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RNA in DNA repair

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ABSTRACT

Our genome is constantly subject to damage from exogenous and endogenous sources, and cells respond to such damage by initiating a DNA damage response (DDR). Failure to induce an adequate DDR can result in increased mutation load, chromosomal aberrations and a variety of human diseases, including cancer. A rapidly growing body of evidence suggests that a large number of RNA binding proteins are involved in the DDR, and several canonical DNA repair factors have moonlighting functions in RNA metabolism. RNA polymerases and RNA itself have been implicated at various stages of the DDR, including damage sensing, recruitment of DNA repair factors and tethering of broken DNA ends. RNA may even serve as a template for DNA repair under certain conditions. Given the vast number of non-coding RNAs in cells, we have barely started to decipher their potential involvement in genomic maintenance and future research on the interrelationship between RNA and DNA repair may open entirely new treatment options for human disease.

1. Introduction

The DDR is not a fixed pathway, but rather a repertoire of pathways from which modules are mobilized depending on the type of damage, type of cell, chromatin context and cell cycle stage [1]. Examples of such pathways are base-excision repair (BER) to correct small base lesions and some mismatches [2], nucleotide excision repair (NER) to remove bulky and helix-distorting adducts [3] and mismatch repair (MMR) to correct replication-induced mismatches [4]. DNA double-strand breaks (DSBs) are repaired via high-fidelity homologous recombination (HR) [5], or by the more error-prone non-homologous end-joining (NHEJ) [6]. The boundaries between the pathways are apparently not fixed and repair of some lesions often involves proteins from more than one pathway [7]. The DDR also encompasses mechanisms to halt the cell cycle and downregulate housekeeping functions, thus contributing to successful DNA repair. Accumulating evidence has revealed an unforeseen and important function of RNA at various stages of the DDR (Fig. 1). Various non-coding RNAs (ncRNAs) are indirectly involved by e.g. transcriptional induction and translational enhancement of DDR proteins, or more directly by tethering DNA repair proteins or DNA breaks. RNA may also act as a template for DNA synthesis during repair of strand breaks. In some cases, ribonucleotides are even transiently incorporated as part of DNA repair patches. Moreover, well-known RNA-binding proteins have emerged as novel factors facilitating DDR directly or by interacting with DNA repair enzymes. This review attempts to summarize current knowledge of RNA involvement at different stages of the DDR. We also include some cases where RNA polymerase is directly involved in damage sensing. Specific aspects within the area have been covered in recent excellent reviews, to which the reader is referred throughout the text. However, for most of these proteins it remains to be established to what degree binding to RNA functionally contributes to the DDR.

2. Many RNA-binding proteins are involved in the DDR

Several studies have aimed to map proteins involved in the DDR, e.g. by employing siRNA screens or affinity-based methods coupled to downstream LC–MS/MS analysis (reviewed in [8]). Bader et al. [9] collectively analyzed data from studies employing various methods [10–12] and found significant enrichment of RNA-interacting proteins, especially proteins involved in transcription and transcript processing. RNA-interacting proteins constituted 54 % of those recruited to damaged chromatin and 39 % of proteins modified in response to DNA damage. RNA-binding is also a key feature of many canonical DNA repair factors (reviewed in [9,13,14]). Collectively, these studies indicate that RNA-binding proteins, including RNA-processing enzymes, play important functions in the DDR. Such proteins have been coined DNA-damage response RNA-binding proteins (DDRBPs) and encompass canonical DNA repair proteins as well as a steadily growing number of other proteins (reviewed in [15]).

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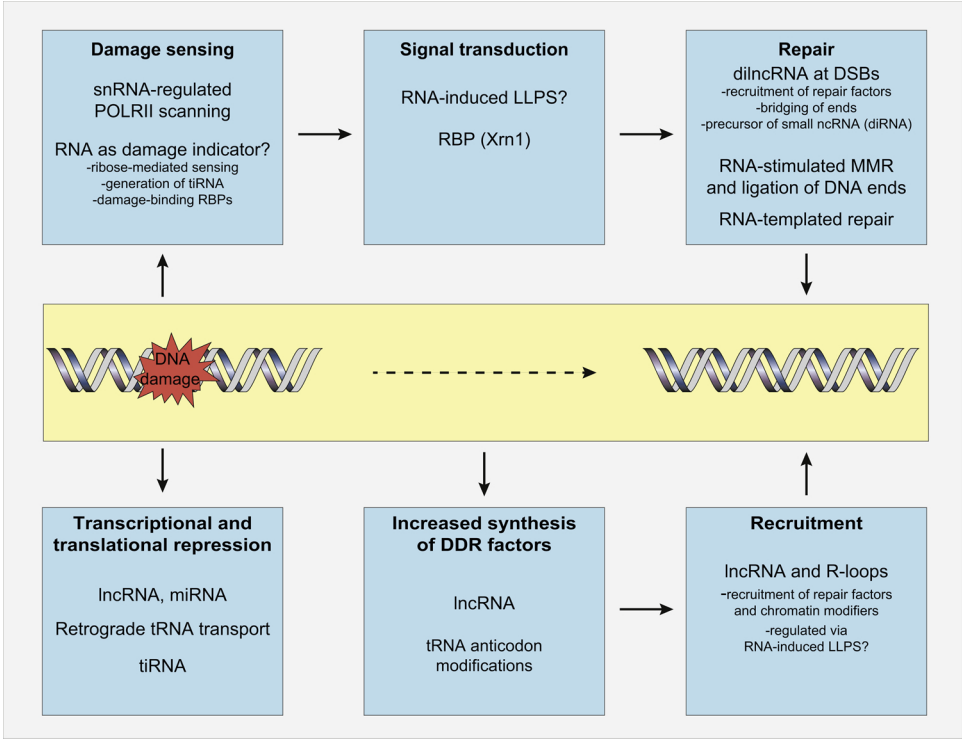

