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The evolving complexity of DNA damage foci: RNA, condensates and chromatin in DNA double-strand break repair

Carel Fijen[1,*], **Eli Rothenberg**[1]

[1]Department of Biochemistry and Molecular Pharmacology, NYU Grossman School of Medicine, New York, U.S.A.

Abstract

Formation of biomolecular condensates is increasingly recognized as a mechanism employed by cells to deal with stress and to optimize enzymatic reactions. Recent studies have characterized several DNA repair foci as phase-separated condensates, behaving like liquid droplets. Concomitantly, the apparent importance of long non-coding RNAs and RNA-binding proteins for the repair of double-strand breaks has raised many questions about their exact contribution to the repair process. Here we discuss how RNA molecules can participate in condensate formation and how RNA-binding proteins can act as molecular scaffolds. We furthermore summarize our current knowledge about how properties of condensates can influence the choice of repair pathway (homologous recombination or non-homologous end joining) and identify the open questions in this field of emerging importance.

Keywords

Double-strand break repair; DNA damage foci; LLPS; lncRNA; biomolecular condensates

1. Introduction

Cells have to deal with continuous insults to their genomes. Environmental factors like radiation and chemicals as well as endogenous sources can cause a host of different types of DNA damage, impacting genome integrity [1]. DNA repair has been a topic of extensive study, which revealed the existence of multiple different pathways, each tailored toward the repair of a specific type of lesion [2]. Repair of the most severe lesion, a double strand break (DSB), starts off with a signaling cascade and the consecutive recruitment of repair factors to the damage site. Central to the DSB signaling and repair processes is the formation of

[*]Corresponding author. carolus.fijen@nyulangone.org.

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Eli Rothenberg: Conceptualization, Writing – review and editing

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a repair focus – a designated volume in which related biochemical reactions are regulated. These foci correspond to an accumulation of damage markers and repair proteins at the damage site and are considered a hallmark of DNA damage and repair, yet the regulation and inner workings of these repair centers remain elusive.

Some foci, for example those created by p53-binding protein 1 (53BP1) or RAD52, have recently been identified as phase-separated compartments in the nucleus, behaving as liquid droplets [3–5]. Condensate formation is common in cells, examples being membrane-less organelles like paraspeckles [6], Cajal bodies [7] and stress granules [8]. These compartments have dedicated functions in cell homeostasis and are not harmful to the cell *per se*. Certain mutations in proteins that induce condensate formation, however, can contribute to formation of pathological aggregates that are thought to underlie neurodegenerative diseases like amyotrophic lateral sclerosis (ALS) [9]. For example, mutations in the intrinsically disordered proteins FUS, EWS and TAF15 (the “FET” proteins) have been identified in ALS patients and are generally associated with an increased tendency to form solid aggregates in neurons that cannot be dissolved [10].

Over the last decades, there has been an increasing body of evidence that RNA can fulfill other roles in the cell in addition to merely being transcripts coding for proteins. RNAs can suppress gene expression by a process called RNA interference; conversely, RNAs of varying lengths can also promote gene transcription with the help of Argonaute proteins [11]. Additionally, the cellular DNA damage response involves regulation of gene expression post-transcription, in which non-coding RNAs and RNA binding proteins play a major role [12]. Recently, members of a class of RNAs known as long non-coding RNAs (lncRNAs), which are not being translated, were shown to be directly involved in DNA repair activities, interacting with repair proteins or even substituting for some [13,14].

Additionally, it was found that transcription of damage-induced lncRNAs (dilncRNAs) takes place in the immediate vicinity of DSBs, and that RNA binding proteins are recruited to the repair site [15,16]. Although transcription is in general suppressed upon detection of a DSB [17], these damage-induced lncRNAs may still be produced since they do not need a promoter [18].

The notion that many of the cell’s membrane-less organelles consist of RNA and RNA-binding proteins, together with the observation of phase-separated repair foci in the nucleus and the discovery of lncRNAs, has led to the suggestion that RNA has a more elaborate role in DNA repair. Since RNAs are complex biopolymers, differing in length, sequence, secondary structure and abundance, this role is likely not limited to a single process. Indeed, it has been shown that RNA molecules can regulate transcription of repair proteins [19], act as template for repair [20–22], aid in the formation of repair foci [3] or modulate the function of specific repair proteins by interacting with them [13,23–35].

This review will focus on the structural role of RNA and RNA-binding proteins in DNA damage foci. We will give a short overview of condensate formation and DSB repair in general, as well as highlight a couple of RNAs and RNA-binding proteins with specific roles

in DNA repair. We will then provide an overview of how condensates and RNA shape the repair process. Finally, we will briefly discuss mechanisms for condensate resolution.

2. Condensate Formation in the Cell

Liquid-liquid phase separation (LLPS) has recently been implicated in the formation of a host of membrane-less organelles in cells. LLPS is the de-mixing of a solution into a dense and dilute phase [36–38]. It originates when weak multivalent interactions between related molecules become more energetically favorable than interactions of these molecules with the solvent (which is often water). In practice, this means that phase separation will occur when the concentration of a component in the cytoplasm or nucleoplasm exceeds a threshold value. Alternatively, computer simulations showed that a slowly diffusing macromolecule or "molecular scaffold" can seed phase separation at lower concentrations if it is able to retain enough other molecules in its immediate vicinity [39].

