The effect of Liquid-Liquid Phase Separation on the DNA damage response to double-stranded breaks

Sophie Schretlen **4/1/21**

Supervised by prof. dr. MATM van Vugt

Abstract

One of the hallmarks of cancer is genomic instability, which encompasses a high rate of genetic alterations during cell division. To ensure proper cell function and faithful transmission of the genome to progeny, cells have evolved sophisticated and strict mechanisms that accurately respond to DNA insults thereby either repairing the affected DNA or drive the cell into apoptosis, summarized as the DNA damage response (DDR). Recently, the emerging topic of membraneless organelles, formed by the process liquid-liquid phase separation (LLPS) is also implicated to be involved in the DDR. LLPS encompasses the process in which molecules, due to their intrinsic disordered properties, can undergo rapid transitions in phase either by self-assembly or by interactions with other proteins. These properties enable multivalent interactions leading to a dynamic mode of action in which particular molecules are included while others are excluded, thereby compartmentalization reactions to a limited amount of space. LLPS plays a major role in the formation of DNA damage foci, which are compartmentalized droplets comprising of secondary DDR factors that stimulate proper DNA repair. The complex relationship between RNA transcription and the DDR is an emerging topic, in which active transcription is increasingly recognized to function in the DDR after DSB exposure. Correspondingly, this transcription has been shown to drive phase separation due to RNA polymerase II activity, RNAs and RNA binding proteins exhibiting the intrinsic potential to drive LLPS in successive proteins, thereby forming DDR foci. Also, LLPS is involved in the localization and clustering of double-stranded breaks (DSBs) to nuclear pores to promote functional repair. In this thesis, I will outline the current knowledge and findings regarding DNA repair processes in response to DSBs involving LLPS to provide insight into how LLPS contributes to the maintenance of genomic integrity.

Table of content

Introduction

Genomic instability is one of the hallmarks of cancer, leading to the accumulation of mutations and continuous positive selection resulting in tumor formation. To ensure proper cell function and faithful transmission of the genome to progeny, preservation of genome integrity is a prerequisite. The genome is constantly subjected to various endogenous (reactive oxygen and nitrogen species (ROS/RNS), inflammation, dietary factors, hypoxia) and exogenous factors (radiation, UV light) that may cause different types of DNA insults [1,2]. Therefore, eukaryotes have evolved sophisticated and highly coordinated networks that control the replication, segregation, and telomeric function during the cell cycle [3,4]. The most comprehensive mechanism ensuring genomic integrity is the DNA damage response (DDR). The DDR includes a subset of DNA repair mechanisms, damage tolerance processes, and cell-cycle checkpoint pathways [2]. Sensors detect DNA lesions, transducers propagate DDR signals to induce repair, and effectors execute the proper response suitable for distinct types of DNA damages [5].

To maintain genomic integrity, these multiple demanding tasks require a strict organization of the simultaneously occurring molecular reactions, responding with high selectivity while mostly occurring in close spatial proximity. Besides organelles, in which biological reactions are enclosed by a lipid bilayer membrane delimiting uncontrolled reactions with its surroundings, also membraneless compartments are currently of increased interest. These compartments are structures separated from their environment by their biophysical properties. A widespread of current studies have suggested that this compartmentalization is due to liquid-liquid phase separation (LLPS), driving molecules into membraneless organelles, also termed droplets or condensates [6]. LLPS refers to the process in which biological compounds are demixed into two or more distinct phases leading to separated coexisting liquid microenvironments. These coherent structures ensure compartmentalization and concentration of selected molecules, thereby stimulating reactions. In recent years, the application of these micron phases separated droplets has increased, now linking this phenomenon to a variety of cellular processes, such as innate immune signaling, microtubule nucleation, actin compartmentalization, transcription, chromosomal colony formation, and adaptive stress responses. Recently, LLPS is also implicated to be required for the formation of DNA damage foci, necessary for the recruitment of secondary DDR factors [7].

DNA damage foci arise from the multivalent interactions of protein-protein reactions creating spatiotemporal and biophysical boundaries. These condensates are found to be involved in DNA repair in double-stranded breaks (DSB), and single-stranded breaks (SSB), by either homologous repair (HR) or non-homologous end-joining (NHEJ). By facilitating compartmentalization, condensates can shield DNA from detection and processing by unwanted repair protein thereby limiting protein assembly to specific DNA damage machinery [7,8]. Therefore, it is proposed that condensates contribute to functional DNA repair by enabling precise coordination of every enzymatic step, by separating different reactions by promoting nucleation, yet being dynamic and reversible.

The emerging and novel discoveries around LLPS have brought new insight into genetic processing in both normal cellular function and stressful circumstances. The notion that LLPS is involved in a variety of processes of the DDR, implicate that LLPS may also play important roles in cancer evolution. Accordingly, a recent study demonstrated the involvement of LLPS in the efficacy of common cancer drugs: LLPS serves as a mediator of proper drug translocation to damaged DNA thereby modulating drug efficacy, confirming the role of condensates in instable genomes[9]. In addition, some types of cancer exhibit an increase in specific types of condensates when compared to healthy cells, suggesting a pathological contribution of LLPS[8,9]. Since impaired DDR signaling frequently underlies several types of cancer, and LLPS is acknowledged to exert different functions in the DDR, it is important to understand in what way LLPS contributes to the DDR thereby ensuring genomic integrity. To address the emerging perspective of the role of LLPS in DNA repair, this thesis revisits the current knowledge concerning the potential roles of condensates that facilitate DNA repair signaling.

LLPS

In nature, molecules can reside in different phases as reflected in water: damp, fluid, and ice. Recently, also macromolecules are recognized to exhibit different phases. Soluble macromolecules can be referred to as the gas stage, showing a highly entropic homogeneous distribution around the environment. The liquid phase, on the other hand, is characterized by droplets of liquid including and excluding specific compartments, exhibiting a fluid texture. Solid phases are referred to as the low entropic stage in which compounds are determined to a specific space, without mobility [7]. When molecules transcend their solubility factor, they enter a different phase in which the entropy of the universe increases when molecules are clustered. In other words, less energy is required when droplet-like clusters exhibiting intermolecular interactions are formed, than the entropy when molecules are homogeneously spread in solution[10].

Inside cells, LLPS drives the concentration of specific molecules, thereby including and excluding molecular content based on their biophysical properties to confine reactions to specific space areas. The surface tension enables this insulation and extraction of molecules forming droplet-like spherical phases that exhibit viscous structures with dynamic fluctuations of shape. Condensate formation is a reversible process, in which the formation and dissociation, and the exchange of components with the external fluid enable the membraneless compartments to easily turn on and off intracellular functions thereby contributing to proper cell signaling[8]. The liquid phase enables dynamic and mobile movements of the included molecules and promotes the exchange of molecules with their environment. This dynamic mechanism of LLPS is ensured by the ability to form multivalent weak interactions, frequently generated by intrinsically disordered regions (IDR) or low-complexity domains (LCDs) in proteins or nucleic acids[11,12]. These regions increase the engagement of multivalent interactions due to their lack of predetermined structure thereby retaining conformational flexibility compared to highly organized protein structures[13].

In the living cell, a couple of distinct droplet types reside in the nucleus, including the nucleolus, nuclear paraspeckles, RNA granules, silent heterochromatin domains, promyelocytic leukemia bodies, chromosome territories, DNA damage foci, and transcriptional condensates[6]. However, when the cell experiences stress for a prolonged period, the droplet formation becomes irreversible exhibiting a liquid-to-solid transition, leading to aggregates. These aggregates are found to play a central factor in many diseases, among which neurodegenerative diseases and cancer[7].

The process of LLPS can be distinguished into three phases, including nucleation, growth (either by coalescence or inclusion), and coarsening. When emphasizing DDR foci formation, the first phase withholds the initiation of phase separation induced by DNA damage, exhibiting small droplet formation surrounding lesions [14]. The second phase encompasses maturation and growth during which foci include successive components, thereby becoming increasingly viscous. Finally, coarsening, or ripening entails the process during which the number of nucleated foci decreases, while the radius and volume of condensates increase[10,15]. During this stage, diffusing smaller droplets coalesce with larger droplets. After maturation, droplets frequently halt their growth showing a constant focus radius, while exhibiting dynamic fluctuations in surface tension caused by rapid exchange of included compartments. This dynamic exchange of molecules of condensates reflects the liquidity of the droplet[16].