Fig. 1. Verified and proposed roles of RNA at various stages of DNA repair. dilncRNA; damage-induced long non-coding RNA, diRNA; small, damage-induced RNA, DSB; DNA double-strand break, LLPS; liquid-liquid phase separation, lncRNA; long non-coding RNA, miRNA; microRNA, MMR; DNA mismatch repair, ncRNA; non-coding RNA, RNAPII; RNA polymerase II holoenzyme, RBP; RNA-binding proteins, snRNA; small nuclear RNA, tiRNA; tRNA-derived stress-induced RNA.

3. Overall and local transcriptional and translational responses to DNA damage

Upon exposure to DNA-damaging agents, cells respond by adapting their protein repertoire. Overall RNA and protein synthesis is downregulated and contributes to cell cycle exit (reviewed in [16,17]). However, mechanisms are in place to ensure that a subset of transcripts are specifically modulated to mediate a targeted DDR via e.g. altered transcriptional rate, splicing and 3′-processing [18]. Several classes of non-coding RNAs (ncRNAs) play crucial functions in the coordinated transcriptional responses, of which microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) appear especially important. miRNAs are a large group of 18–25 nt ncRNAs that regulate about 60 % of all human mRNA molecules through posttranscriptional mRNA degradation and suppression of protein translation. A significant number of these miRNAs also modulate the expression or function of DDR proteins (reviewed in [19]). LncRNAs constitute another large group of ncRNAs encompassing about 170 000 members in humans (http://www.noncode.org/) and are involved in a plethora of gene-regulatory mechanisms (reviewed in [20,21]). Many lncRNAs have been identified that directly affect the responses to different DSB-inducing agents and thus constitute promising biomarkers in anti-cancer therapy (reviewed in [22]). Although most of these lncRNAs act via transcriptional transregulation, a growing number has been found to directly bind to and modulate DNA repair proteins. Among these, DDSR1 binds and recruits hnRNPUL1 to DSBs while concomitantly restricting accumulation of the BRCA1/RAP80 complex, thus favoring HR [23]. LINP1 is an integral component of the synaptic complex of NHEJ that stabilizes the structure of Ku80–DNA-PKcs interaction and promotes NHEJ [24]. GUARDIN acts as a scaffold required for association between BRCA1 and BARD1 and is necessary to activate both HR and NHEJ [25]. HITT binds to ATM and blocks association between ATM and NBS1, thereby inhibiting HR [26]. These topics have been subject to recent comprehensive reviews [18,21,27].

Ongoing transcription is locally downregulated at DNA lesions. This is commonly mediated by DDR kinases such as ATM and DNA-PK. ATM modulates RNA polymerase II (RNAPII) itself [28], elongation factors [29], cohesin [30] and chromatin modifiers such as the PBAF subunit BAF180 [31] and ZMYND8-NuRD [32] as well as the poly(ADP-ribose) polymerase PARP1 [10]. Concomitantly, activation of DNA-PK may promote eviction of RNAPII from repair sites [33]. These studies have revealed several and complex mechanisms contributing to repression of ongoing transcription in the vicinity of DNA lesions (reviewed in [34,35]).

Genotoxic stress also induces a translational reprogramming that favors translation of certain stress-related genes while slowing down global translation. Transfer RNA (tRNA) seems to have a key role in this reprogramming. First, the tRNA pool accessible for translation is rapidly modulated by active and reversible transport of certain cytoplasmic tRNA species into the nucleus in a process called tRNA retrograde transport [36]. Secondly, tRNA coding properties are altered by enzymatic modification of its nucleotides to increase translation of critical DDR protein transcripts that use specific degenerate codons and codon biases (reviewed in [37]). Third, stress induces cleavage of specific tRNAs to produce a novel class of small regulatory RNAs called tRNA-derived stress-induced RNAs (tiRNAs) [38] which will be further addressed in Section 11. These factors are thought to collectively contribute to the translational adaptation upon stress, adding another layer of RNA-mediated regulation of the DDR.

