound to Rfa1 and explore the entire volume of the focus. Importantly, the diffusion coefficient of Rad52 changes sharply when the protein crosses the boundary of the focus [64]. The restriction of protein diffusion across the boundary of the condensate indicates the presence of a surface tension resulting from phase separation. In conclusion, a model emerges whereby the

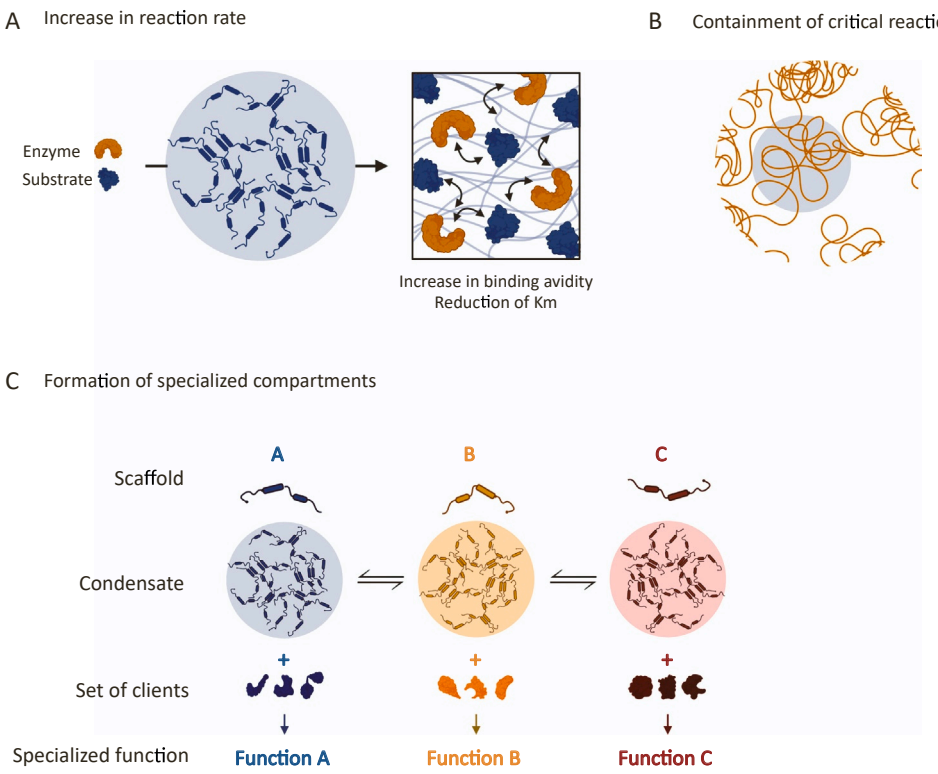

Fig. 3. Functions of biomolecular condensates. A. Condensates are thought to fulfill a variety of functions. In response to DNA damage, some evidence (see text) suggests that protein condensation provides spatiotemporal control of the biochemical reactions that underlie DNA damage signaling and DNA repair. The concentration of sequence-specific protein interaction sites within condensates directs the local accumulation of effector proteins at DNA damage sites. The local and selective concentration of proteins favors protein rebinding after dissociation, increasing avidity between enzymes and substrates and increasing reaction rates. B. Condensates ensure spatiotemporal control over the proper organization of biochemical reactions in space and time to minimize unwanted and harmful effects on neighboring components. C. The function of condensates is determined by their composition, which depends on the specific protein-protein interaction domains present in the condensates. Changes in scaffold stoichiometries and scaffold combinations, or scaffold valency through post-translational modifications, can modulate the composition of the condensate promoting a distinct function. Created with Biorender.com.

recruitment of Rad52 by Rfa1 seeds the formation of a membrane-less compartments within which the large majority of Rad52 molecules diffuse freely [64]. However, the protein scaffold(s) that drives the assembly of Rad52 containing foci has not been formally identified. Fascinating work lies ahead also to understand whether and how the high local concentration and the dynamics of recombination factors within condensates facilitate homologous recombination.