Cellular components that can phase separate are typically macromolecules without a well-defined secondary structure, such as single-stranded RNAs and intrinsically disordered domains of proteins [40]. The phase-separated compartments that they form are often referred to as "biomolecular condensates" or "liquid droplets". Their stability is strongly dependent on environmental factors like salt concentration and pH; moreover, they can grow in size and fuse with other droplets, properties that make them highly versatile and give cells a toolbox to react to all kinds of environmental stress [38]. There are other advantages of phase separation as well: by locally increasing the concentration of certain proteins, enzymatic reactions may go faster. Condensates can also act as a sieve, providing selective access to the proteins that are needed for a specific reaction while keeping others out [41]. However, phase separation is not without risk for the cell. Biomolecular condensates can undergo a process called maturation, during which the increasing density in the core of the droplet will result in a liquid-to-solid phase transition. These can be toxic for the cell if they cannot be resolved. Indeed, mutated versions of LLPS proteins that are more prone to form aggregates are linked to neurological disorders like ALS [37]. An example is the previously mentioned FUS protein, which contains RNA-binding domains and a so-called "prion-like domain" which is intrinsically disordered [42]. The ability of the prion-like domain to establish weak multivalent interactions is thought to drive LLPS. However, pathological mutations of FUS are found in both the RNA-binding domains and the prion-like domain. These are often simple missense mutations, increasing the overall tendency of the protein to aggregate [10].

As pointed out before [43], we cannot automatically assume that formation of a certain membrane-less organelle is due to LLPS. There are more ways a cell can compartmentalize proteins, such as binding to polymeric scaffolds like DNA or RNA. In this review, we will use the overarching term "condensate" to avoid confusion.

Importantly, biomolecular condensates in cells are not homogeneous structures. Instead, they adopt some degree of organization. For example, histone protein 1a (HP1a) undergoes LLPS in *Drosophila* and humans to create heterochromatic domains, which were found to consist of mobile and immobile parts [44,45]. The inner immobile parts are likely

caused by direct interactions between HP1a and DNA, whereas weak multivalent protein-protein interactions dominate in the outer more dynamic regions. Similarly, super-resolution imaging showed that paraspeckles, which are condensates found in the nucleus, consist of a core and shell [46]. The cores of paraspeckles consists of RNA binding proteins from the *Drosophila behavior/human splicing* (DBHS) protein family. In humans, this family consists of SFPQ (Splicing Factor, Proline- and Glutamine-rich), NONO (Non-POU domain-containing Octamer-binding protein) and PSPC1 (Paraspeckle Protein Component 1) [47]. DBHS proteins are often characterized as molecular scaffolds: they contain RNA binding motifs and intrinsically disorder regions, and are capable of forming heterodimers and oligomers [48,49]. Their ability to bind RNA as well as DNA provides them with a multitude of different roles in the cell, including in RNA splicing, transcriptional regulation and DNA repair, as we will see later [50]. Here, they contribute to the architecture of the paraspeckle by occupying the core, where they interact with the middle region of lncRNA *NEAT1* [46,51]. Using fluorescence *in situ* hybridization (FISH), the authors were able to show that the 5' and 3' tails of *NEAT1* are pointing outwards, forming the shell of the paraspeckle. Paraspeckles are thought to have a role in gene regulation and function by sequestering certain gene transcripts. They are able to retain these RNAs exactly because of their weak multivalent interactions.

In DNA damage repair, the protein 53BP1 is recruited to DSBs to form foci. These foci were recently identified as biomolecular condensates, showing droplet-like behavior [3,4]. Thus, while only a limited number of 53BP1 proteins can bind to the actual break site, there is apparently a physiological advantage to create a far larger cellular compartment around the DSB lesion. The large (1972 amino acids [52]) 53BP1 protein interacts specifically with modified histones through its Tudor and BRCT domains at sites of DNA damage, whereas its oligomerization domain is necessary to induce phase separation [4,53–55]. This results in an architecture in which interactions between the Tudor domain and the break site govern the center of the focus, while protein-protein interactions through the oligomerization domain dominate the outer shell [4].

In the nucleolus, there appear to exist "phases within a phase", forming the sub-compartments of this membrane-less organelle. This organization originates from differences in surface tension between the individual condensates [56].

Thus, although liquid-liquid phase separation in the cell is stimulated by weak multivalent interactions between disordered domains of proteins and RNA, the resulting biomolecular condensate can adopt a structure with a well-defined architecture. Because of the multitude of factors involved in the formation of these structures *in vivo*, in combination with technological difficulties to visualize them at high spatial and temporal resolution, our knowledge about how that architecture contributes to function is unfortunately limited.

3. Pathways for the Repair of Double-Strand Breaks

DSBs pose an immediate threat to genomic integrity and cell viability. Extensive studies on DSB repair found a tightly regulated signaling cascade that quickly activates the repair machinery through phosphorylation and ubiquitylation of target proteins. Two main

pathways for repair were identified: homologous recombination (HR) [57,58] and non-homologous end-joining (NHEJ) [59,60]. Repair of the break often proceeds through one of these two.

HR uses the homologous DNA sequence of a sister chromatid to facilitate error-free repair. This mode of repair is only possible when a sister chromatid is present, and can thus only be employed during the S or G2 phase. It adopts a mechanism in which the DNA around the break site is first resected by nuclease activity from the MRE11-RAD50-NBS1 (MRN) complex, which recognizes DNA ends, and the nucleases EXO1 and DNA2 [61–64]. The BRCA1 protein helps coordinate resection by binding to the resection factor CtIP [65]. It also antagonizes binding of 53BP1, a protein that limits end resection [66,67]. The exposed ssDNA is protected by RPA, followed by initial loading and nucleation of RAD51 recombinase, which is mediated by RAD52 [68]. This is followed by the formation of RAD51 filaments stabilized by BRCA2, to support efficient homology search and strand invasion activities. The protein SFPQ, which we described before as a molecular scaffold, was found to increase homologous pairing and strand exchange at low RAD51 concentrations, while inhibiting these activities at higher RAD51 concentrations [69]. Once a homologous sequence is found, polymerases such as Pol δ use it as a template for repair of the break, thus restoring the original sequence [70]. Repair is completed by ligation.

NHEJ, on the other hand, involves direct alignment and ligation of the broken DNA ends. It is therefore much faster and more efficient than HR and serves as the predominant cellular repair process for DSB repair; however, it is considered more error-prone than HR, often resulting in insertions or deletions. It is active throughout the cell cycle and dominant in G1.