4. Dangers and benefits of misincorporated ribonucleotides in DNA

Ribonucleotides constitute primers of Okazaki fragments and transiently make up about 5 % of the nascent lagging strand during replication [39]. In addition, ribonucleotides are frequently misincorporated by replicative polymerases [40]. It has been estimated that more than one million ribonucleotides are incorporated in mammalian genomes during each cell division, likely making this the most frequent source of cellular DNA damage [41]. Whereas single ribonucleotides in DNA can be tolerated up to a certain threshold, several consecutive ones may constitute a threat to viability and genomic stability (reviewed in [42]). Misincorporated ribonucleotides can be removed from the nuclear genome via a mechanism called ribonucleotide

excision repair (RER) [43], while mitochondria apparently lack this pathway [44]. In RER, RNase H2 recognizes single as well as consecutive ribonucleotides in dsDNA and incises the DNA backbone at the dNMP-rNMP junction. Pol δ/ε then perform displacement synthesis from the 3′−OH end, the flap is removed by FEN1 and the single-strand nick sealed by DNA ligase (reviewed in [45]). Interestingly, eukaryotic RNase H2 cannot repair damaged ribonucleotides such as abasic ribosephosphates and 8-oxo-7,8-dihydroguanine (8-oxoG) in DNA, whereas this repair function was recently assigned to APE1 [46].

Defects in RNase H2 lead to genomic instability and is associated with multiple diseases, including cancer (reviewed in [45]). This may be caused by an alternative mode of processing genomic ribonucleotides. In the alternative pathway, the ribonucleotide is removed via a topoisomerase 1 (TOP1)-dependent mechanism involving a covalent intermediate of TOP1 and the 3′-terminal phosphate of the ribonucleotide [47]. Based on work in yeast, completion of repair may then proceed via several mechanisms, some of which may lead to small deletions and DSBs (reviewed in [45]). However, further work is needed to understand the relative contribution of TOP1-mediated genomic ribonucleotide cleansing in humans and its contribution to genomic instability. Nevertheless, it was shown that RNase H2-deficient cells were also highly sensitive to PARP-inhibitors [48]. The authors proposed that RER and TOP1 compete for removal of ribonucleotides. In RER-deficient tumors, a shift towards TOP1-mediated removal would generate PARP-trapping intermediates, thus rendering the cells highly sensitive to PARP inhibition. Recent work in yeast suggests that RER-deficiency may also be highly toxic in situations of low dNTP concentrations, e.g. resulting from hydroxyurea treatment. In such situations, the translesion synthesis (TLS) polymerase Pol η (POLH) can rescue replication by incorporating longer stretches of ribonucleotides and allow full genome duplication. This, however, becomes harmful if the ribonucleotides subsequently cannot be removed by RNase H activities [49].

It may seem paradoxical why evolution should allow a large fraction of ribonucleotides to slip through replicative proofreading without associated benefits. One potential benefit emerged in 2013, when the Kunkel and Jiricny labs reported that ribonucleotides misincorporated during replication facilitate specific scission of the newly synthesized strand during eukaryotic MMR [50,51]. This would be especially important in the leading strand, which has much fewer strand scissions associated with Okazaki fragments. However, it is not likely that ribonucleotides account for all MMR-initiation events, since they are incorporated on average every 6−8 kb during replication [51,52], which is considerably longer than most MMR tracts.

Finally, incorporation of ribonucleotides by Pol μ (POLM) and the terminal deoxynucleotidyltransferase TdT (DNTT), was recently assigned a role in DNA repair [53]. This will be described in more detail in the context of DSB repair.

5. Transcription-coupled NER and RNA polymerase as DNA damage sensor

Roles of RNA in DNA repair must also encompass RNA polymerases and preferential repair of transcribed genes. The first indications of such a mechanism came from the Hanawalt laboratory, by demonstrating that UV-lesions in the DHFR gene were repaired much more efficiently than in the overall genome [54] and that preferential repair occurred in the transcribed strand [55]. This type of transcription-coupled NER (TC-NER, or simply TCR) has later been described in many species and the underlying mechanisms have been mapped in considerable detail (reviewed in [56]).

The central DNA damage sensor in TC-NER is the transcribing RNAPII, which has been referred to as “the most specific damage recognition protein” [57]. Specific structural elements and DNA lesions delay progression of RNAPII to varying degrees and some bulky lesions, such as CPDs, constitute virtually complete blocks when present in the transcribed strand [58]. Stalling induces a ternary RNAPII/RNA/DNA complex with a half-life of approximately 20 h *in vitro* [59,60], providing ample time to recruit factors that can relieve blockage. According to the ENCODE project [61], this type of DNA damage detection can protect up to 80 % of the human genome. Apparently, DNA repair factors are not directly recruited by the stalled polymerase itself, but rather mediated by transcription-repair coupling factors (TRCF in bacteria). In humans, the ATP-dependent translocase CSB (ERCC6) serves this function. Lesions that cannot be bypassed mediate tighter binding of CSB to RNAPII and the complex induces DNA bending that signals recruitment of CSA, UVSSA and TFIIH [62] and downstream NER factors as well as chromatin modifiers (reviewed in [56]).