6. Double-strand break repair compartments: Focus on 53BP1

53BP1 is a key protein scaffold that drives the formation of nuclear compartments in response to the formation of DSBs. 53BP1 limits the 5′ to 3’ resection of the broken DNA ends, notably in the G1 phase of the cell cycle. 53BP1 is recruited to DSBs through site-specific recognition of ubiquitinated lysine 15 of histone 2 A (H2AK15ub) and dimethylated lysine 20 of histone 4 (H4K20me2)[65,66]. At the macroscopic level, 53BP1 condensates exhibit liquid-like properties: spherical shape and sensitivity to osmotic stress, temperature changes and 1,6 hexanediol treatment [67,68]. These properties suggest that cooperative interactions drive the assembly of 53BP1 mesoscale structures. The direct contribution of 53BP1 to the assembly of DDR condensates in living cells has been shown using an optogenetic system [67]. This system exploits the photoreceptor cryptochrome 2 from *Arabidopsis thaliana*, which adopts a tetrameric state when activated by 488 nm light [69]. When fused to a multivalent scaffold that contributes directly to the assembly of biomolecular condensates, Cry2 photoactivation nucleates their assembly with high temporal precision, within a few seconds, often less than one minute, in absence of DNA damage [67,70,71]. The oligomerisation domain of 53BP1, and to a lesser extent its BRCT domain, are essential for the condensation of 53BP1 [67]. This highlights the critical role of sequence-specific interactions in this process. Remarkably, upon optogenetic activation, the W1495A mutant 53BP1 protein that has lost recognition of the H4K20me2 histone mark still forms foci at a DSB induced by an endonuclease, suggesting that the optogenetic and localized seeding events combine to trigger 53BP1 condensation at the DNA break, specifically [67]. A fluorescence fluctuation spectroscopy (FFS) study has shown that preformed 53BP1 dimers first relocate to the DSB via recruitment to H2AK15ub and then immobilize when the dimer engage with H4K20me2 [72]. The capture of 53BP1 on chromatin then triggers 53BP1 oligomerization and foci maturation [72]. 53BP1 condensation may modulate the surrounding chromatin environment. Consistent with this, using diffractive optics and photoactivatable chromatin probes, a recent study reports that the motions of chromatin microdomains near DSBs are more correlated with one another than domains in undamaged regions and this phenomenon is dependent on 53BP1 [73].

Below the diffraction limit of light, super-resolution imaging revealed that 53BP1 condensates are composed of an assembly of 53BP1 nanodomains (nanometer-sized structures) arranged circularly around an active DNA repair site [74]. These 53BP1 nanodomains co-localize with topologically associating domain (TAD) sequences, suggesting that 53BP1 compartments define a multi-TAD assembly [74]. RIF1 and cohesins stabilize this circular architecture, notably after DNA breakage. Hence, the organization of 53BP1 compartments is determined, at least in part, by chromatin architecture. Indeed, high-resolution 4 C-seq analyses show that pre-established TADs determine the formation of DDR condensates containing γH2AX and 53BP1 proteins [75]. Cohesins accumulate at DSBs and catalyze a one-sided loop extrusion on either side of the break. This promotes the spreading of H2AX phosphorylation by ATM, as the cohesin ring pulls through the chromatin fiber. The process terminates when a TAD border element is reached [75]. DSB-containing TADs cluster in an ATM-dependent manner and forms γH2AX/53BP1 compartments insulated from the surrounding chromatin [76]. 53BP1 cooperates with microtubules and the LINC complex to increase the mobility of DSBs [77]. In addition, the Schlafen family protein SLFN5 regulates the mobility of DSBs and the organization of 53BP1 into microdomains through interactions with 53BP1 and with LINC complex subunits [78].