Briefly, in NHEJ the DNA ends at the break site are recognized by the Ku heterodimer, which acts as a scaffold for other NHEJ proteins to bind to. Ku binding is followed by pairing, or synapsis, of the broken DNA ends, an essential intermediate stage enabling for the alignment and further processing of the ends and subsequent ligation. Due to its transient nature, the first study where the end synapsis step was directly measured required the use of a single-molecule FRET assay. This allowed for monitoring of the DNA ends during a reconstituted NHEJ reaction using purified human proteins [71]. In this study, as well in subsequent reports [72–74], we and others have shown that synapsis is facilitated by the scaffolding proteins XLF and XRCC4, which together with DNA ligase 4 (Lig4) were shown to form extended filaments in vitro and in cells [71,75,76]. Interestingly, it was recently found that a heterodimer of SFPQ and NONO can substitute for XLF (Figure 1A) [77,78]. When NONO was depleted, repair through NHEJ decreased while repair through HR increased [79].

Another key NHEJ factor, DNA-PKcs, is a PIKKs family kinase and binds Ku to form the DNA-PK holoenzyme. While it was initially speculated that DNA-PKcs contributes to synapsis, the specific roles of DNA-PKcs in NHEJ, and especially in the mediation of end synapsis, are a matter of debate [80]. Single-molecule FRET studies using purified human proteins have consistently shown that DNA-PKcs does not contribute to the synapsis stage, and that efficient synapsis is achieved in the presence of Ku, XRCC4-Lig4 and XLF [71,74]. These findings were further supported by optical tweezers experiments, showing

that complexes of XRCC4 and XLF can efficiently bridge neighboring DNA molecules [81]. In contrast, a study utilizing an *Xenopus laevis* egg extract system to reconstitute NHEJ measured by smFRET assays, revealed that synapsis and NHEJ in this system involves an intermediate step that heavily relies on the presence and activity of DNA-PKcs [82].

When the damage is too extensive for direct rejoining of the two ends, additional processing may take place. Proteins like Artemis (a nuclease), WRN (a helicase) and Polμ (a polymerase) are typically involved in this [83,84].

Beyond the canonical NHEJ proteins, the foci forming protein 53BP1 was also proposed to facilitate end synapsis based on observations of foci coalescence [85]. As discussed briefly before, 53BP1 is a key player in regulating DSB pathway choice by preventing excessive resection. It keeps the DNA ends mostly intact during the repair process as part of the pro-NHEJ Shieldin complex [86]. It will be important to determine how 53BP1 contributes to or affects the biochemical properties of the end synapsis step.

The repair pathways themselves are not set in stone, with a recent study showing a mechanism for NHEJ that surprisingly depends on resection of the DNA ends [87]. Other work showed how NHEJ in non-cycling cells can be error-free when homologous RNA transcripts are used as a template [20,88]. How individual enzymes steer pathway choice, however, is still unclear. Although pathway choice is mainly dependent on cell cycle, recent studies have painted a more nuanced picture with a crucial role for phase-separated domains, which we will discuss later in this review.

4. The Structural Roles of RNA Transcripts in Double-Strand Break Repair

RNA polymers are ideal candidates to participate in phase separation, because of their ability to form transient interactions with other RNAs (in a sequence-specific context) and RNA binding proteins [89]. Moreover, they can act as polymeric scaffolds to compartmentalize proteins. As discussed earlier, they are an important component of paraspeckles and stress granules [46,9] and likely support phase separation in other membrane-less organelles such as the nucleolus [56,90,91].

According to the central dogma of molecular biology, RNA serves as an “assembly guide” for proteins. However, cells contain many long transcripts that are never translated into proteins: so-called long non-coding RNAs (lncRNAs). While initially overlooked, it is now widely accepted that these lncRNAs serve dedicated functions in the cell, mostly related to chromatin architecture and transcription regulation [92,19]. The sequence-dependent secondary structure of these long RNA molecules allows them to specifically interact with target proteins or localize to a specific location in the genome.

Here we will briefly discuss the structural roles that RNA can play in DNA damage repair. We will therefore focus on the RNAs that accumulate at the damage site, rather than the lncRNAs that work at the level of transcription regulation.

4.1 LncRNAs showing direct interactions with repair factors

Over the last decade, a number of lncRNAs have been identified to interact directly with repair factors around the break, modulating their activity. We provide a brief overview of these lncRNAs, subdividing them in those that promote HR, those that promote NHEJ and those that are involved in other aspects of the DNA repair response. We also provide a short overview in tabular form (Table 1).

Pro-HR—*DDSR1* is a lncRNA that directly interacts with BRCA1 and hnRNPUL1, and is induced upon DNA damage in multiple different cancer cell lines [27]. hnRNPUL1 is a regulator of end resection. Indeed, upon depletion of DDSR1, less end resection is detected. Moreover, loss of DDSR1 lead to excessive accumulation of BRCA1 and repair factor RAP80 at DSBs, and thereby negatively affects the efficiency of HR.

Another lncRNA, *BGL3*, is recruited to DSBs by PARP1 at an early stage, interacting with its DNA-binding domain [32]. It also binds BARD1, a binding partner of the HR protein BRCA1, promoting the retention of BRCA1-BARD1 complexes at DSBs and enhancing the binding of BARD1 to other repair proteins. *BGL3* therefore acts as a molecular scaffold during HR. Indeed, *BGL3* deficiency led to reduced HR efficiency, while not significantly affecting NHEJ.

PRLH1 is a lncRNA expressed in p53-deficient or mutant cells (p53 is the main protein that protects genome stability in humans) [31]. *PRLH1* then interacts with RNF169, displacing 53BP1 from ubiquitinylated chromatin, and paving the way for end resection and homologous recombination. Finally, telomeric repeat containing RNAs (*TERRA*) are a group of lncRNAs that are transcribed from regions near chromosome ends. They are targeted to short telomeres through a UUAGGG-repeat sequence motif, where they associate with RAD51 to form R-loops [35]. These R-loops trigger telomere fragility, replication stress and recombination events. HR-like repair at chromosome ends is considered undesired, since it eventually contributes to alternative lengthening of telomeres, which is a mechanism for some cancer cells to overcome telomere shortening [93]. Interestingly, NONO and SFPQ have been shown to suppress the formation of these R-loops [94], and thereby help control telomere length in cancer cells.