Upon genotoxic stress cells shift to a “safe mode” of RNAPII elongation. This includes a wave of synchronous RNAPII release from promoter-proximal pausing sites into gene bodies of virtually all active genes [63]. RNAPII generally pauses 20–100 nt from transcription start sites (TSSs). It can be unleashed by pTEFb, a dimer consisting of the CDK9 kinase and Cyclin T1/T2. pTEFb is normally sequestered within the 7SK snRNP ribonucleoprotein complex [64] that in humans contains a ~330 nt small nuclear RNA (snRNA). Genotoxic stress induces release of pTEFb from 7SK snRNP, enabling it to phosphorylate POLR2A (at S2) and the negative transcription factors NELF and DISF. NELF then dissociates from RNAPII, NELF turns into a positive transcription factor and RNAPII is released This activates transcription of

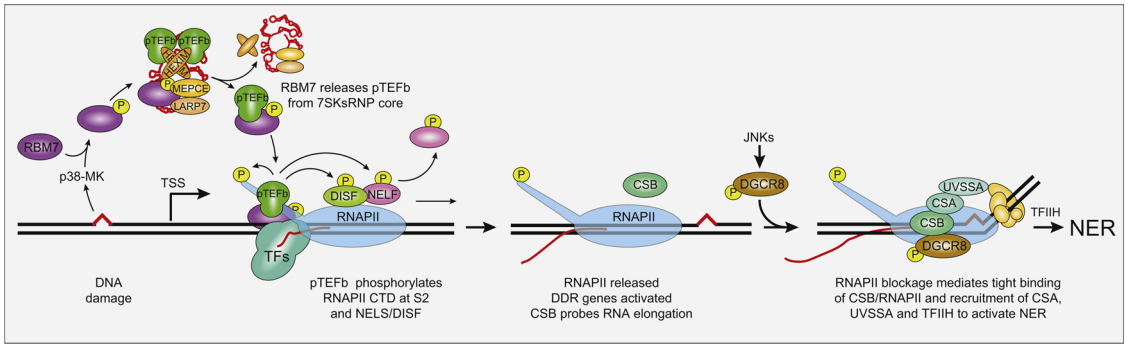

Fig. 2. Transcribing RNAPII, 7SK snRNA and the miRNA processing factor DGCR8 are involved UV lesion sensing and TC-NER. Genotoxic stress activates p38MAPK-MK, which phosphorylates RNA-binding protein 7 (RBM7) and triggers its binding to the 7SK snRNP. The core 7SK RNP complex consists of a ~330-nucleotide snRNA (red) transcribed by RNAPIII, the La related protein LARP7, the methylphosphate capping enzyme MEPCE and HEXIM1. Binding of phosphorylated RBM7 releases pTEFb and allows binding to transcription factors (TFs). The CDK9 kinase of pTEFb phosphorylates the RNAPII CDT at S2 as well as the negative TFs DISF and NELF. This promotes dissociation of NELF and turns DISF into a positive TF, releases the RNAPII complex to continue transcription and allows DNA damage scanning [64]. When a transcription blocking lesion is encountered, CSB binds tightly to RNAPII, recruits CSA, UVSSA, TFIIH and downstream TC-NER factors. UV-stress also mediates activation of the miRNA processing factor DGCR8 that promotes TC-NER through a yet unknown mechanism.

several key DDR genes and several classes of ncRNAs that induce cell cycle arrest and concomitantly allows RNAPII-mediated detection of DNA lesions that can be repaired by TC-NER [64] (Fig. 2). pTEFb-mediated release of RNAPII has been found to be dysregulated in many cancers and has highlighted CDK9 as a potential novel therapeutic target [65].

miRNA-processing factors have also been implied in UV-induced DDR. A subset of cellular DICER accumulates in the nucleus upon UV damage and facilitates chromatin decondensation that stimulates the global genomic (GG) NER pathway [66]. DICER mediates recruitment of the methyltransferase MMSET to the damage site, resulting in histone H4 K20 dimethylation. This stimulates GG-NER and involves the scaffolding protein XPA. DGCR8, which normally functions in DROSHA in an early step of miRNA biogenesis, also interacts with RNAPII and CSB and has an important function in TC-NER in a manner independent of miRNA processing. Apparently, RNAPII stalling phosphorylates DGCR8 and this, via a yet unidentified mechanism, promotes TC-NER [67]. It should be noted, however, that UV mediates several types of DNA lesions, and it is presently not clear exactly which type lesion that engages the miRNA-processing factors.

6. RNA in base excision repair

A damage-sensing function of RNAPII was recently also reported in BER. Alkyladenine DNA glycosylase AAG (also known as MPG) forms a complex with RNAPII via direct interaction with the Elongator complex subunit ELP1 and inhibition of transcript elongation inhibits AAG-initiated BER. In addition, AAG-ELP1 interaction co-regulates expression of a specific set of genes, implying a role of methylbase lesions in gene regulation [68]. Others have reported the involvement of transcription in repair of oxidized bases. CSB, which is essential for TC-NER, was recruited in a transcription-dependent manner to sites of oxidative DNA damage *in vivo* [69]. Transcription-dependent recruitment was also observed for the BER scaffold protein XRCC1 [70]. It is likely that at least some of the BER-enhancing effects of ongoing transcription rely on chromatin decondensation since chromatin remodeling is apparently required to gain access to some base lesions [71].