The DNA damage response

The genome is constantly challenged by different types of insults and stress types, ranging from obstacles in the DNA molecule, exogenous and endogenous factors affecting DNA, and intrinsic inefficiency of replication-related factors. These DNA damages can cause mutations that impair normal cellular functioning, causing diseases such as cancer. To recognize and repair these DNA damages, eukaryotes have evolved sophisticated networks of rapid-acting DNA repair machinery, damage tolerance processes, and cell-cycle checkpoint pathways, summarized as the DNA damage response (DDR). The damages or stress-specific responses can vary based on the type of insult: distinct mechanisms are induced as a response to single-stranded DNA (ssDNA) breaks (SSB), while others are induced when double-stranded breaks (DSB) occur. The core of the DDR pathways involves the base excision repair (BER), nucleotide excision repair (NER), both acting in response to SSBs. Homologous recombination (HR), and non-homologues end-joining (NHEJ) are induced after exposure to DSBs. Together, these processes can repair the vast majority of insults affecting the genome, thereby maintaining genomic stability [2]. As the two major DNA repair processes of double-stranded breaks in eukaryotes, I will outline how LLPS is involved in homologous recombination and nonhomologous end-joining.

Double-stranded breaks

When both strands are affected by damaging factors such as radiation, reactive oxygen species, DNA replication errors, and agents that induce inter-strand cross-links (ISCLs), the lesions are more difficult to repair. The response to DSBs can be grossly divided into two distinct mechanisms in which their activation is predominantly dependent on the stage of the cell cycle. The most common pathways, homologous recombination (HR) and non-homologous end-joining (NHEJ), act in the S- / G2 phase and the post-mitotic G1 phase, respectively [17]. The distinction thus depends on the presence or absence of a sister chromatid containing the information of base pairs that should be present in the lesion. The initial steps in these pathways involve the instant recognition of double-stranded breaks (DSB) and the generation of accessible chromatin.

Homologous recombination

During the S- and G2 phases, the HR pathway is predominantly activated to repair DSBs. The initial steps after DNA damage exposure are decisive for the DNA repair pathway, in which the end resection of exposed DNA ends triggers HR repair. Within seconds, PARP1 recognizes DNA strand interruptions and triggers the addition of poly(ADP)-ribose units forming polymers at the DSB site, termed PARylation. This PARylation results in relaxation of chromatin allowing access of the helicase-nuclease "sensor" Mre11-Rad50-Nbs1 (MRN) complex to the site of damage via the association with PAR chains. The assembly of the MRN complex holds the broken strands in place and activates ATM, after which the MRN-ATM collaboration recruits and provides a structural basis for successive repair factors BRCA1, CtIP nucleases, EXO1[18,19]. Simultaneously, ATM predisposes the histone γH2AX thereby modulating chromatin for hundreds of kilobases from the damaged site. Following the initial recognition, end resection of the broken DNA ends is induced, which occurs in a two-step mechanism thereby generating an intermediate for HR strand invasion [20]. The MRN-CtIP interaction initiates DNA-end resection of both double-stranded breaks, which catalyzes the nucleolytic degradation of the broken ends in the 5' to 3' direction thereby removing the first 50-100 bp [21]. In the second step, recruited EXO1 and/or DNA2 nucleases generate long 3' ssDNA tails by extending the resected tracts [22,23]. The 3' directional ssDNA, or overhang, provides the structural platform for proteins that participate in the subsequent homologous recombination repair.

Strand invasion

Ubiquitous and abundant ssDNA binding protein RPA binds to the exposed ssDNA region after which the RPA is exchanged for RAD51 nucleoprotein filament mediated by the PALB2-BRCA2 effector complex, upstream TopoisomeraseII (Top2) and BRCA2-interacting protein C-terminal helicase 1(BRIP1). The RAD51-ssDNA filament leads to strand invasion into the homologous sister chromatid, generating a temporarily triplex-DNA intermediate, in which strand information exchange occurs [20,24]. The Rad51 nucleoprotein is essential for the search to the homologous sequence on the complementary strand of a sister, or donor DNA duplex, followed by resolution of branched DNA structures, DNA synthesis, and ligation [23]. DNA repair is ensured by three distinct mechanisms, including break-induced replication (BIR). Synthesis-dependent strand annealing (SDSA) withholds that the 3'end strand responsible for invasion is extended by DNA synthesis followed by D-loop formation and inversion of the primary break site to re-establish the missing sequence at the breakpoint. Due to the D-loop inversion of the synthesized DNA extension, the annealing with the resected end in 5'-3'direction is promoted [25]. The latter process is mediated by RAD52 and promoted by homologue sequences annealing culminating in the reconnection of the two broken ends. The last process involved in HR is double Holliday junctions (dHJ). This involves the BTR complex containing four proteins, the Bloom's syndrome helicase BLM, Topoisomerase IIIa, RMI1, and RMI2. The BTR complex promotes the migration of two HJs to produce the formation of a hemicatenane structure, which is a junction between two complementary strands of double-stranded DNA [19].

Non-homologous end-joining

Double-stranded pathological breaks, as well as physiological regulated breaks, require proteins that recognize, resect, polymerize, and ligate the DNA ends, which predominantly occurs during the G1 phase of the cell cycle. Non-homologous end joining (NHEJ) is recognized as an error-prone process since the repair pathway involves the direct ligation of DNA ends without or with little homology in which nucleotides can be easily gained or lost at the DNA ends before ligation [20,26]. In addition, direct end-joining of DSBs on different chromosomes can result in chromosome translocations [20]. The initiation of NHEJ requires the recognition of DSB by Ku70-Ku80 (Ku) heterodimer, which, when recognized, acts as a loading protein to which other proteins are recruited, simultaneously protecting the ends from degradation. The DSBs consist of two incompatible DNA ends that hinder direct ligation. The recruited complexes involve NHEJ polymerase, nuclease, and ligase machinery. These factors perform in concordance multiple rounds of resection and addition of nucleotides aiming at creating micro-homology between the two DNA ends to ensure end-joining [27]. DNA-dependent protein kinase (DNA-PK) is the central checkpoint kinase regulating NHEJ, which interacts with Ku. Two DNA-PK complexes are directly activated at the two DNA ends, thereby recruiting other factors to the site of damage, such as Artemis, XRCC4/ligase IV/XLF, DNA polymerase λ, and μ [17,28]. The next step of human NHEJ involves the DNA polymerase μ (pol μ) and Pol λ, which interact with Ku through their N-terminal BRCA1 C terminus domains. After resection, the polymerases incorporate either dNTPs in both template-dependent and template-independent matter [29]. Subsequently, the DNA-PK complex recruits XRCC4/DNA ligase IV (Dnl4/Lig4) and ligate the newly incorporated nucleotides [30]. The choice of end-joining pathways distinguishing between HR and NHEJ relies on the balance between resection proteins, Ku, and other DSB recognition proteins [31,32]. The extent of end resection is a decisive factor for which pathway is induced. The process is asserted: longer end resection is likely more beneficial for HR because the affinity of Ku to DNA strands decreases when resected strands are longer. Overhangs of homology (~4 nucleotides) induce NHEJ, in contrast to end resection of larger fragments (~30 nucleotides) if HR is favored[27,28]. In response to PARylation, MRN and Ku are simultaneously recruited to the DSB strand in which they either antagonize each other. However, the exact roles remain elusive and are highly dependent on the damage site, a time point in the cell cycle, and type of damage as well as the damaging agent [3334].

Chromatin remodeling and DDR foci formation

In eukaryotes, genetic information is packaged into chromatids, in which the DNA is wrapped around chromatin repeating units of nucleosomes connected by linker DNA. The core components of these nucleosomes are histone octamers, which are catalyzed by histone modification enzymes establishing posttranslational modifications to regulate chromatin conformation and gene expression. As a response to DNA damage, posttranslational signaling pathways remodel the chromatin in the vicinity of the DNA lesion and shut down the transcriptional activity nearby promoters. One of the first events following DNA damage is the phosphorylation of H2AX into γH2AX by ATM, DNA-PKs, or replication stress-induced ATR to amplify local DDR signaling at the lesion site leading to γH2AX foci formation. Simultaneously, γH2AX foci formation leads to decondensation of chromatin, accompanied by a widespread of other types of chromatin modifications, which spreads over a large region from the DNA break site, thereby recruiting successive DDR factors: When HR is induced, γH2AX is required for the accumulation and retention of Rad50, Rad51, BRCA1 and MDC1[35]. In terms of NHEJ, DNA-PK mediated histone modifications result in the secondary recruitment of factors involved in the inhibition of end resection by 53PB1. The amplification of DDR involved factors, in turn, leads to the recruitment of more MRN-ATM or Ku-DNA-PK axes and successive histone modification, establishing a positive feedback loop[36]. Therefore, the spreading of γH2AX and histone modification induces a secondary wave of DDR factors to the damage site, thereby increasing the local density, thus, establishing cytologically detectable DDR foci [21,35].