Pro-NHEJ—The Ku heterodimer is known to be able to interact with RNA, specifically with certain sequence motifs [95,96]. It is therefore not surprising that two lncRNAs have recently been identified that link this ability to DNA damage repair: *LRIK* and *LINP1*. The 5’ region of *LRIK*, which indeed contains the AATG and CATGA motifs, binds to the Ku heterodimer [33]. The interaction increases Ku’s affinity for DSBs and facilitates the efficient recruitment of downstream repair factors like XRCC4. *LINP1*, which is overexpressed in multiple cancers, forms condensates by itself, but adopts a filamentous structure when bound to Ku [13,97]. The Ku-*LINP1* complex is still capable of interacting with XLF, but binding with another accessory protein, PAXX, is abrogated. Interestingly, Ku-*LINP1* can still participate in end joining; in fact, *LINP1* stabilized the synaptic complex more than PAXX did (Figure 1B). Insulin-like growth factor binding protein-3 (IGFBP3), a protein that modulates NHEJ in triple-negative breast cancer, binds NONO and SFPQ

but needs *LINP1* for complex formation [98,99], further cementing a role for *LINP1* in promoting NHEJ.

Expression of the lncRNA *HITT* was found to be reduced in tissues from colon cancer, thyroid cancer and chromophobe renal cell carcinoma [24]. Subsequently, *HITT* was shown to prevent recruitment of ATM to sites of DNA damage [100]. ATM is a kinase involved early in the DSB response, and regulates the activity of many downstream repair factors. *HITT* blocks ATM's binding site for the MRN complex, specifically the NBS1 protein, thus disfavoring HR.

Small Nucleolar RNA Host Genes (SNHG) are a subset of lncRNAs which have received attention due to their oncogenic role in cancer [101]. *SNHG12* was identified as a factor that is overexpressed in atherosclerosis [23]. In this disease, cells near plaque build-up often show signs of senescence, which could be due to persistent DNA damage. *SNHG12* binds to DNA-PK and seems to mediate the interaction of this repair factor with Ku. Knocking down *SNHG12* leads indeed to higher levels of DNA damage in the cell, indicating a role for *SNHG12* in NHEJ. Another member of this class of lncRNAs, *SNHG17*, was shown to be upregulated upon *H. pylori* infection [34]. Overabundant *SNHG17* recruits NONO to DSBs and upregulates RING1, which in turn induces RAD51 breakdown. These combined effects cause a shift from HR to NHEJ. This change in DNA repair pathway is thought to contribute to the development of gastric cancer that is often associated with chronic *H. pylori* infection.

Other relevant repair-associated lncRNAs—There are a couple of repair-related lncRNAs that, to our current knowledge, are not necessarily pro-HR or pro-NHEJ but are more involved with cell fate decisions in general. Here, we describe a couple of lncRNAs that work at different levels to steer the cell's response to DNA damage.

NEAT1, which we briefly discussed before as a major component of paraspeckles, is a multifunctional lncRNA that was also found to be involved in the transcriptional activation of HR genes [102]. Another study found that activation of p53 triggers the formation of paraspeckles [103]. *NEAT1* further modulates ATR signaling, thereby preventing DSBs from occurring. Combined, these studies suggest a role for *NEAT1* in protecting genome stability. The exact mechanisms through which NEAT1 and paraspeckles contribute to this remain unclear at the moment, but the sequestration of harmful transcripts and proteins inside biomolecular condensates is not unthinkable.

NEAT2, also known as *MALAT1*, was first identified in non-small cell lung cancer [104], but is upregulated in many other cancers [105–107]. In multiple myeloma, it forms a complex with PARP1 and Ligase 3 to promote alternative NHEJ, a process that involves minimal resection and subsequent alignment of microhomologies around the break site [30]. It is also reported to bind SFPQ and thereby release the oncogene PTBP2 from SFPQ/PTBP2 complexes [108]. Given the previously described role of SFPQ in DNA repair, it would be interesting to know if the interaction of *NEAT2* with SFPQ has any effect on the function of SFPQ in those repair complexes.

Linc00312, a lncRNA first found in nasopharyngeal carcinoma, is associated with cell apoptosis [109]. It inhibits DNA repair by binding to DNA-PKcs and preventing its recruitment to Ku80 [28]. Overexpression of *linc00312* also downregulates MRN expression, although the molecular mechanism is unknown. LncRNAs that are associated with preventing apoptosis include *lnc-bc060912* and *HITTERS* (unrelated to *HITT*). *lnc-bc060912* binds to nucleophosmin (NPM1), which is a nucleolar protein involved in DNA damage repair, and to PARP1 [29]. *HITTERS* is upregulated in certain cancer cells by endoplasmic reticulum stress [25]. It works as a scaffold to promote binding of MRE11 to RAD50, thereby supporting DNA repair.

GUARDIN is a p53-responsive lncRNA that acts on at least two different levels to protect genome integrity [26]. It prevents end-to-end fusion of chromosomes by binding to microRNA23a, which has a partially complementary sequence. microRNA23a normally suppresses TRF2 expression, which is part of the shelterin complex found at chromosome ends. *GUARDIN* thus indirectly helps to protect telomeres from undesired fusion events. On a different level, *GUARDIN* aids in BRCA1-BARD1 complex formation by directly interacting with both proteins. Depletion of *GUARDIN* leads to degradation of BRCA1. Additionally, the authors showed that depletion of *GUARDIN* also leads to impaired repair through both HR and NHEJ.