Interestingly, single-strand selective monofunctional uracil DNA glycosylase (SMUG1) can recognize and process 5-hydroxymethyluridine (hmU) in both DNA and RNA [72]. SMUG1 also interacts with the DKC1-containing H/ACA ribonucleoprotein complex and is required for maturation of telomeric RNA component (hTERC) through regulating the levels of modified bases in a region involved in DKC1 binding. SMUG1-deficient cells exhibit telomerase deficiency, leading to impaired bone marrow proliferation in Smug1$^{-/-}$-mice [73]. RNA-metabolizing functions have also been assigned to APE1, the main AP endonuclease in BER [74] and also a redox coactivator of transcription factors [75]. Evidence of an RNase H-like activity in APE1 was first reported in 1995 [76] and it has been implicated in removal of damaged ribonucleotides from DNA [46]. APE1 is also involved in several RNA metabolic processes, including mRNA splicing and miRNA processing (reviewed in [77,78]). Although it is still unknown to what degree these non-canonical functions of APE1 may modulate BER, it is tempting to speculate that both the redox- and the splicing modulating activities may indirectly contribute to fine tune the DDR subsequent to genotoxic stress.

7. RNA in DSB repair

DSBs are considered the most cytotoxic DNA lesions if left unrepaired [79]. DSB repair (DSBR) occurs via two major pathways conserved between prokaryotes and eukaryotes. Homologous recombination (HR) depends on the presence of a homologous sequence, commonly spanning hundreds of nucleotides present in a sister chromatid, and is thus mainly restricted to the late S- to G2/M-phases of the cell cycle [5]. Conversely, the much faster classical non-homologous end-joining (C-NHEJ or simply NHEJ) pathway ligates two ends of dsDNA without the need for extensive resection and can operate in any phase of the cell cycle (reviewed in [80]). In addition, more slow alternative end-joining (a-EJ) and single-strand annealing (SSA) pathways rely on various degrees of resection and microhomology and are generally regarded error-prone (reviewed in [81]). The engagement of either of these pathways at a DNA break may not be based on an early choice, but rather by hierarchical interrogation of available pathways, with the highest fidelity pathway on top [81]. The pathway employed is influenced by the complexity of the broken ends [82], the pre-existing chromatin status [83] and the transcriptional activity [84] (reviewed in [85]).This would explain why several modes of DSBR can be observed even within the same cell cycle phase (reviewed in [86]). Emerging evidence suggest that RNA plays critical roles in several DSBR subpathways. The different methods employed to induce DSBs as well as their pros and cons in terms of obtaining various types of mechanistic information, were recently reviewed [87].

7.1. Ribonucleotides mediate ligation of complex ends in NHEJ

DSBs often contain chemically complex ends that are not directly compatible with ligation. This poses a potential problem for NHEJ, in which there is very limited end-resection and thus little complementarity. In such cases, the X-family polymerases Pol μ (POLM) and TdT (DNTT) can add nucleotides to the 3′-overhangs to form ligatable ends. TdT is template-independent, expressed in lymphocytes and contributes to V(D)J recombination whereas Pol μ is more ubiquitously expressed and contributes to both NHEJ and V(D)J recombination (reviewed in [88]). Pol μ can act in both template-dependent and -independent ways, and typically uses the second end as template to incorporate an incoming nucleotide triphosphate. Thus, Pol μ may bridge short gaps between the broken ends in a DSB [89]. Compared to most other DNA polymerases, TdT and Pol μ discriminate about 1000-fold less against ribonucleotides [90] and a recent study indicates that as much as 65 % of NHEJ may involve transiently embedded ribonucleosides [53]. Somewhat surprisingly, incorporation of ribonucleotides, but not deoxyribonucleotides, effectively promoted ligation by LIG4. They proposed a triple strand break model in which the first strand is repaired via RNA and the opposite strand is repaired via DNA only. After both strands are ligated, ribonucleotides remaining in the first strand are then repaired via RNase H2-mediated RER (Fig. 3). It is presently unknown which polymerase operates during second strand repair. It should also be noted that if ribonucleotides are incorporated during second strand repair, RNase H2-mediated RER would re-introduce a DSB unless ribonucleoside removal is timely separated in the two strands.