LLPS in DNA repair

The local amplification of DDR factors requires spatial and temporal coordination of the dynamic interactions of repair proteins and chromatin, which is accomplished by liquid-liquid phase separation. The spatiotemporal focal assemblies of secondary DDR factors are called DNA repair foci and ensure compartmentalization of distinct repair pathways by clustering reactions to limited space areas. Therefore, it may be assumed that LLPS plays a major role in the DDR. Despite some indirect regulatory aspects of LLPS in the DDR, such as the organization of chromosome territories and silencing, enhancing genes encoding cell cycle proteins, and/or specific DDR machinery, here, I will review all aspects in the formation of DDR foci that directly involve LLPS driven reactions.

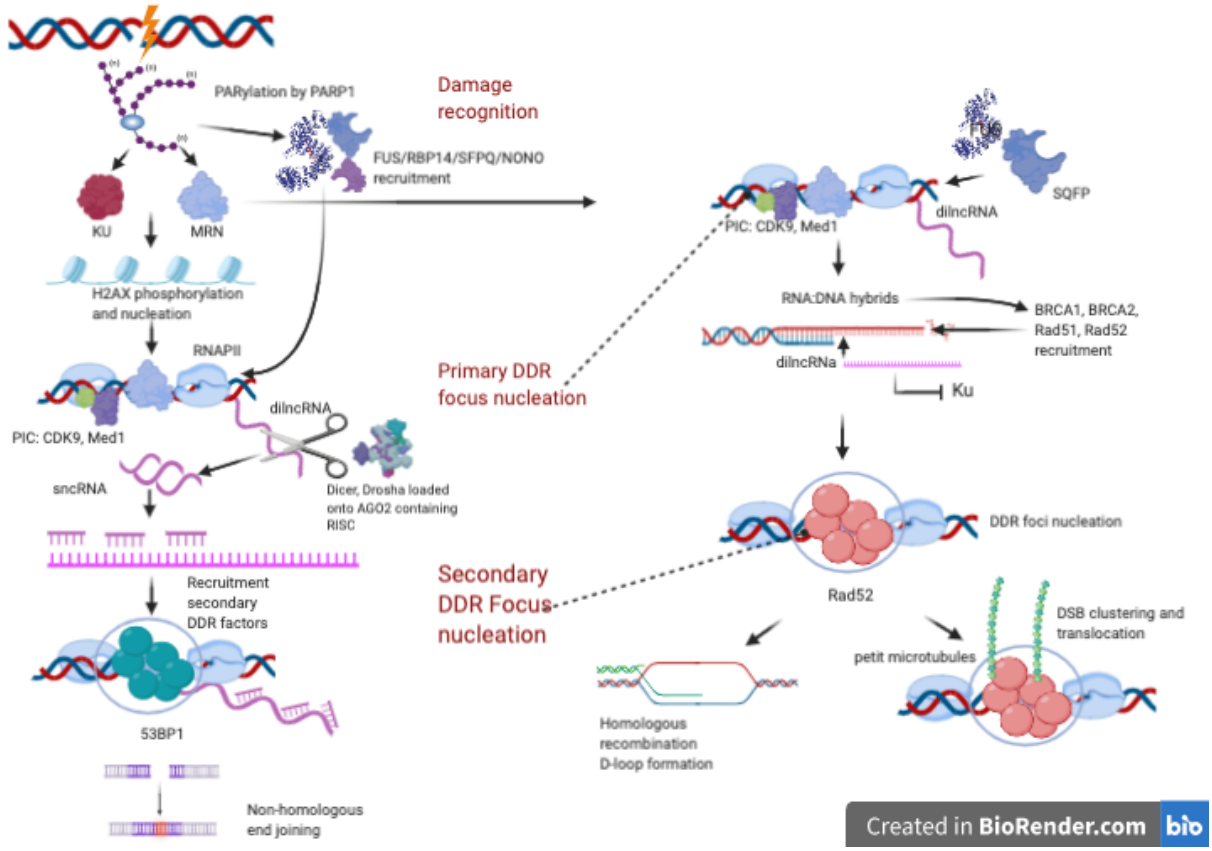

Fig 1. The processes of the DDR after DSB exposure involving liquid-liquid phase separation. Following DSB exposure, PARylation of DSB ends results in the recruitment and assembly of MRN and the stabilization of Ku, resulting in H2AX phosphorylation. RBPs including FUS is also immediately recruited towards DNA break ends in a PAR dependent matter forming liquid separated condensates, which mediates this γH2AX foci nucleation. γH2AX foci serve as beacons onto which successive DDR factors can assemble. Besides the DSB break end itself reminiscent of promotors or enhancers, FUS and RBM14 mediate the recruitment of transcription factors, CDK9, Med1, PIC, and RNAPII. Their activation results in the transcription of lncRNA, which is subsequently processed further into DDRNA by Dicer and Drosha. DDRNAs are found to be necessary for the site-specific recruitment and assembly of secondary DDR factors, including 53BP1, and are guided towards DSBs by their precursor dilncRNA. In parallel, dilncRNA transcription at DSB sites drives phase separation of the secondary DDR factor 53BP1 forming DDR foci[15,37–40]. When repair is favored by HR, Rad52 is recruited in a transcription-dependent matter and regulates the clustering of DSBs forming DNA repair centers. Interaction of Rad52 with petDIMs results in phase separation, thereby ensuring functional relocation of the DSBs to NPCs. The formation of DDR foci reflects the importance of LLPS in biological processes, since the condensate formation of DDR factors and generated RNAs are necessary to ensure functional repair signaling. Created with BioRender.com

PAR

PAR is a polymer of ADP-ribose units resembling nucleic acid in nucleobases, ribose sugars, and phosphates. In contrast to DNA and RNA, PAR covalently binds to proteins thereby inducing post-translational modifications termed poly(ADP)-ribosylation (PAR), to define the protein structure and folding. The modifications are added by poly(ADP-ribose polymerases (PARP) while removed by poly(ADP)-ribose glycohydrolase (PARG) [32]. PARylation results in branching patterns consisting of 2-200 units, exhibiting a high local density of negative charges that function as a platform to recruit positively charged domains. Proteins susceptible to undergo phase separation have been recognized to contain a high frequency of repetitive arginine and glycine-glycine (RGG) motifs, as well as RNA binding domains (RBD), thereby allowing dynamic multivalent protein-protein interactions. PARylation has been shown to seed phase separation of these intrinsically disordered proteins: The negatively charged PAR-chain exhibits low complexity dynamics due to its repetitive surface of the nucleic acid. Therefore, PARylated proteins serve as a multivalent platform for non-covalent bindings of proteins, which promotes in vitro phase separation[41]. In conclusion, PAR modifications act as concentrating agents and nucleation points that initiate the spatiotemporal phase separation of proteins confining reactions into specific droplets[42].

DNA damage ends exhibit also enrichment of negative charges which triggers PARP-1 recruitment and activation [42]. Therefore, this model reflects the earliest stages of the DDR: DSBs induce rapid recruitment of PARP1 which in turn causes PAR modifications conferring a negatively charged area, termed nucleation, attracting positively charged LCD containing residues, such as FUS, EWS, and TAF15 [43]. The structural flexibility of positively charged repetitive elements of proteins therefore promotes their multivalent electrostatic interactions with PAR chains at DNA ends to promote LLPS [10]. The transient nature of liquid droplets is thought to be ensured by phosphorylation, which reverses the phase separation by introducing negative charges. Indeed, phosphorylation of proteins concentrated within the liquid droplets results in disruption of the liquid-liquid phase-separated state [6].

In the context of the DDR, PARP1 has been shown to recognize both SSBs and DSBs. PARP-1 activation precedes the recruitment of both the MRN and the Ku complex, implicating that PARP-1 functions as the primary DSB sensor of both HR and NHEJ. The corresponding PARylation may therefore guide and concentrate Ku and MRN complexes at DSB sites which in turn phosphorylate H2AX thereby facilitating DNA repair foci formation[34]. PARylation leads to the assembly of HR involved factors, inducing end resection. In contrast, PARP-1 inhibition reduces the loading of 53BP1 at DDR foci, implicating that PARP-1 is also a critical factor involved in NHEJ regulation(fig. 1)[41]. The outcome of the ambiguous mode of action of PARP1 in early DDR responses is dependent on successive associating proteins, such as FUS and noncoding RNA (ncRNA), 53BP1, and Rad52 which has been shown to rapidly accumulate at DNA damage sites in a PAR-dependent matter (fig. 2).