Often, only parts of the lncRNAs physically interact with the DNA repair factors. This suggests that the remainder of the RNA polymer can be used for other interactions. This may indeed include interactions with other proteins, rendering such lncRNAs molecular chaperones, or an architectural role in a condensate like the previously described *NEAT1* in paraspeckles. In this context, interactions with proteins from the DBHS family are particularly interesting. As pointed out before, these proteins can complement or even substitute dedicated DNA repair proteins, and may also help with lncRNA recruitment to the break site.

4.2 Break-induced transcription

Another source of lncRNAs is break-induced transcription. Although transcription in general is downregulated upon detection of a DSB [17], it was reported that transcription in the immediate vicinity of the break still occurs [16,110]. The resulting damage-induced lncRNAs (dilncRNAs) are therefore produced from sequences directly around the break site.

A general approach to study break-induced transcription involves integrating an artificial construct with a unique cut site into the genome, which can then be used to induce a DSB site-specifically, such that the sequences flanking the break are known. The extent to which break-induced transcription occurs in genetically unperturbed cells, however, has been debated. The main criticism is that the artificial locus would not resemble the natural state of the chromatin. A single-molecule approach shows that break-induced transcription indeed depends on the chromatin landscape, with intragenic regions showing nucleosome depletion and bidirectional transcription upon induction of a DSB [111]. Another study using next-generation sequencing was not able to detect transcription when the break was induced in genic or intergenic regions, but the authors did find break-induced transcription at ribosomal DNA loci [112]. A third study could not find proof for generation of small RNAs

around break sites either, but did show a role for the RNA processing enzyme DROSHA in generating DNA:RNA hybrids around the DSB [113]. Despite these concerns, *de novo* production of dilncRNAs was found to be crucial for formation of repair foci [3,114]. We will therefore provide a brief overview of the literature supporting break-induced transcription.

Break-induced transcription does not seem to be associated with promoters: by inducing a DSB at a specific location in the genome and using single-molecule FISH and RT-qPCR to analyze the dilncRNAs that were formed, it was shown that transcription takes place to and from the break site [114]. The polymerase involved was identified as RNAPII, which is recruited to the DSB through the MRN complex and the transcription pre-initiation complex [3]. A potential mechanism for this break-induced transcription came from the observation that the interaction between RNAPII and the MRN complex alone can stimulate RNA synthesis *in vitro* [18,114]. One would perhaps expect that the nuclease activity of the MRN complex would create the ideal ssDNA substrate needed for transcription. Surprisingly, however, the nuclease activity of MRN does not play a role in this. Instead, the underlying mechanism is thought to rely on the ability of MRN to melt DNA ends, creating an opportunity for RNAPII to start transcription from the break site inwards (Figure 1C). This is not difficult to imagine conceptually, since local DNA melting is always a prerequisite for transcription initiation. The transcript itself can exist in the form of an RNA:DNA hybrid, which offers a potential explanation for the observation that such hybrids exist near break sites, and are therefore not necessarily the result of interrupted transcription that occurred before the damage. Eventually, these transcripts are processed by DICER and DROSHA to create smaller DNA damage-induced RNAs, coined DDRNAs [15,115]. Their sequence allows these DDRNAs to be site-specifically recruited to break sites. DDRNAs, DICER and DROSHA were all found to be necessary for formation of repair foci [3,113,114].

4.3 Transcription-associated DSB repair

Although break-induced transcription is consistent with a repair model that involves phase separation, it is unclear if there is an additional use for the sequence information stored in RNA transcripts. For example, it has been suggested that RNA can aid in homology-based repair. In yeast, both synthetic RNAs and endogenous transcripts can indeed act as a template in DNA repair if the sequence is homologous to the break site [116,117], while RAD51 and RAD52 were shown to be able to participate in strand exchange between DNA and single-stranded RNA [21,118]. In human cells, however, that process seems to be less efficient than when DNA is used [22]. In 2015, support for transcription-associated HR came from a study that showed that in the G0/G1 phase of the cell cycle, proteins associated with HR are preferentially recruited to damage sites where transcription takes place. Additionally, this recruitment was found to rely on Cockayne Syndrome Protein B [119]. One year later, Chakraborty *et al.* showed that NHEJ proteins preferentially associate with transcribed genes, and can use the nascent RNA as template for error-free repair [20]. Very recently, RNA transcripts from around the break site were shown to stimulate HR in human cells [120], a process that is dependent on RAD51-associated-protein 1. Despite these findings, much remains unknown. For example, the molecular mechanisms behind RNA-templated repair are not well understood. It is also still unclear to what extent this type

of repair is applied in human cells, and how it depends on cell type and phase. Considering the scope of this review, it would also be interesting to know if the increased accessibility of the transcribed genomic region plays a role in the recruitment of specific repair factors that guide pathway choice.

5. Nucleation and Development of DNA Damage Foci

The DNA damage response is a tightly organized sequence of events, starting off with a signaling cascade that helps the cell in isolating, identifying, and repairing the lesion. Although much is known about the order in which repair factors are recruited, we would like to give an overview that emphasizes the role that damage-induced lncRNAs and condensates can play in this process.

5.1 PARylation as the Initiation of Phase Separation in DNA Repair

One of the earliest responders to DNA damage is the poly (ADP-ribose) polymerase PARP1 [121]. This highly expressed protein has many functions in the cell, ranging from DNA repair to chromatin maintenance. Its enzymatic activity, the production of long and branched poly(ADP-ribose) (abbreviated as *PAR*) chains from NAD^+, is a form of a post-translational modification and is strongly enhanced by binding to damaged DNA [122,123]. PARP1 can PARylate itself (automodification), other proteins and also DNA [124]. The resulting PAR chains are a signal for DNA repair proteins, facilitating their recruitment to the site of damage. Indeed, many DNA repair proteins contain PAR-binding motifs [123]. The importance of PARP1 activity for recruitment of downstream repair factors was illustrated by treatment with the PARP inhibitor talazoparib, which altered recruitment of many repair factors in HeLa cells, delaying the ones that are known to interact with PAR [121].