The clustering of DSBs, however, comes with the inherent risk of an increased rate of translocations. Remarkably, a subset of DNA damage genes, including p53 target genes, are recruited to DSB-induced compartments for optimal activation in response to DNA breaks [76].

Consistent with this, the tumor suppressor p53 co-localizes with both optogenetic and endogenous 53BP1 condensates [67], which may reflect the re-localization of DNA damage responsive genes [76]. These observations suggest that 53BP1 compartments may ensure spatiotemporal coordination of DNA repair with expression of damage-responsive genes [67,76]. In addition, the scaffold protein AHNAK restrains the condensation of 53BP1 through direct interaction with 53BP1 oligomerization domain, thereby controlling the network of p53 target genes [79]. Furthermore, 53BP1 co-assembles condensates with HP1α independently of DNA damage. 53BP1-HP1α condensates are required to maintain the structural integrity and the transcriptional repression of heterochromatin [80]. Here also, the oligomerization domain of 53BP1 is the minimal region required for 53BP1-HP1α condensation [80]. Taken together, these findings highlight the importance of understanding the functions of membrane-less compartments formed by DNA damage response factors to elucidate the mechanisms that ensure cell homeostasis.

7. Fused in Sarcoma

Fused in Sarcoma (FUS) is involved in multiple stages of RNA metabolisms, including transcriptional regulation and pre-mRNA splicing. FUS belongs to the FET family proteins, like EWS and TAF15. FET proteins contain an intrinsically disordered Prion-like domain (PLD) and an RNA recognition motif. The PLD of FUS is often translocated with the DNA binding domain of transcription factors in sarcomas [81]. In the context of oncogenic fusion proteins, the PLD of FET proteins strongly activates transcription. FUS and TDP-43 are also present in stress granules, and in pathological cytoplasmic inclusions of neurons in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) [82]. In addition, FUS promotes DSB repair in neurons via the recruitment of the histone deacetylase 1 (HDAC1) at DSBs, which promotes chromatin modifications required to facilitate DNA repair [83].

FUS recruitment to chromatin is induced by poly (ADP-ribosyl)ation. *In vitro*, FUS forms DNA-rich compartments upon PARP-1 activation [84]. In living cells, PAR seeds the condensation of FET family proteins at DNA lesions induced by laser micro-irradiation [85,86]. In fact, more than 120 proteins localize to damaged chromatin in a PAR-dependent manner, among which a great proportion are RNA binding proteins, including FET proteins [87]. Five minutes after laser irradiation, FUS and TAF15 are excluded from damaged sites [88]. Their prolonged exclusion is dependent on the kinase activities of ATM, ATR and DNA-PKcs, and is accompanied by the removal of RNA-DNA hybrid structures called R-loops [88]. Consistent with this, PARP1 activity promotes the removal of both nascent RNA and elongating RNA polymerase II from sites of DNA damage to facilitate repair [89]. Thus, the transient recruitment of FET proteins at DNA damaged sites may promote a chromatin environment conducive to DNA repair. One study proposes that FUS LLPS tethers DNA ends [31], on the basis that the recruitment of the splicing factor SFPQ and of Ku80 at sites of laser micro-irradiation is slightly delayed, on perturbation of DNA damage responses by osmotic stress and 1,6 hexanediol treatment, and on a subtle γH2AX clustering defect in FUS-KO cells.

Intense studies of FET proteins, FUS in particular, have led to the notion that prion-like domains (PLDs), and, more generally, intrinsically discorded regions, can drive LLPS [90–93]. PLDs - driven condensates are held together by amino acids that act as stickers, interspaced by amino acids spacers that ensure flexibility of the disordered domain. In the particular case of FET proteins, phase separation is driven by cation-π interactions between tyrosines in the PLD and arginines in the RNA binding domain [94]. In the spacer, glycine residues promote flexibility and dynamicity of the droplets, while serine and glutamine in spacers reduce liquidity causing the hardening of the droplets [94]. Disease mutations in the PLD will cause FUS to turn into insoluble aggregates [92]. This highlights the danger of exceeding the solubility limit, and underlines the need to distinguish pathological aggregation and physiological mechanisms that underlie the formation of functional condensates.