RNA-binding proteins: SFPQ, RBM14, NONO

RBPs are disordered proteins that can form condensates due to their enriched LCDs and PLDs enabling both multivalent interactions with RNA molecules and PAR chains, and homotypic self-assembly[44]. Besides their roles in RNA metabolism, including mRNA splicing, RNA biogenesis, modifications, and mRNA processing, a subset of RBPs have also been implicated to impart direct roles in the DDR by regulating DDR foci formation, including SFPQ, RBM14, NONO, and FUS. Therefore, RBPs can be assigned as one of the crucial factors in controlling the balance between transcription and DNA damage machinery at DNA damage sites. Following DSB exposure, RBPs are rapidly recruited towards the PARylated break ends of the DNA lesion where they undergo PAR-dependent phase separation.

Attenuation of either SFPQ or NONO results in decreased resolution of γH2AX foci formation, thereby counteracting the secondary DDR wave[45]. Purified and isolated SFPQ plays roles in HR repair, exhibiting re-annealing, and strand invasion capacity, contributing to D-loop formation [41,46]. Though, SFPQ plays a role in NHEJ as well[47]. SFPQ interactions with NONO, but also purified NONO apart from SFPQ, explicitly stimulate canonical NHEJ (cNHEJ) during the secondary damage response, by substituting the core NHEJ protein XLF ligation activity thereby promoting end-to-end joining [41]. The interaction of NONO and SFPQ has also been shown to stimulate side-by-side end-joining evolving in loops that contribute to ligation by increasing the DNA and DNA ligase IV XRCC4 concentration. Overall, as these factors contain IDRs, they drive LLPS of higher-order protein complexes after their assembly. In addition, these domains bind to long noncoding RNAs (lncRNA) by their shared globular domain [45]. Therefore, NONO and SFPQ have also been implicated to mediate RNA dependent DNA repair thereby driving DDR foci formation, further elucidated in the chapter 'FUS' (fig. 2).

RBM14 interacts with Ku, thereby promoting the recruitment and assembly of ligase complex IV XRCC4 and XLF promoting cNHEJ[13]. Ku assembles in response to the direct PARylation of DNA ends after DSB induction, followed by lncRNA transcription by RNAPII. The lncRNA transcription at DSB sites in transcriptionally active genes as in intergenic regions is promoted by RBM14 in a Ku-dependent matter, by acting as a co-activator of the RNAPII[48]. The RBM14 dependent transcription at DSB sites generates noncoding RNAs that serve as platforms for further assembly of DDR factors. Due to the intrinsically disordered domains, RBM14 is phase-separated when interacting with NONO and SFPQ, which is downstream of RNA synthesis, closing the circle (fig. 1)[49].

FUS

The RBP Fused-in-sarcoma (FUS) as part of the FET-family is reported to be a multifunctional factor involved in DNA/RNA binding, including transcription, pre-mRNA splicing, mRNA transport, and translation. Recently, FUS is also recognized to be involved in the DDR. Lack of FUS both *in vivo* and *in vitro* sensitizes cells to DNA damage or results in inefficient DNA repair, both reflected in increased and prolonged H2AX phosphorylation. Collisions between replication and transcription machinery can result in the generation of RNA:DNA hybrid, or R-loops, that can be processed to DSBs [50]. FUS is implicated to counteract R-loop formation by stimulating the formation of D-loops between complementary DNA molecules. Therefore, FUS is thought to prevent the aberrant pairing of the nascent RNA to the template DNA strand and thus, the generation of DSBs.

Besides its role in preventing DNA damage, FUS plays also a direct role in the DDR. Following DNA damage, FUS is recruited early in a PAR-dependent matter. In turn, FUS is required for the higher-order spatial clustering of γH2AX foci during the first steps of the DDR thereby establishing a platform for assembly of secondary DDR factors. Indeed, upon synthetic DSB induction in FUS KO cells, decreased γH2AX foci were observed compared to FUS WT which in turn correlated with a delayed assembly of 53BP1. Thus, FUS is recruited early at sites of DNA damage as a substrate of ATM and DNA-PK and in turn, stimulates protein assembly of secondary DDR factors such as PB531 by orchestrating the organization of the γH2AX nano foci required for DDR foci formation[8].

The D-loop formation mediated by FUS preventing DSB induction, is also one of the first steps of HR *in vitro*, suggesting that FUS mediates HR induction. The dilncRNA after RNAPII transcription pairs with DNA, followed by FUS binding, thereby inducing excessive end resection [51]. On the other hand, Ku assembly to DNA broken ends was impaired upon DSB induction when FUS was knocked out, showing a shorter Ku peak and reduced Ku accumulation compared to WT cells. Therefore, FUS is also implicated to be involved in Ku retention thereby competing against MRN regulated end resection.

Indeed, when Ku retention was impaired, NSB1 levels were increased. Overall, these results suggest that FUS plays a role in the regulation of the NHEJ or HR repair pathways.

Interestingly, FUS contains highly conserved PLDs and LCDs that give rise to homotypic multivalent intermolecular interactions resulting in FUS self-assembly[52]. In a FUS dependent matter, multiple RBPs are recruited, implicating a direct role of RNA in the DDR. At physiological concentration, FUS is shown to drive LLPS via multivalent protein-protein with SPFQ/NONO or RNA-protein interactions, constituting higher-order structures exhibiting higher complexity in terms of shape and reversible dynamics[52,53]. Besides PAR, FUS clustering is also dependent on RNA transcription, showing that KO of RNAPII impairs FUS droplet formation[54]. Vice versa, FUS mediates RNAPII recruitment towards DSB ends [55]. Inhibiting FUS-induced liquid droplet formation by aliphatic alcohols and ammonium acetate impaired the formation of γH2AX foci and indirectly 53BP1 foci, implicating that FUS-dependent LLPS is required to ensure efficient DDR foci formation. The LLPS -generated DDR foci formation is tightly regulated, which is reflected in the observation that DNA-PK phosphorylates FUS thereby inhibiting phase separation and assembly of secondary DDR factors. These results imply that FUS-driven LLPS occurs at sites of DNA damage and that these droplets are required for the formation of DNA damage foci and the activation and retention of the DDR signaling cascade (fig.1)[36].

Noncoding RNA

Over the past decade, growing attention has been drawn to the complex interplay between RNA transcription and DNA repair. Despite the proposition that DSB-induced damage repair withholds transcription by affecting promoters located near the DSB, mounting evidence indicates that DNA in the vicinity of the DSB still is transcribed into distinct types of RNA without the requirement of promoters, among which noncoding RNA (ncRNA) is the most common type[15]. NcRNAs are sequences transcribed while not encoding a protein, representing the vast majority of all transcripts from the nuclear genome in eukaryotes. NcRNAs involved in the DDR are divided into two major groups based on their size: small ncRNAs and long noncoding RNAs. The ncRNAs transcribed in the vicinity of DSBs exert different functions in DNA repair in various ways [56]. First, ncRNAs have been shown to regulate the abundance of DNA repair proteins (1) [57]. Secondly, evidence showed that HR repair is guided by ncRNAs (2) [58]. Last, the ncRNAs serve as an intact copy used as a template for DSB repair (3) [59]. Since RNA, DNA and PAR-chains contain both LCDs and PLD with the intrinsic high multivalent capacity to interact with several RBPs and RBDs[60]. Therefore, the low-complex nature of RNA allows modulation of the dynamicity and the phase separation of biomolecular condensates[60].