Altmeyer *et al.* showed that the production of PAR chains leads to phase separation around the damage site (Figure 2A,B) [125]. They made the interesting suggestion that this phase separation allows the cell to strictly control the earliest response to DNA damage by restricting the proteins that can reach the break. The negative PAR chains allow early access into the phase separated domain for certain proteins with intrinsically disorder regions (IDRs), which can then actively participate in phase separation through weak multivalent interactions with each other. Among the proteins encountered in this early phase-separated domain are the RNA-binding FET proteins. The implication of these proteins in DNA repair is intriguing, since they are known to have functions in transcription, are associated with chromosomal rearrangements in cancer and have a role in neurological disorders like ALS [10,126]. Indeed, there is a link between familial FUS mutations that promote pathological protein aggregation and increased DNA damage [127,128]. In this early stage, proteins with PAR-binding motifs are recruited early as well, as for example XRCC1 [121]. NONO, which interacts with PAR chains through its RNA recognition motif, is also recruited [79], although the timing is unclear. Interestingly, the important DNA repair factor 53BP1 is initially kept out [125].

The presence of the RNA-binding FET proteins makes it tempting to speculate that transcription of damage-induced long non-coding RNAs (dilncRNAs) also occurs at this very early stage. As shown recently, members of the transcription apparatus were found

to interact with the MRN complex, which is an early responder to DNA damage [3]. As discussed earlier, the MRN complex was found capable of stimulating transcription around break sites [18]. The presence of RNAPII at DSBs may be further stabilized by the ability of its C-terminal domain to participate in phase separation [129,130]. In fact, it was shown that the IDRs of the FET proteins form polymeric fibers that interact with the RNAPII C-terminal domain in a manner that correlates with transcriptional activation [131]. Further support for an early onset of transcription comes from cells in which H2AX was knocked down, halting DNA repair before γH2AX foci formation. In these cells, small non-coding RNAs localize to the break site in a sequence specific manner [114]. These RNAs could contribute to phase separation due to their negative charge and chemical resemblance to PAR chains.

It has been shown *in vitro* that automodification of PARP1 will promote dissociation of PARP1 from the break site, likely due to steric hindrance or electrostatic repulsion [132], making the break site available for further processing.

5.2 Chromatin Relaxation increases the Accessibility of Proteins to the Damage Site

Building further on this model for the role of phase separation in DNA repair, the next step involves ATM phosphorylating histone H2AX (Figure 2C). This spread in phosphorylation is thought to occur in 3D space, rather than in a linear fashion along the DNA, within the confines of a topologically associated domain (TAD), which can have a size in the Mbp range [133]. Phosphorylation does not extend beyond the CTCF binding sites that form the border of the TAD [134], suggesting a natural limitation exists to how large a γH2AX focus can grow. It has been shown that ATM silences transcription around DSBs [17], although ATM inhibition does not seem to affect dilncRNA production [114]. Regardless, the spread of γH2AX promotes chromatin decondensation [135,136], which fundamentally changes the phase-separated compartment around the break site, allowing more proteins in. A study that has not yet been peer-reviewed at the time of writing suggests that FUS is necessary for the organization of small γH2AX foci into larger clusters [137], suggesting FUS is still present at this point. MDC1 binds γH2AX [138], recruiting the E3 ligases RNF8 and RNF168. These two proteins then ubiquitylate the chromatin around the break site [139]. The RNA processing enzyme DROSHA is also required at the break site around the same time, preceding pathway choice [113].

5.3 53BP1 Focus Formation

The recruitment of the large protein 53BP1 takes place downstream of ubiquitylation by RNF8 and RNF168 (Figure 2D). Its multi-domain structure allows 53BP1 to interact with the damage site in multiple different ways. First of all, the 53BP1 Tudor domain is able to bind dimethylated histone H4K20 [55]. This histone modification is also present in undamaged chromatin; however, decondensation of the chromatin around the break site is thought to make it more accessible. The Tudor domain was also shown to interact with dilncRNAs and DDRNAs in cells in which damage was induced at specific loci [114]. Additionally, the 53BP1 UDR domain interacts with ubiquitylated histone residue H2A(X)K15 [54]. Using a system in which light can trigger phase separation of a 53BP1-fusion protein, Kilic *et al.* showed that the oligomerization and BRCT domains of the protein

are important for its phase-separating capabilities, while the disordered N-terminal domain is surprisingly dispensable [4]. Nucleation and growth of 53BP1 foci is stalled by RNAPII inhibitors, suggesting these processes are dependent on *de novo* transcription, which may indeed be the previously described break-induced transcription [3]. Moreover, treatment with RNase A results in dissipation of the foci [140], as does treatment with anti-sense oligonucleotides against regions around the break [114]. As pointed out above, RNAPII itself may participate in phase separation [129,130], and an active transcription apparatus could perhaps increase focus stability.

5.4 Choice of Repair Pathway and DSB mobility

A critical question to which we do not have a full answer yet, is in what way the development of a DNA repair focus contributes to the choice of repair pathway.

A study by Lemaître *et al.* showed that the nuclear position where the break occurs dictates pathway choice in yeast [141]. If a break occurs in the heterochromatin region of the nucleus, HR is impaired. The nuclear lamina, a dense fibrillar network, also suppresses HR. Conversely, the recruitment of NHEJ proteins to the break was not delayed, resulting in repair through an end-joining mechanism. Another example of the importance of chromatin compaction for pathway choice comes from work by Aymard *et al.*, that shows that breaks in transcriptionally active genes (in euchromatic regions) are preferentially repaired through HR, while breaks in inactive regions are typically repaired through NHEJ [142,143]. As discussed before, Chakraborty *et al.* showed that NHEJ proteins are preferentially recruited to transcribed genes [20,88]. This seems at odds with the idea that active chromatin is repaired through HR. It is important to point out here that non-replicating cells, for example neurons, cannot apply classical HR due to the absence of a sister chromatid. The involvement of the NHEJ machinery and the ability to use nascent RNAs as a template can therefore be the mechanism of choice for error-free DNA repair in these cells.