7.2. Small miRNA-like RNAs are formed at DSBs, but their role in repair remains controversial

An early response to DSBs in actively transcribed genes is rapid and transient downregulation of transcription surrounding the DSB, likely to avoid conflict between transcription and repair [34]. However, while transcription is paused, promoter-independent synthesis of ncRNA may be induced at the DSB and contribute to DSBR (reviewed in [9,91]). These ncRNAs can be divided into two main classes based on their sizes. Transcripts > 200 nt are called damage-induced long non-coding RNAs (dilncRNAs) [92], whereas small, miRNA like RNAs of about 21 nt are known by different names such as diRNAs [93], DDRNAs [94], qiRNAs [95] or endo-siRNAs [96]. These small ncRNAs are probably formed by processing of double-stranded dilncRNAs [96] analogous to the biosynthesis of miRNAs. Thereby the diRNAs could serve in a degradation mechanism to protect the cell from truncated mRNA [96]. In support of this, proteins normally contributing to miRNA biogenesis, like DICER, DROSHA and AGO2, are also involved in the formation of diRNAs [92,97–99]. A considerable body of evidence suggests that diRNAs

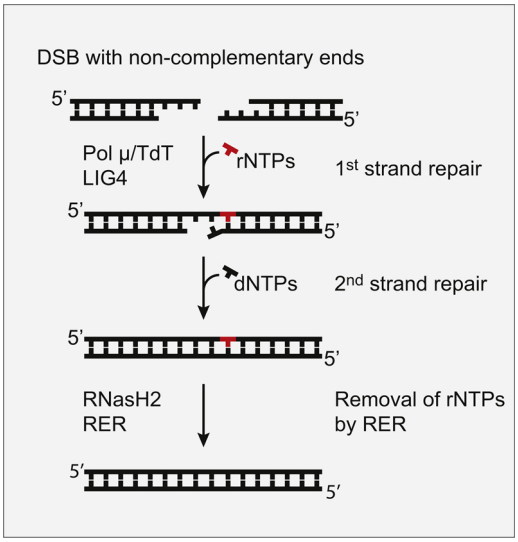

Fig. 3. Triple strand break model for NHEJ of DSBs with incompatible ends [53]. Pol μ or TdT elongates 3′-end of the 1st strand by incorporating ribonucleotides, stimulating ligation by LIG4. The 2nd strand gap is filled in with deoxyribonucleotides by an unknown polymerase and ligated by LIG4. Remaining ribonucleotides in the 1st strand are replaced by RNase H2-mediated RER. Pol μ or TdT can also act as template-independent DNA/RNA polymerases, potentially able to bridge gaps prior to templated insertion of ribonucleotides by Pol μ.

contribute directly to DSBR. Production of diRNAs in the vicinity of DSBs was shown to promote repair via HR and depletion of either DICER or AGO2 mediated significantly reduced repair [93]. This was apparently mediated by the ability of AGO2, guided by diRNA, to recruit RAD51 to DSBs [100]. diRNA-associated AGO2 recruits the chromatin remodelers MMSET and Tip60 to DSBs, promoting chromatin decondensation and recruitment of the HR factors RAD51 and BRCA1 [99]. Conversely, inactivation of DICER or DROSHA reduced the number of repair foci containing 53BP1, suggesting diRNAs promote NHEJ [94]. RNase A-treatment of γ-irradiated and permeabilized cells reduced the number of repair foci, whereas addition of exogenous, size-fractionated, cellular RNA of 20–35 nt as well as synthetic, locus-specific DDRNAs selectively restored the 53BP1-containing repair foci [94]. DICER may also have a diRNA-independent function in the DDR by sequestering the histone deacetylase SIRT7 in the cytoplasm, thereby increasing the level of acetylated histone H3 (H3K18Ac) at DSBs and promoting recruitment of NHEJ factors Ku70/80 and 53BP1 [97,101].

Collectively, these studies strongly suggest that components of the miRNA biogenesis machinery are involved in DSBR. A direct role of diRNAs remains, however, more controversial. In *Drosophila*, perturbed synthesis of damage-induced siRNAs did not affect DSBR [102]. Some studies suggest that the small RNAs are rather by-products of the experimental systems employed, often consisting of endonuclease-cleavable reporter transgenes as well as highly repeated sequences. Repetitive DNA and DSBs have previously been shown to induce siRNA production when both are present [103]. In *Arabidopsis*, the level of diRNA correlated with RNAPII-transcription of a CRISPR/Cas9-targeted transgene, whereas no diRNAs were observed when cleavage was induced within endogenous genes [104]. Lu et al. [105] employed IR and an inducible restriction enzyme system to induce DSBs in the endogenous genome of human cell lines. They found no diRNAs at any of the endogenous cleavage sites. Bonath et al. [106] employed the homing endonuclease I-PpoI to induce DSBs in the repetitive 28S DNA as well as in unique genic and intergenic loci. Whereas dilncRNAs were readily detected at all loci, they observed substantial levels of diRNAs only at the repetitive 28S locus. Thus, additional studies are needed to resolve the involvement of diRNAs and non-canonical DSBR functions of proteins normally involved in miRNA biogenesis.