NcRNAs generated in response to DNA damage, have been implicated to function as platforms for secondary DDR factors. In response to DSB exposure, PARylated DNA ends to recruit the MRN and Ku complexes leading to H2AX phosphorylation. In a γH2AX dependent matter, MRN recruits the transcription machinery PIC, Med1, and CDK9, followed by RNAPII to the DSBs in a site-specific manner, reminiscent of transcriptional promoters or enhancers. After assembly of this transcription machinery, the bidirectional RNA synthesis is initiated from and towards exposed DNA ends. These transcribed RNAs carrying the sequence of the DNA flanking the DSB are termed damage-induced lncRNA (dilncRNA)[15,61]. Following the transcription of long noncoding sequences, the precursors are further processed into small non-coding RNA (sncRNA) by the RNA interference pathway (RNAi)-dependent factors, Dicer, and Drosha, which load onto the RNA-induced silencing complex (RISC) containing Argonaute-2 (AGO2)[37]. SncRNAs that are transcribed from sequences in the vicinity of the damage site is termed DSB-induced small RNAs (DDRNAs). Previous studies showed that the recruitment of secondary DDR factors to sites of DNA damage relies on this Dicer- and Drosha-dependent generation of DDRNAs [39]. Moreover, Drosha and Dicer are not involved in the initial recognition of DSBs, but rather function similar to H2AX by providing scaffolds for RNA-protein interactions thereby retaining DDR factors in proximity to DNA lesions (fig. 2)[38,39].

Instead of hybridizing with DNA at the damaged locus, the pairing of DDRNAs with their precursor dilncRNA allows site-specific localization of DDRNAs at the damaged sites. These *de novo* generated DDRNAs are required for the site-specific recruitment and accumulation of secondary factors, such as 53BP1 and Rad52, at DNA damage sites in the form of DDR foci [15,38]. Interestingly, DNA repair-involved transcription is found to drive LLPS of biomacromolecules. First, after phase transition, FUS recruits RNAPII and RBPs (see: 'RBM14') together forming liquid droplets, thereby regulating transcription [6]. Second, RNA itself is a crucial factor to drive phase separation of RNA-protein condensates. Research showed that dilncRNA, in a sequence-dependent matter, can increase the viscosity of nucleating foci and increase the exchange rate thereby establishing condensates, as is shown in 53BP1 and Rad52 foci [15,58,62]. Therefore, dilncRNA transcription controls the condensate evolution over time by accelerating maturation and ripening of LLPS condensates, stressing the property of RNA to modulate condensate sizing and composition [12]. Importantly, inhibiting DDRNA or lncRNA formation by antisense oligonucleotides or RNAPII inhibition prevents recruitment of secondary DDR factors [35]. Moreover, only in the presence of H2AX-containing nucleosome arrays and subsequent RNAPII and PIC recruitment, dilncRNA:DDRNA interactions lead to the recruitment and phase separation of secondary DDR factors 53PB1 and MDC1 (fig. 1)[61]. Therefore, the local transcription of dilncRNA guiding DDRNAs, together with H2AX phosphorylation, is required for the recruitment and phase separation of secondary DDR factors in the form of DDR foci.

53BP1

53BP1 is a key DNA repair factor since, predominantly involved in the choice of pathway. Association of 53BP1 with DSBs promotes NHEJ in G1. 53BP1 contains interaction surfaces for numerous DSB-responsive proteins constituting a platform for the recruitment of other repair factors. Therefore, 53BP1 is assigned as an adaptor/mediator for processing of the DDR signal[63]. Almost all factors exhibiting LLPS converge on the assembly of 53BP1. First, the binding of 53BP1 is dependent on the initial PARylation of the DSB ends, since blocked Parylation results in reduced 53BP1 assembly. Secondly, H2AX foci formation is determinant for 53BP1 assembly, reflected in reduced 53BP1 recruitment when H2AX phosphorylation is impaired. Thirdly, DDRNAs:dilncRNAs interactions drive the secondary assembly of 53BP1. Finally, 53BP1 foci formation is dependent on FUS. Thus, the axis from PARylation of DSB break ends to secondary recruitment of 53BP1 is for the majority dependent on LLPS (fig.1,2).

Since 53BP1 contains intrinsically disordered domains capable of forming multivalent protein-protein interactions, it is demonstrated that 53BP1 drives liquid separation of DDR foci. Localization of 53BP1 to damaged sites is driven by the Tudor domain which specifically binds histone H4 dimethylated lysine-20 (H4K20me2) [64]. When bound, 53BP1 recruits successive proteins establishing a competitive assembly of HR or NHEJ dependent factors. NHEJ of DSBs is promoted by 53BP1-RIF1 REV7 and the Shielding complex complexes that protect the DSB ends from exonuclease processing [65]. Loading of the MRN-CtIP-BRCA1 complex onto DSB ends initiates HR repair. Instead of blocking MRN assembly, 53BP1 antagonizes the initial BRCA1 accumulation at damaged sites thereby counteracting HR induction by preventing end resection[65,66]. Since NHEJ is promoted by 53RB1 but antagonized by CtIP/BRCA1, the abundance and accessibility of these critical factors are highly dependent on the timing during the cell cycle and is therefore highly CDK dependent [21]. Importantly, 53BP1 is an adaptor/mediator for DNA damage signaling which accurately responds to DNA damage where it forms nuclear foci. Rather than inducing repair itself, 53BP1 acts as a transducer of the DNA damage checkpoint signal through regulation of p53 accumulation [67]. Indeed, the increased local density of p53 has been found in droplets formed by 53BP1 phase separation.

BP531 foci formation is owed to the BRCT and OD domain showing strong liquid separating properties. The BRCT and OD domains found in the arginine and tyrosine rich C-terminus are implicated to enable self-assembly of 53BP1 thereby amplifying secondary DSB responses. The contribution of BRCT was

surprising since it mainly antagonizes 53BP1 activation. The ambiguous roles of BRCT in DSB repair can be explained by the observation that BRCT is recently found to stabilize p53, thereby conducting tumor-suppressive motion. Therefore, the BRCT domain contributes most to the transient 53PB1/p53 interaction after which p53 relocates to exert its gene regulatory function. Sorbitol treatment, which previously has been shown to disrupt 53BP1 foci formation, impaired 53BP1 self-assembly, and impaired p53 stabilization while leaving the insult recognition and accumulation compartments MDC1 as well as H2A unaffected, implicating that p53/53RB1 interactions require phase condensation[68]. Consistent with previous reports, the p53 and p21 activation are dependent on 53BP1 accumulation, resulting in checkpoint defects. Therefore, phase separation of 53BP1 is thought to integrate initial recognition reactions after DNA injury with effector proteins, thereby coordinating the detection of DNA insults with global alterations in gene expression maintaining cell cycle checkpoints.

Rad52

To prevent deleterious translocations, which occur when two DSBs are abnormally rejoined inducing incorrect genomic rearrangements, cells have evolved strict mechanisms regulating DSB repair ensuring proper strand invasion of the 3'-end that search for correct homologous sequences. Additionally, heterochromatin domains may inhibit DSB repair due to their dense conformation. Therefore, to ensure proper DSB end-joining, to escape repair-repressive heterochromatin domains, and to localize the DSBs to repair conducive nuclear pore complexes (NPC), movement of DSBs is required to promote functional DNA repair. Furthermore, DSBs are clustered into DNA repair centers thereby ensuring concentration of repair enzymes while excluding interfering protein machinery.
The delocalization of these DNA repair centers to NPCs is required to increase the accessibility of repair machinery to DNA lesions [25]. DSBs are transported onto intranuclear microtubule filaments (DIMs) emanating from the microtubule-organizing center (MTOC) by kinesin-14 motor proteins Kar2 and Cik1 motor proteins.
It has been shown that the DIMs associate with Rad52 foci, in which the capturing of multiple Rad52 foci with smaller DIMs (petDIMs) is favored thereby promoting droplet fusion and DSB clustering. Rad52 contains intrinsically disordered regions and domains driving phase-separated foci formation. Furthermore, computational fluid dynamics simulations revealed that the generated petDIMs assemble and disassemble at the same speed as Rad52 foci appearance and disappearance, further substantiating the observation that petDIMs enable droplet fusion at DSB sites. Therefore, the petDIMs bind to Rad52 thereby driving focus formation followed by the fusion of multiple Rad52 driven condensates forming DNA repair centers. Accordingly, disruption of these short DIMs resulted in decreased fusion and fission velocities of Rad52, together with excessive DDR signaling. The formed repair center droplets formed by DIMs in concordance with Rad52 concentrate tubulin thereby projecting an aster-like microtubule filament. The filament-containing droplet then associates with long DIMS, after which the captured droplet moves along the long DIM enabling translocation to the perinuclear space to promote DNA repair at NPCs (fig. 1, 2).