In yeast, DNA damage foci that are rich in the HR protein RAD52 were found to behave like liquid droplets, with their movement and fusion mediated by nuclear filaments [5]. Clustering of these damage sites is thought to occur to facilitate repair in dedicated repair centers. The extent to which DSBs move in mammalian cells, however, has been debated before [144]. In the work by Aymard *et al.*, discussed above, breaks in transcribed regions of the chromatin were found to be clustered in a mechanism that depends on the MRN complex, and on the actin and microtubule organizers FMN2 and the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex [143]. The involvement of FMN2 and the LINC complex suggests active transport of breaks across the nucleus; the involvement of MRN, on the other hand, suggests resection may be necessary for this to occur. The authors suggest these DSBs are sequestered to make sure they are repaired in an error-free manner in a later stage of the cell cycle.

Another possible reason for DSB mobility is that it may aid in the homology search. However, this can increase the chances of mis-rejoining if repair droplets fuse, particularly in an environment where NHEJ is the preferred pathway. A system based on dysfunctional telomeres, which are often joined together through NHEJ, showed that these telomeres exhibited increased mobility in search for other telomeres, which indeed results in mis-

rejoining [145]. This behavior extended to IR-induced DSBs, and was found to depend on 53BP1, the LINC complex and dynamic microtubules. Importantly, the role of 53BP1 seems disconnected from its role in resection [146], which opens the possibility that the observed DSB mobility is due to the tendency of 53BP1 to phase separate, irrespective of whether the break requires HR or NHEJ. The authors propose that DSB mobility may be beneficial for the cell when the number of breaks is low, since that lowers the chances of mis-rejoining.

In conclusion, HR seems to be the preferred pathway if the chromatin is less condensed and therefore more mobile (particularly during S/G2), while NHEJ occurs in denser regions and non-dividing cells (Figure 2E). DNA repair proteins that are able to form condensates, like 53BP1, are able to confer mobility to the break site. Although phase separation alone can in principle explain the fusion of such repair centers, the involvement of the LINC complex and nuclear filaments suggests that the cell retains some autonomy on the formation of larger clusters.

5.5 Condensate Resolution

The importance of condensates for DNA repair and cellular function in general begs the question how the cell regulates not only their formation, but also their dissipation. Here we will briefly discuss some of the mechanisms employed by the cell to dissolve condensates, with a focus on DNA repair foci.

The PAR chains that constitute the original condensate are eventually broken down by the enzyme PAR glycohydrolase (PARG) [147] (Figure 2F). Together with the tendency of PARP1 to dissociate upon autoPARylation [132], this prevents excessive growth of the phase-separated compartment.

Another way for the cell to control the size of the condensate is by post-translational modifications, for example phosphorylation (Figure 2F). Kinases such as ATM and DNA-PK have central roles in the DNA damage signaling cascade, Indeed, phosphorylation can have a pronounced effect on the tendency of intrinsically disordered proteins to phase separate or aggregate [148]. The aggregate formation of the FUS protein has been well studied because of its role in neurological disorders. Wild-type FUS was shown to be less prone to aggregation when the protein was phosphorylated [149]. Likewise, phosphorylation of the disordered C-terminal domain of RNAPII, which produces dilncRNAs, also affects its ability to phase separate [129,130]. Moreover, one of the factors triggering chromatin condensation is thought to be the phosphorylation of HP1a [45]. Phosphorylation of histone H2AX is of course responsible for chromatin decondensation in a DNA damage context. The dephosphorylation of γH2AX, which occurs during or after repair, is performed by protein phosphatase 2A and WIP1 [150,151], and presumably helps with bringing the chromatin back to its native state, resolving the focus. Thus, a small modification like phosphorylation may be a tool for the cell to change the larger physical behavior of a repair focus.

Another post-translational modification is ubiquitylation, which can lead to the breakdown of misfolded proteins that may exist in the core of the condensate. An example of a protein that assists in this type of degradation is the segregase VCP, also known as p97 (Figure

2F). It is recruited to DNA damage sites, where it removes K48-ubiquitylated proteins to facilitate recruitment of 53BP1, BRCA1 and RAD51 [121,152]. An inhibitor of VCP was shown to induce an accumulation of ubiquitylated proteins and cell death [153,154]. It is not unthinkable that large DNA repair foci contain misfolded proteins at their core, which may be toxic if the cell cannot dissolve them. Whether such aggregates exist, and what role segregases like VCP play in resolving these, remains to be seen.

6. Concluding Remarks

Over the years it has become clear that RNA polymers can fulfill more roles than merely being an assembly guide for proteins. LncRNAs have emerged as modulators of processes like transcription and DNA repair through direct interactions with key proteins. We now know that RNA polymers, through their ability to form weak multivalent interactions and to act as scaffolds, are also ideal building blocks for biomolecular condensates. Indeed, membrane-less organelles such as stress granules, paraspeckles, nucleoli and Cajal bodies all contain large amounts of RNA.

Biomolecular condensates are dynamic yet organized structures that are highly sensitive to environmental conditions, rendering them an ideal tool for the cell to respond to stress. Although DNA damage is a major stress-inducing event, the importance of condensate formation for DNA repair has until recently received only limited attention. Canonical DNA repair pathways describe repair as a tightly orchestrated sequence of events. Condensate formation, starting with PARylation and supported by damage-induced transcription, offers a compelling explanation for the timely recruitment and spatial organization of repair factors.

The main challenge exists in determining the structure-function relationship of the biomolecular condensates of DNA repair. This starts with the identity of repair foci: are they indeed phase-separated compartments, or would another description be a better fit? This is not just semantics, since the behavior of the compartment is integral to its function and may enhance our understanding. The development of repair foci over time may hold crucial information about pathway choice. How is focus growth and eventual dissipation regulated by the cell, and what is the interplay with the different repair pathways? Not much is known about the influence of condensates on the biochemical functions of the repair enzymes. How are the kinetics of the repair process affected by the dense environment? It may well be possible that certain enzymes or substrates are excluded from the condensate. How can we best simulate those conditions in the lab?