7.3. Potential roles of dilncRNAs in DSBR

There is currently increased focus on the formation and potential roles of dilncRNAs beyond being precursors for diRNAs. Michelini et al. [92] found that the MRE11-RAD50-NBS1 complex recruits RNAPII to DSBs to synthesize dilncRNAs from and towards the DNA ends. However, it is still unknown how RNAPII initiates transcription of dilncRNAs in the absence of any promoter. Deep sequencing has shown that dilncRNAs emerge only a few nucleotides away from the DSB [92,106]. Thus, a canonical pre-initiation complex is apparently not involved, since this would leave a non-transcribed sequence near the break [107]. Likely, chromatin status plays an important role, as suggested by a recent study employing single-molecule imaging [108]. Here, a DSB was introduced in an RNAPII-transcribed region either proximal to the promoter or within an exon of a reporter gene. At the promoter-proximal DSB, full-length transcription was stably suppressed within about 10 min and recruitment of mediator of DNA damage checkpoint 1 (MDC1) was observed at about 12 min. When the DSB occurred within an exon, a complete shutdown of preexisting transcription initiation was observed whereas ongoing RNAPII elongation continued until the break site. In contrast to the promoter-proximal DSB, full-length transcription recovered in nearly half of the cells harboring an intra-exon DSB after 30 − 40 min in a manner dependent on LIG4, suggesting that repair by NHEJ was necessary for repair of these DSBs prior to transcription recovery. Importantly, in a somewhat smaller fraction of these cells, break-induced transcription from the DSB was induced immediately after suppression of the pre-existing transcription. This occurred stochastically in either, although only one, direction from each DSB. The authors propose that this was facilitated by formation of a promoter-like nucleosome-depleted region around the DSB [108]. In support of this, they analyzed previous DSBCapture and transcriptome data [109], which revealed significantly higher antisense transcription at DSBs in intragenic regions compared to random genetic regions, and which overlapped with nucleosome-depleted regions [108].

Although the above studies shed novel light on the formation of dilcRNAs, their precise function in DSBR remains poorly understood. Several lines of evidence suggest, however, that their propensity to form RNA:DNA hybrids is important.

7.4. RNA:DNA hybrids in DSB repair

RNA:DNA hybrids have primarily been studied in the context of R-loops, which are three-stranded structures composed of an RNA:DNA hybrid and a displaced ssDNA strand [110]. R-loops cover up to 5 % of mammalian genomes [111] and play crucial functions in many biological processes (reviewed in [112,113]). Uncontrolled R-loops can, however, pose a threat to genomic stability and mechanisms must be in place to ensure their timely resolution. This may occur by RNase H activities that degrade RNA hybridized to DNA, or by various helicases such as Senataxin (SETX), Aquarius (AQR), DDX23 or DHX9 (reviewed in [114–116]). R-loops can be induced by different genotoxic agents, including ROS or laser micro-irradiation [117,118] and endonuclease-mediated DSB induction [105,119–124]. A flurry of recent studies also indicate that RNA:DNA hybrids and R-loops have direct roles in DSBR (reviewed in [9,91,113,125,126]). However, it is still unclear to what extent these hybrids are formed by dilncRNAs, by nascent canonical transcripts or by transcripts produced prior to a DSB in the corresponding gene. Theoretically, both sense and antisense transcripts may occur simultaneously at a DSB, since R-loops formed by nascent sense transcripts can act as intrinsic RNAPII promoters and seed *de novo* transcription of lncRNAs in the antisense direction [127]. Nevertheless, a prevailing view is that RNA:DNA hybrids mediate recruitment of repair proteins, including DNA end resection and chromatin remodeling

factors [9,91,117,124,128]. Studies in *Saccharomyces pombe* suggest that RNA:DNA hybrids formed by dilncRNAs may constitute a mechanism to regulate DSB end resection and thus dictate the repair pathway [124]. The authors suggest two models, in which dilncRNAs synthesis either follows the strand resection process or actively promotes resection by opening the chromatin and the DNA strands ahead of the exonucleases. In the latter model, the degree of end resection would resemble the length and speed of dilncRNA transcription. This would depend on several factors, including the propensity of the dilncRNAs to form RNA:DNA hybrids and to expose ssDNA. G-rich sequences in the non-template DNA form especially stable RNA:DNA hybrids [129]. However, strong RNA:DNA hybrids may also hamper further transcription [130,131] and must be resolved by helicases or degraded by RNases to allow transcript elongation. In agreement with this, decreased length of RPA-covered ssDNA was observed around the DSB in an RNase H1-deficient strain, whereas overexpression of RNase H1 resulted in extra-long ssDNA segments [124]. A more recent study in *S. pombe* suggests, however, that RNase H is not needed for efficient DSB repair, but rather to eliminate RNA:DNA hybrids that trigger replication fork collapse [122]. One study in human cells demonstrated that dilncRNAs-mediated RNA:DNA hybrids promoted HR by recruiting BRCA1/2 and RAD51, but without affecting DNA-end resection [120]. Conversely, another study found that depletion of the exosome subunit EXOSC10 mediated accumulation of dilncRNAs and RNA:DNA hybrids, accompanied by increased DNA end resection and impaired recruitment of RPA to the DSBs [91]. Whereas normal DNA resection could be restored by transcription inhibitors, overexpression of RNase H1 normalized recruitment of RPA. Potentially, Senataxin cooperates with the exosome to resolve the RNA:DNA hybrids [123]. Very recently, Senataxin was shown to limit RNA:DNA hybrid formation at DSBs in budding yeast to control DNA end resection and repair fidelity [132]. The emerging picture is that balanced induction and resolution of dilncRNA-induced RNA:DNA hybrids is important for faithful repair of DSBs by HR, but additional studies are needed to understand the molecular mechanisms involved and their dependence on genomic context and cell cycle status.