Discussion

In this essay, I reviewed the most recent data and current knowledge regarding the role of LLPS in the DNA damage response to double-stranded DNA breaks (fig.2). LLPS is predominantly involved in the formation of DNA damage foci, comprising of secondary DDR factors. When proposing a model to explain how LLPS is involved in the DDR, LLPS plays predominantly roles in the recruitment and retention of effector molecules. The primary recognition and subsequent remodeling of chromatin, including γH2AX foci nucleation, predisposes the DNA break to the assembly of successive factors to form DDR foci. Only recently, RNA has been implicated in the DDR, and LLPS helps to explain in what way RNA contributes to accurate signaling ensuring DNA repair. Important to notice is the interdependence between LLPS and DDR signaling factors in every step of the DDR signaling pathway converging on 53PB1 phase separation (Fig. 2).

The *de novo* transcription of dilncRNA has been shown to recruit and drive phase separation of secondary DDR factors forming DDR foci[61]. Therefore, liquid phase separation constitutes a link between DDR signaling and RNA transcription. The implication that RNA synthesis drives phase separation[15], indicates that more proteins undergo phase separation during DNA repair, provided that the interacting molecules exhibit LLPS properties. In addition to the RNA:RNA binding driving 53BP1 phase separation in response to DNA damage, dilncRNAs also pairs with DNA forming RNA:DNA hybrids, or R-loops. Studies in yeast showed that locally produced dilncRNA hybridizes with the DNA template forming D-loops thereby inhibiting Ku assembly while promoting the assembly of BRCA1, BRCA2, Rad51, and Rad52, thus stimulating repair via HR [69] (fig. 1). Moreover, Rad52 recruitment to invading intermediates is shown to be dependent on lncRNA: DNA hybrids, in addition to PALB2-BRCA complexes[58]: Rad52 has been shown to interact with RNAPII, followed by the binding to RNA, forming a sequence-directed ribonucleoprotein Rad52-RNA complex, thereby promoting DNA repair utilizing RNA as a template (fig. 2)[25]. However, whether these RNA:DNA hybrids drive LLPS, or whether these Rad52-RNA complexes are liquid droplets, remains unknown. Given that RNAPII, Rad52, and RNAs all contain the intrinsic potential to form demixed liquid droplets, further investigation is needed to determine whether dilncRNA in concordance with Rad52 binding also drives phase separation in HR repair.
In line with the function of Rad52 to cluster and relocate DSBs as DNA repair centers, also 53BP1 is postulated to provide a mechanical buffer between repair compartments and undamaged areas of the genome thereby ensuring compartmentalization. The outer shell of 53BP1 generates these mechanical forces[68], thereby pushing undamaged chromatin regions away from the break site, thus rearranging chromatin. This mode of action is similar to that observed in Rad52, suggesting an interrelationship or collaboration between both mechanisms while regulating distinct pathways.

As was shown in recent studies in 53PB1, the formerly observation that the N-terminus predominantly drives phase separation, turned out to a precipitated conclusion[68]. The intrinsically disordered N-terminus was dispensable when compared to the contribution of the tyrosine and arginine-rich C-terminus, exhibiting electrostatic interaction- and pi-pi interaction- potential. Phase condensation detection is frequently based on the IDR mediated simple coacervation, which encompasses the phenomenon in which IDRs that lack charged residues phase separate due to a preference of homotypic self-assembly over free movement in suspension[44]. Indeed, pi-pi bonding and electrostatic interactions are overlooked over the past decade when investigating phase separation, while comprising great potential to form multivalent interactions[70]. Therefore, the presence of disordered sequence stretches alone may not be a good predictor for phase separation. Even though low complexity-based self-assembly constitutes an important source of LLPS, it may not be the primary factor responsible for liquid phase separation, or it may be overshadowed by other known or not known properties associated with LLPS[71]. Therefore, research conducted in the last decade analyzing components with phase separating properties based on simple coacervation may need to be revised.

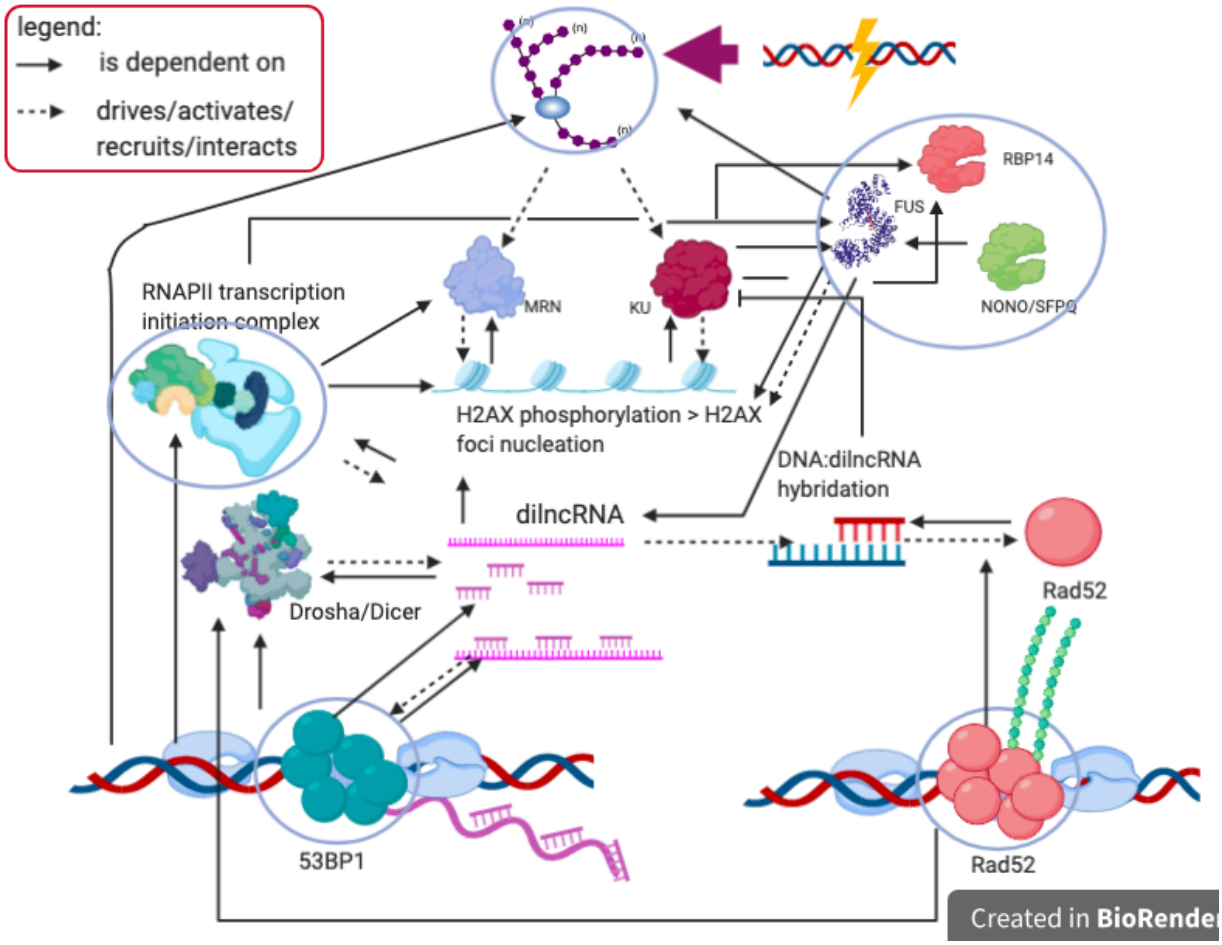

Figure 2: The interdependence of nucleated condensates and DDR factors: Parylation of DSB break ends recruits a subset of proteins: PAR recruits and activates MRN and Ku. Its phase condensation state is necessary to drive phase separation of RBPs FUS, RBM14, and NONO/SPFQ analogs. MRN and Ku activation results in the phosphorylation of H2AX and the organization of clustered H2AX foci, thereby forming a beacon for successive factors to assembly. In turn, this reorganization is dependent on FUS activity. Ku activity is retained, but not activated, by FUS and RBM14 activity. RNAPII, PIC, CDK9, and Med1 assembly in response to DSB exposure, in an MRN and γH2AXdependent matter, mediated by FUS and RBM14. Transcription of dilncRNA, carrying sequences of the vicinity of the DSBs are generated by RNAPII and corresponding transcription factors. Drosha and Dicer dependent slicing of dilncRNA results in DDRNA, which are relocated to the DSB by its guidance dilncRNA. In turn, secondary DDR factor 53BP1 is site-specifically recruited by DDRNAs. DilncRNA binds to 53BP1 thereby driving phase condensation forming a DDR focus. DDR foci are directly or indirectly dependent on PARylated DSB break ends, RNAPII, Drosha/Dicer, and resulting dilncRNA. When damaged is repair utilizing HR, dilncRNA hybridizes with DNA thereby blocking Ku assembly, stimulating end resection of BRCA and associated proteins. In turn, Rad52 is recruited and binds in an RNA:DNA-dependent matter. Binding of petDIMs to Rad52 drives phase separation thereby clustering DSB breaks and relocate these DNA repair centers to NPCs. Created with BioRender.com