There is a considerable amount of literature on condensate formation in artificial systems. Additionally, more and more studies are being published on other membrane-less organelles that may well behave very similar to DNA repair foci. In combination with the significant progress that has been made in imaging techniques, such as super-resolution microscopy, they should provide us with the tools to answer these intriguing questions.

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Highlights

- We discuss the current knowledge about the function of biomolecular condensates in DNA repair
- We describe the role of RNA and RNA binding proteins in the formation of repair foci
- We highlight several long non-coding RNAs that perform dedicated tasks at the damage site

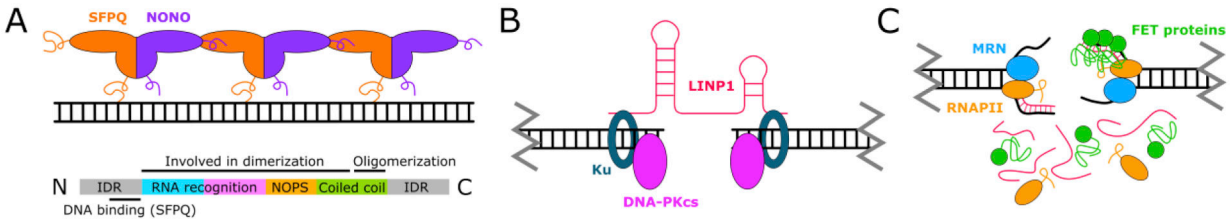

Fig. 1.

Emerging Factors in DNA Repair. A) Proteins from the DBHS family are molecular scaffolds. They contain domains that recognize nucleic acids and domains that allow them to dimerize and oligomerize. Furthermore, their intrinsically disordered N- and C-terminal domains may participate in phase separation. Here, the SFPQ-NONO dimer forms a filament along the DNA that can substitute XLF in NHEJ. IDR = intrinsically disordered region; NOPS = NonA/Paraspeckles domain (involved in dimerization). B) LncRNAs modulate repair processes. Here, LINP1 directly interacts with Ku to promote synapsis in NHEJ. C) Components of the transcription apparatus participate in condensate formation. RNAPII interacts with the MRN complex to produce dilncRNAs. The FET proteins may be involved in stimulating RNAPII activity. The intrinsically disordered domains of RNAPII and the FET proteins form weak multivalent interactions with RNA and with each other, supporting focus formation.

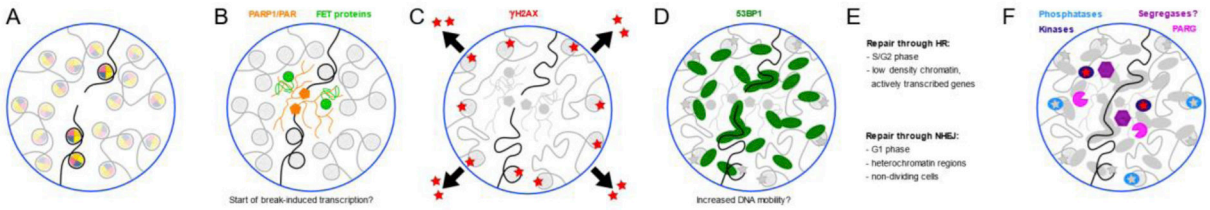

Fig. 2.

Development of DNA repair foci. A) A double strand break occurs. B) PARP1 seeds a phase-separated domain by synthesizing PAR chains. Proteins with a PAR binding motif and several RNA binding proteins (FUS, EWS and TAF15) are recruited in the phase-separated domain. Break-induced transcription may start. C) ATM phosphorylates histone H2AX in a confined volume around the break, leading to local chromatin decondensation. D) 53BP1 is recruited to the break site, interacting with the chromatin and the DNA. E) Repair proceeds through HR or NHEJ. How condensates aid in pathway choice is not fully understood, but HR seems to be preferred in low-density chromatin. F) Dissipation of the focus can occur through post-translational modifications and the breakdown of PAR chains. What role segregases like VCP play is not known exactly.

Table 1.

Overview of lncRNAs discussed in this article.

lncRNA	Interactions and Functions	Promotes…	References
BGL3	Binds PARP1 and BARD1, promoting retention of the BRCA1-BARD1 complex at DSBs	HR	[32]
DDSR1	Interacts with BRCA1 and hnRNPUL1 to regulate HR	HR	[27]
PRLH1	Interacts with RNF169, replacing 53BP1 from ubiquitinylated chromatin	HR	[31]
TERRA	Interacts with RAD51 at telomeres to form R-loops	HR (at telomeres)	[35,94]
HITT	Blocks the ATM binding site for the MRN complex	NHEJ	[24,100]
LINP1	Binds to Ku to stabilize the synaptic complex	NHEJ	[13,97]
LRIK	Interacts with Ku, enhancing the efficiency of NHEJ	NHEJ	[33]
SNHG12	Mediates the interaction between DNA-PK and Ku	NHEJ	[23]
SNHG17	• Binds NONO • Causes upregulation of RING1, which induces RAD51 degradation	NHEJ	[34]
GUARDIN	• Prevents chromosome end-to-end fusion by sequestering microRNA23a • Promotes BRCA1-BARD1 complex formation	HR and NHEJ	[26]
HITTERS	Promotes binding of MRE11 to RAD50	Cell survival	[25]
Linc00312	Binds to DNA-PKcs to prevent recruitment to Ku80	Apoptosis	[28]
Lnc-bc060912	Interacts with PARP1 and NPM1	Cell survival	[29]
NEAT1	• Binds DBHS proteins and FUS • Forms paraspeckles upon activation of p53 • Is involved in transcriptional activation of DNA repairgenes	Genome stability	[46,51,102,103]
NEAT2/ MALAT1	• Forms a complex with PARP1 and LIG3 to aid in alternative NHEJ • Binds SFPQ, which renders it unable to sequester oncoprotein PTBP2	Cell survival	[30,108]