Yet, it has been proposed that the "grammar" of PLDs-driven LLPS can be deduced from the number and patterning of stickers, enabling mutations of stickers or spacers for structure function analysis [94]. The amino acids sticker model implies that in order to change the phase behavior of the PLD, multiple mutations would be necessary to target efficiently protein multivalency, without affecting other functions of the protein. To our knowledge, separation of function mutants demonstrating the physiological function of FUS LLPS driven by amino acids stickers in cells have not yet been reported.

Data from the laboratory of Steve McKnight suggest that the physiological form of FUS PLD is a hydrogel assembled via structurally-specific cross-β interactions that are labile to dissolution [16,95]. FUS hydrogels bind the carboxyl terminal domain (CTD) of RNA polymerase II, consistent with reports by the Tomas Cech laboratory [96,97]. Furthermore, a mutation in the FUS PLD that enhances RNA Pol II CTD binding to FUS hydrogels in vitro also enhances the capacity of the FUS PLD domain to activate transcription in live cells [98].

On the other hand, below Csat, FUS, EWSR1 and TAF15 form clusters with an heterogenous distribution of sizes [82]. Endogenous protein concentrations are often lower than the concentrations required for protein phase separation in vitro. Because FUS clusters reflect the behavior of eukaryotic transcription factors that assemble local high concentration hubs via multivalent interactions [99], it is likely that FUS protein networks formed below Csat are functionally relevant entities in live cells. It would be interesting to probe if the recruitment of FUS at DNA damage sites promotes activation of DNA damage responsive genes within damage-induced nuclear foci.

8. Activation of the DNA damage response: Focus on TopBP1

The DNA damage response is implemented by spatiotemporally controlled steps of DNA lesion sensing, followed by the amplification and transmission of the DNA damage signal. TopBP1 promotes activation of the master checkpoint kinase ATR in S phase [100]. ATR signaling proteins sense RPA-covered single-stranded (ss) DNA and double-stranded (ds)/single stranded (ss) DNA junctions that accumulate as a result of blockages in the progression of replication forks [101–104]. ATR is recruited to stalled forks through ATRIP recognition of RPA-ssDNA [101], whereas TopBP1 is recruited to the ATR-activating structure by the MRN complex [105]. In addition, TopBP1 has a specific affinity for DNA containing bulky adducts [106]. TopBP1 binds RPA [107], ATRIP [108], and the 9–1–1 complex to activate ATR [109,110].

The subsequent amplification step of ATR activation can be decoupled from DNA damage sensing. The ATR-activation domain of Rad4^{TopBP1} functions to amplify the checkpoint signal independently of RPA-covered ssDNA and ssDNA/dsDNA junctions [111]. Nuclear internalization of an ATR-activating carboxyl-terminal fragment of TopBP1 activates ATR/Chk1 signaling in the absence of DNA lesions [112], and artificial tethering of TopBP1 to DNA activates ATR without the requirement for DNA damage [113,114]. More generally, the stable association of DNA damage response factors with chromatin, rather than the recognition of DNA lesions per se, is the critical step in triggering the DNA damage response [115]. This suggests that sensing and activation of the DDR are distinct events and that the former provides spatial control over DNA repair events.

So how does the signal amplification work? In principle, the accumulation of ATR signaling DNA structures can determine the level of ATR activity. However, a defined ATR-activating DNA substrate with a ssDNA gap of only 70 nucleotides is sufficient to induce robust activation of endogenous ATR/Chk1 signaling in human nuclear extracts [104]. TopBP1 is a multivalent scaffolding protein [116]. We recently reported that TopBP1 promotes the assembly of DDR condensates [117]. The exquisite temporal precision of optogenetics revealed a direct correlation between TopBP1 condensation and the amplification of ATR

activity. Furthermore, a W1145R substitution in the AAD disrupts both the function of TopBP1 in ATR activation and the ability of TopBP1 to assemble nuclear condensates [100,117,118]. Thus, TopBP1 condensation acts as a molecular switch that enhances ATR activity. Since TopBP1 condensation is dependent on its phosphorylation by ATR, the signal is amplified by a TopBP1-ATR positive feedback loop that generates nuclear condensates [117].