However, a broad range of reports deliberates on the reliability of the phenomenon of LLPS itself. The compartmentalization is widely accepted, while still receiving some resistance regarding the process in which compartmentalization is accomplished[71]. Moreover, the extent to which the LLPS governs nuclear organization remains unclear. It is important to criticize whether a confined reaction is the result of LLPS or whether other compartmentalizing systems depending on weak interactions are involved. For instance, cellular compartmentalization can be accomplished by different types of phase separation, including polymer-polymer separation, which encompasses the process in which proteins bind to more than one nucleic acid site at the same time, leading to cross-links between binding sites. When the

density of these cross-links transcends a certain level, the nucleic acid transits from an extended coil into a collapsed globule, causing hydrogels. Another phenomenon driving phase separation of membraneless organelles comprises the spreading of proteins along a polymer scaffold, leading to local enrichments of delineated proteins. Distinguishing between hydrogels, dispersed states, and liquid phase separation is therefore important to understand how membraneless organelles arise [71,72].

Despite that the DDR is widely described and investigated, lots of questions remain unanswered. Because LLPS is believed to be involved in a widespread of DDR processes, further research is necessary to fully understand the formation of liquid separated condensates and how they affect DNA repair pathways. This would confer important results since it is postulated that the formation of condensates may exert critical roles in the choice of DDR pathways because of their biophysical properties to include and exclude proteins. Moreover, the capacity of liquid droplets to compartmentalize reactions may give insight into specific DDR processes, in which the detailed interactions are still elusive. Overall, the upcoming implication of LLPS may open doors to further understand the exact mechanism in which the DDR ensures genomic integrity by providing new perspectives. Unraveling how LLPS derived condensates control DNA repair will deepen our understanding of the pathogenesis of cancer, thereby simultaneously providing the bases for the development of relevant and innovative therapeutic strategies.

References:

1. Galhardo, R. S., Hastings, P. J. & Rosenberg, S. M. Mutation as a stress response and the regulation of evolvability. *Crit. Rev. Biochem. Mol. Biol.* **42**, 399–435 (2007).

2. Wei, W., Cheng, Y. & Wang, B. Chapter 27 - Cancer and Genomic Instability. in (eds. Kovalchuk, I. & Kovalchuk, O. B. T.-G. S.) 463–486 (Academic Press, 2016). doi:https://doi.org/10.1016/B978-0-12-803309-8.00027-6

3. Gaillard, H., García-Muse, T. & Aguilera, A. Replication stress and cancer. *Nat. Rev. Cancer* **15**, 276–280 (2015).

4. Rodgers, K. & McVey, M. Error-Prone Repair of DNA Double-Strand Breaks. *J. Cell. Physiol.* **231**, 15–24 (2016).

5. Ray, P. D. & Fry, R. C. Chapter 2 - The Cell: The Fundamental Unit in Systems Biology. in (ed. Fry, R. C. B. T.-S. B. in T. and E. H.) 11–42 (Academic Press, 2015). doi:https://doi.org/10.1016/B978-0-12-801564-3.00002-X

6. Peng, L., Li, E.-M. & Xu, L.-Y. From start to end: Phase separation and transcriptional regulation. *Biochim. Biophys. Acta - Gene Regul. Mech.* **1863**, 194641 (2020).

7. Pessina, F. *et al.* DNA Damage Triggers a New Phase in Neurodegeneration. *Trends Genet.* (2020). doi:https://doi.org/10.1016/j.tig.2020.09.006

8. Levone, B. R. *et al.* FUS-dependent liquid-liquid phase separation is an early event in double-strand break repair. *bioRxiv* 798884 (2020). doi:10.1101/798884

9. Klein, I. A. *et al.* Partitioning of cancer therapeutics in nuclear condensates. *Science (80-.).* **368**, 1386–1392 (2020).

10. Laflamme, G. & Mekhail, K. Biomolecular condensates as arbiters of biochemical reactions inside the nucleus. *Commun. Biol.* **3**, 773 (2020).

11. Hawley, B. R., Lu, W.-T., Wilczynska, A. & Bushell, M. The emerging role of RNAs in DNA damage repair. *Cell Death Differ.* **24**, 580–587 (2017).

12. Garcia-Jove Navarro, M. *et al.* RNA is a critical element for the sizing and the composition of phase-separated RNA–protein condensates. *Nat. Commun.* **10**, 3230 (2019).

13. Altmeyer, M. *et al.* Liquid demixing of intrinsically disordered proteins is seeded by poly(ADP-ribose). *Nat. Commun.* **6**, 8088 (2015).

14. Hyman, A. A., Weber, C. A. & Jülicher, F. Liquid-Liquid Phase Separation in Biology. *Annu. Rev. Cell Dev. Biol.* **30**, 39–58 (2014).

15. Pessina, F. *et al.* Functional transcription promoters at DNA double-strand breaks mediate RNA-driven phase separation of damage-response factors. *Nat. Cell Biol.* **21**, 1286–1299 (2019).

16. Schuster, B. S. *et al.* Identifying Sequence Perturbations to an Intrinsically Disordered Protein that Determine Its Phase Separation Behavior. *bioRxiv* 2020.01.06.894576 (2020). doi:10.1101/2020.01.06.894576

17. Neal, J. A. *et al.* Inhibition of homologous recombination by DNA-dependent protein kinase requires kinase activity, is titratable, and is modulated by autophosphorylation. *Mol. Cell. Biol.* **31**, 1719–1733 (2011).

18. Blackford, A. N. & Jackson, S. P. ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response. *Mol. Cell* **66**, 801–817 (2017).

19. Dinkelmann, M. *et al.* Multiple functions of MRN in end-joining pathways during isotype class switching. *Nat. Struct. Mol. Biol.* **16**, 808–813 (2009).

20. Symington, L. S. Mechanism and regulation of DNA end resection in eukaryotes. *Crit. Rev. Biochem. Mol. Biol.* **51**, 195–212 (2016).

21. Quennet, V., Beucher, A., Barton, O., Takeda, S. & Löbrich, M. CtIP and MRN promote non-homologous end-joining of etoposide-induced DNA double-strand breaks in G1. *Nucleic Acids Res.* **39**, 2144–2152 (2011).

22. El-Khamisy, S. F. *et al.* Synergistic decrease of DNA single-strand break repair rates in mouse neural cells lacking both Tdp1 and aprataxin. *DNA Repair (Amst).* **8**, 760–766 (2009).

23. Liu, T. & Huang, J. DNA End Resection: Facts and Mechanisms. *Genomics. Proteomics Bioinformatics* **14**, 126–130 (2016).

24. Beucher, A. *et al.* ATM and Artemis promote homologous recombination of radiation-induced DNA double-strand breaks in G2. *EMBO J.* **28**, 3413–3427 (2009).

25. Nogueira, A., Fernandes, M., Catarino, R. & Medeiros, R. RAD52 Functions in Homologous Recombination and Its Importance on Genomic Integrity Maintenance and Cancer Therapy. *Cancers (Basel).* **11**, 1622 (2019).

26. Chiruvella, K. K., Liang, Z. & Wilson, T. E. Repair of double-strand breaks by end joining. *Cold Spring Harb. Perspect. Biol.* **5**, a012757–a012757 (2013).

27. Chang, H. H. Y., Watanabe, G. & Lieber, M. R. Unifying the DNA end-processing roles of the artemis nuclease: Ku-dependentartemis resection atbluntdnaends. *J. Biol. Chem.* **290**, 24036–24050 (2015).

28. Löbrich, M. & Jeggo, P. A Process of Resection-Dependent Nonhomologous End Joining Involving the Goddess Artemis. *Trends Biochem. Sci.* **42**, 690–701 (2017).

29. Pryor, J. M. *et al.* Essential role for polymerase specialization in cellular nonhomologous end joining. *Proc. Natl. Acad. Sci. U. S. A.* **112**, E4537—45 (2015).