One recent study proposes that the RPA2 subunit drives the assembly of condensates at telomeres [119]. Whether RPA contributes to the assembly of condensates in other contexts, such as ATR/Chk1 signaling, remains to be explored.

Analyses of TopBP1 condensates by super-resolution STED microscopy indicate that TopBP1 foci are globular clusters of small condensates [117]. Because the diameter of these small condensates is ranges between 150 and 200 nm, we call these structures nanocondensates. Of note, chromatin provides a surface for the formation of small condensates at concentrations below the saturation concentration required for liquid-liquid phase separation [41,42]. This ultrastructural organization appears to be conserved among DDR foci [71,74,120,121]. One study shows that each γH2AX cluster contains a single DSB, suggesting that the cluster rather than the elementary γH2AX nanofocus represents the functional structure [121]. Similarly, 53BP1 forms functional, micrometer-sized modules consisting of a circular arrangement of 53BP1 nanodomains around a single DSB [74]. Likewise, the assembly of globular clusters of TopBP1 nanocondensates activates ATR signaling, thus the clusters are the functional structure. The precise mechanism by which nanocondensates clustering amplifies biochemical reactions remains to be elucidated. Biophysical analyses of protein behavior using single particle tracking analysis should provide new insights into the molecular organization of DDR clusters, and into how the functions arise from the assembly of DDR clusters.

9. Protein modifications and DNA processing reactions: Focus on SLX4

SLX4 is a largely unstructured scaffolding protein essential for maintaining genome stability [122,123]. SLX4 mutations have been associated with Fanconi anemia and have been found in many cancers, highlighting the critical tumor suppressor role of SLX4 [124,125]. SLX4 coordinates the regulated action of structure-specific nucleases for the resolution of DNA secondary structures in a variety of DNA repair and recombination mechanisms [126–136]. In addition, the SLX4 complex exhibits SUMO E3 ligase activity [137]. Importantly, the functions of SLX4 are dependent on its ability to form nuclear foci [138].

We found that SLX4 plays a central role in the assembly of chromatin-bound condensates [139]. These membrane-less structures appear as globular clusters of nanocondensates in super-resolution STED microscopy [139]. SLX4 condensation depends on its BTB (Bric-a-brac, Tramtrack and Broad complex) oligomerization domain and its SUMO interaction motifs. The condensation of SLX4 is induced by SLX4 conjugation to SUMO molecules [139], consistent with a localization-induction model in which post-translational modifications increase protein network connectivity [140]. We found that SLX4 condensates concentrate components of the SUMOylation as well as the SUMO-targeted ubiquitin ligase RNF4 [139]. We suggest that the composition of SLX4 compartments is determined by the local accumulation of the sequence specific protein - binding interfaces of SLX4, in combination with accessible SUMO conjugates in SLX4 condensates. For example, RNF4 is endowed with SUMO-interacting motifs to target SUMOylated proteins.

The SUMOylation/RNF4 pathway promotes the extraction of DNA-protein crosslinks (DPCs) from chromatin, including topoisomerase 1, DNMT1 and PARP1 - DNA protein crosslinks [141–144]. Remarkably, the compartmentalization of the SUMOylation/RNF4 pathway by SLX4 promptly triggers the modification of substrate proteins and the extraction of TOP1-DPCs from chromatin. Thus, SLX4 ensures spatiotemporal control over protein modification and degradation. Furthermore, the condensation of SLX4 triggers the degradation of newly replicated DNA [139], consistent with previous studies showing that SLX4 and RNF4 promote replication collapse in ATR-deficient cells [145]. The combination of SUMO ligases, RNF4 and structure-specific nucleases is required for DNA resection, suggesting that SUMOylation and RNF4-mediated protein modifications make the DNA substrate accessible to the SLX4-associated structure-specific nucleases [139]. In conclusion, we propose a model in which SLX4 controls a cascade of enzymatic reactions in space and time through the assembly of membrane-less compartments.

10. A working framework for the study of DDR condensates

As suggested before [146], protein condensation at DNA damage sites is a multistep process (Fig. 4). This concept may be relevant in the context of other nuclear transactions, such as DNA replication and splicing.

First, DDR proteins are recruited to DNA damage sites through highly regulated and sequence-specific interactions. In a second step, scaffolding proteins that combine structurally defined protein interaction motifs and intrinsically disordered motifs promote the assembly of mesoscale structures via cooperative, dynamic and adaptable interaction surfaces [140]. During the DNA damage response, posttranslational modifications often induce an abrupt change in the connections of molecular elements that form the protein network. This second step corresponds to an associative transition [44].

Several examples illustrate that disruptive mutations of non-redundant sequence-specific interactions interfere with the process of protein condensation. Mutation of a single phosphorylated serine residue (S1138A) in the ATR activation domain of TopBP1 is sufficient to impair TopBP1 condensation [117]. Likewise, mutation of the SUMO interaction motifs in SLX4 abolishes SLX4 condensation [147]. Furthermore, a single point mutation (F708R) in the dimerization domain of SLX4 impairs SLX4 condensation [147]. The folded oligomerization domain of 53BP1 is necessary for 53BP1 focus formation [72,148]YY1258,1259AA mutation in 53BP1 diminishes its capacity to dimerize and form cellular condensates [72]. These observations suggest that reduction of the valence of a protein by one is sufficient to shift the balance between a condensed and a diffuse state. The precision of point mutations coupled with the temporal precision of protein condensation by optogenetic activation offers the opportunity to explore the functions that arise specifically from protein condensation. This is illustrated by a study on TopBP1, which activates ATR/Chk1 signaling through condensation [117], and by a recent study on SLX4 showing that SLX4 condensation triggers protein modification by SUMO and ubiquitin, the extraction of TOP1-DNA crosslinks from chromatin, and the degradation of nascent DNA [147].

The function of condensates is determined by their composition, which depends on the presence and abundance of a variety of binding interfaces within the condensates that may not be necessary for condensation but required for the recruitment of effector proteins to the foci. Since condensation proteins such as 53BP1, TopBP1, and SLX4 often associate in a cell cycle regulated manner, different combinations of scaffold proteins may yield a rich repertoire of compartments with specific compositions and functions in the DDR. Last, structures within condensates may provide a scaffolding platform that controls the spatial organization of biochemical processes.

Non-redundant sequence-specific interactions necessary for the assembly and the composition of condensates assembled in response to DNA damage provide an opportunity to explore the properties and functions that result from the collective action of protein components of DDR condensates in living cells. Thus, DDR condensates may become the objects of a new generation of structure-function analyses that integrate molecular mechanisms and cell physiological responses to DNA damage.