30. Giglia-Mari, G., Zotter, A. & Vermeulen, W. DNA damage response. *Cold Spring Harb. Perspect. Biol.* **3**, a000745–a000745 (2011).

31. Serrano, M. A. *et al.* DNA-PK, ATM and ATR collaboratively regulate p53–RPA interaction to facilitate homologous recombination DNA repair. *Oncogene* **32**, 2452–2462 (2013).

32. Smith, J., Mun Tho, L., Xu, N. & A. Gillespie, D. *The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer*. *Advances in Cancer Research* **108**, (Elsevier Inc., 2010).

33. Wang, M. *et al.* PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. *Nucleic Acids Res.* **34**, 6170–6182 (2006).

34. Cheng, Q. *et al.* Ku counteracts mobilization of PARP1 and MRN in chromatin damaged with DNA double-strand breaks. *Nucleic Acids Res.* **39**, 9605–9619 (2011).

35. Feng, Y.-L. *et al.* H2AX facilitates classical non-homologous end joining at the expense of limited nucleotide loss at repair junctions. *Nucleic Acids Res.* **45**, 10614–10633 (2017).

36. Natale, F. *et al.* Identification of the elementary structural units of the DNA damage response. *Nat. Commun.* **8**, 15760 (2017).

37. Lu, W.-T. *et al.* Drosha drives the formation of DNA:RNA hybrids around DNA break sites to facilitate DNA repair. *Nat. Commun.* **9**, 532 (2018).

38. Bonath, F., Domingo-Prim, J., Tarbier, M., Friedländer, M. R. & Visa, N. Next-generation sequencing reveals two populations of damage-induced small RNAs at endogenous DNA double-strand breaks. *Nucleic Acids Res.* **46**, 11869–11882 (2018).

39. Francia, S., Cabrini, M., Matti, V., Oldani, A. & d'Adda di Fagagna, F. DICER, DROSHA and DNA damage response RNAs are necessary for the secondary recruitment of DNA damage response factors. *J. Cell Sci.* **129**, 1468 LP – 1476 (2016).

40. Sheu-Gruttadauria, J. & MacRae, I. J. Phase Transitions in the Assembly and Function of Human miRISC. *Cell* **173**, 946-957.e16 (2018).

41. Krietsch, J. *et al.* PARP activation regulates the RNA-binding protein NONO in the DNA damage response to DNA double-strand breaks. *Nucleic Acids Res.* **40**, 10287–10301 (2012).

42. Huang, R.-X. & Zhou, P.-K. DNA damage response signaling pathways and targets for radiotherapy sensitization in cancer. *Signal Transduct. Target. Ther.* **5**, 60 (2020).

43. Thandapani, P., O'Connor, T. R., Bailey, T. L. & Richard, S. Defining the RGG/RG Motif. *Mol. Cell* **50**, 613–623 (2013).

44. Pak, C. W. *et al.* Sequence Determinants of Intracellular Phase Separation by Complex Coacervation of a Disordered Protein. *Mol. Cell* **63**, 72–85 (2016).

45. Jaafar, L., Li, Z., Li, S. & Dynan, W. S. SFPQ•NONO and XLF function separately and together to promote DNA double-strand break repair via canonical nonhomologous end joining. *Nucleic Acids Res.* **45**, 1848–1859 (2017).

46. Rajesh, C., Baker, D. K., Pierce, A. J. & Pittman, D. L. The splicing-factor related protein SFPQ/PSF interacts with RAD51D and is necessary for homology-directed repair and sister chromatid cohesion. *Nucleic Acids Res.* **39**, 132–145 (2011).

47. Simon, N. E., Yuan, M. & Kai, M. RNA-binding protein RBM14 regulates dissociation and association of non-homologous end joining proteins. *Cell Cycle* **16**, 1175–1180 (2017).

48. Jang, Y. *et al.* Intrinsically disordered protein RBM14 plays a role in generation of RNA:DNA hybrids at double-strand break sites. *Proc. Natl. Acad. Sci.* **117**, 5329 LP – 5338 (2020).

49. Kai, M. Roles of RNA-Binding Proteins in DNA Damage Response. *Int. J. Mol. Sci.* **17**, 310 (2016).

50. Knijnenburg, T. A. *et al.* Genomic and Molecular Landscape of DNA Damage Repair Deficiency across The Cancer Genome Atlas. *Cell Rep.* **23**, 239-254.e6 (2018).

51. Ohle, C. *et al.* Transient RNA-DNA Hybrids Are Required for Efficient Double-Strand Break Repair. *Cell* **167**, 1001-1013.e7 (2016).

52. Sukhanova, M. V, Singatulina, A. S., Pastré, D. & Lavrik, O. I. Fused in Sarcoma (FUS) in DNA Repair: Tango with Poly(ADP-ribose) Polymerase 1 and Compartmentalisation of Damaged DNA. *Int. J. Mol. Sci.* **21**, 7020 (2020).

53. Aleksandrov, R. *et al.* Protein Dynamics in Complex DNA Lesions. *Mol. Cell* **69**, 1046-1061.e5 (2018).

54. Patel, A. *et al.* A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation. *Cell* **162**, 1066–1077 (2015).

55. Baradaran-Heravi, Y., Van Broeckhoven, C. & van der Zee, J. Stress granule mediated protein aggregation and underlying gene defects in the FTD-ALS spectrum. *Neurobiol. Dis.* **134**, 104639 (2020).

56. Durut, N. & Mittelsten Scheid, O. The Role of Noncoding RNAs in Double-Strand Break Repair . *Frontiers in Plant Science* **10**, 1155 (2019).

57. Bader, A. S. & Bushell, M. DNA:RNA hybrids form at DNA double-strand breaks in transcriptionally active loci. *Cell Death Dis.* **11**, 280 (2020).

58. McDevitt, S., Rusanov, T., Kent, T., Chandramouly, G. & Pomerantz, R. T. How RNA transcripts coordinate DNA recombination and repair. *Nat. Commun.* **9**, 1091 (2018).

59. Chakraborty, A. *et al.* Classical non-homologous end-joining pathway utilizes nascent RNA for error-free double-strand break repair of transcribed genes. *Nat. Commun.* **7**, 13049 (2016).

60. Gotor, N. L. *et al.* RNA-Binding and Prion Domains: The Yin and Yang of Phase Separation. *bioRxiv* 2020.01.14.904383 (2020). doi:10.1101/2020.01.14.904383

61. Michelini, F. *et al.* Damage-induced lncRNAs control the DNA damage response through interaction with DDRNAs at individual double-strand breaks. *Nat. Cell Biol.* **19**, 1400–1411 (2017).

62. Elbaum-Garfinkle, S. *et al.* The disordered P granule protein LAF-1 drives phase separation into droplets with tunable viscosity and dynamics. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 7189–7194 (2015).

63. Gupta, A. *et al.* Role of 53BP1 in the regulation of DNA double-strand break repair pathway choice. *Radiat. Res.* **181**, 1–8 (2014).

64. Chan, Y. W. & West, S. C. A new class of ultrafine anaphase bridges generated by homologous recombination. *Cell*

Cycle **17**, 2101–2109 (2018).

65. Dimitrova, N. & de Lange, T. Cell cycle-dependent role of MRN at dysfunctional telomeres: ATM signaling-dependent induction of nonhomologous end joining (NHEJ) in G1 and resection-mediated inhibition of NHEJ in G2. *Mol. Cell. Biol.* **29**, 5552–5563 (2009).

66. Gorgoulis, V. G. *et al.* Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature* **434**, 907–913 (2005).

67. Ma, A. & Dai, X. The relationship between DNA single-stranded damage response and double-stranded damage response. *Cell Cycle* **17**, 73–79 (2018).

68. Kilic, S. *et al.* Phase separation of 53BP1 determines liquid-like behavior of DNA repair compartments. *EMBO J.* **38**, e101379 (2019).

69. D'Alessandro, G. *et al.* BRCA2 controls DNA:RNA hybrid level at DSBs by mediating RNase H2 recruitment. *Nat. Commun.* **9**, 5376 (2018).

70. Vernon, R. M. *et al.* Pi-Pi contacts are an overlooked protein feature relevant to phase separation. *Elife* **7**, e31486 (2018).

71. A, P. & Weber, S. C. Evidence for and against Liquid-Liquid Phase Separation in the Nucleus. *Non-coding RNA* **5**, 50 (2019).

72. Taiana, E. *et al.* LncRNA NEAT1 in Paraspeckles: A structural scaffold for cellular DNA damage response systems? *Non-coding RNA* **6**, (2020).