Maturation of DDR condensates may result from the coupling

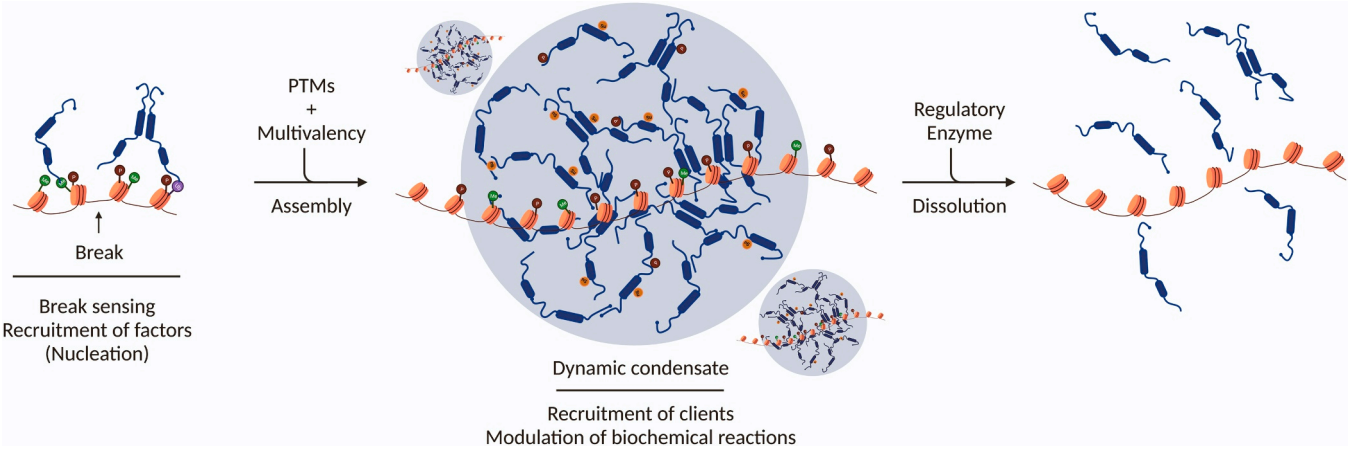

Fig. 4. A working framework for the study of DDR condensates. We propose that the formation of condensates in response to DNA damage is a multistep process. In the first step, multivalent scaffolding proteins are recruited to DNA damage sites via interactions with sensor proteins and histones modified by specific post-translational modifications. This local accumulation represents a nucleation event. Next, posttranslational modifications of scaffolding proteins induce an associative transition that yields an extensive and selective DNA damage protein network. Last, a segregative transition may ensure maturation of the DDR foci. Thus, DDR condensates are held together by reversible cooperative interactions and dependent on protein posttranslational modifications. Specific protein clients are recruited and reaction rates are enhanced until specific enzymes accumulate in the condensate and weaken the interactions, leading to condensate dissolution and termination of the DNA damage response. Created with Biorender.com.

between associative and segregative transitions [44]. Segregative transitions belong to the realm of thermodynamics. Our understanding of crosstalks between associative and segregative transitions in live cells will necessitate long-standing collaborations with physicist, and may depend on the development of novel modeling concepts that integrate the complexity of the intracellular environment. Furthermore, a number of sophisticated microscopy approaches offers complementary means to study quantitatively the biophysical properties of condensates in living cells. For instance, Fluorescence Correlation Spectroscopy methods can determine diffusion constants, oligomerization states, and protein concentrations in the dilute and condensed phases. Dedicated Fluorescence Recovery After Photobleaching (FRAP) methods can measure interfacial properties of condensates. PALM (Photo-Activated Localization Microscopy) and single particle tracking (spt)PALM in live cells offer the means to measure small diffusion constants, for example within a confined space, and track the trajectories of individual protein molecules. In addition, STORM (Stochastic Optical Reconstruction Microscopy) in fixed cells yields super-resolution localization maps of specific proteins.

Explorations of the mechanisms of assembly and the regulation of DDR condensates open new perspectives for targeting DNA damage responses for therapeutic purposes. Modification of the composition or dynamic properties of DDR condensates by condensate-modifying drugs that alter key interaction surfaces may sensitize cancer cells to intrinsic and chemotherapy-induced DNA lesions.

CRediT authorship contribution statement

Emile Alghoul: Conceptualization, Writing – original draft, Writing – review & editing, Visualization. **Jihane Basbous:** Conceptualization, Writing – review & editing. **Angelos Constantinou:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

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

Data Availability

No data was used for the research described in the article.

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