Finally, if a DSB occurs within an already transcribed gene, an RNA:DNA hybrid spanning the break could offer error-free, RNA-templated DSBR of the break. This would likely be favored by DSB-induced transcriptional pausing, which has been shown to enhance the formation of R-loops [133].

7.5. RNA-templated DSBR

In 2003, Rothkamn et al. quantified contribution of HR and NHEJ to repair IR-induced DSBs through the mammalian cell cycle [134]. These experiments confirmed that NHEJ was dominant in G1, consistent with the idea that HR was not a major contributor due to lack of a sister chromatid template. Nevertheless, they found that in G1, HR-deficient cells were more sensitive to IR than wild-type cells, suggesting that an unknown template for HR was present. Building on these findings and previous studies in yeast, Trott and Porter [135] proposed that this unknown template could be RNA and suggested alternative models whereby nascent RNA transcripts could serve such a function. RNA-templated repair of transcribed genes would make biological sense for at least two reasons: Firstly, enhanced frequency of DSBs has been observed at nucleosome-depleted regions at transcriptional start sites, and correlates with transcription rate [136–139]. Secondly, highly transcribed genes likely constitute the most “valuable” parts of the genome and templated DSBR via the nascent transcript or pre-mRNA could allow error-free repair of these genes in the absence of a DNA template. It is also tempting to speculate that such RNA-templated DSBR might cover a larger part of the genome than merely highly transcribed genes. The finding that genotoxic stress induces global unleashing of transcription elongation from promoter-proximal pausing sites [63] (Fig. 2) indicates that nascent transcripts or pre-mRNAs covering a large part of the genome will be available for transcript templated repair shortly after the initiating lesion.

Experimental evidence supporting transcript-templated DNA repair emerged in 2007, when Storici et al. demonstrated that RNA could serve as template for DNA synthesis during DSBR in yeast and that yeast DNA polymerases α and δ could copy short RNA templates *in vitro* [140]. In 2011, they showed that a DSB could be repaired, although a low frequency, by transfection of an oligoribonucleotide homologous to the broken DNA ends [141]. Shortly thereafter, the HR factor Rad51 was found to promote formation of RNA:DNA hybrids in yeast [142]. A separate study demonstrated that a yeast transcript mediated preferential repair of a DSB in the same gene. In the absence of RNase H activity (that would degrade the RNA in RNA:DNA hybrids), the repair apparently proceeded via a Rad52-facilitated annealing mechanism in which the RNA served either as a template to bridge the broken ends or to initiate single-strand annealing via RT-mediated extension of the broken DNA ends [143]. They also demonstrated that RAD52 could promote annealing of RNA to DNA, and that RNA:DNA annealing was even more efficient than DNA:DNA annealing in the presence of RPA. Aymard et al. [83] identified an “HR-prone” subset of DSBs located in actively transcribed genes. These DSBs selectively recruited RAD51, underwent resection, and were dependent on RAD51 for efficient repair. In nondividing U2OS cells, BRCA1/2, CtIP and NBS1 were equally recruited to DSBs in both transcribed and non-transcribed loci. Conversely, the recombination factors RPA1, RAD51C and RAD52 were selectively recruited to the transcribed loci and their dissociation correlated with the completion of repair. Notably, their recruitment was dependent on CSB, but not ATM. Mazina et al. [144] found that yeast and human Rad52 were able to promote inverse strand exchange between homologous dsDNA and RNA. This would allow repair to proceed in the absence of exonucleolytic resection of the broken ends unless such resection is necessary to provide ligatable termini.

Although the above experiments indicate that transcript-templated DSBR occurs largely through HR, NHEJ factors are apparently also involved. Chakraborty et al. found that upon DSB induction, NHEJ proteins formed a multiprotein complex with RNAPII and selectively promoted repair in transcribed genes. They termed this transcription-coupled NHEJ (TC-NHEJ) and demonstrated that a DSB-containing plasmid lacking several nucleotides within the *E. coli LacZ* gene could be repaired in an error-free manner in control- but not NHEJ-deficient mammalian cells [145]. Very recently, the same group reported that the mammalian RNAPII forms a megadalton complex together with ATXN3, PNKP and LIG4 [146]. They propose a model of TC-NHEJ in which RNAPII pauses at DSBs, the large RPB1 subunit is monoubiquitinylated for functional assembly of repair proteins and strand invasion of the nascent RNA to form an RNA:DNA hybrid. ATXN3 then activates PNKP to generate 3′−OH ends, from which an RNA-dependent DNA polymerase can restore the missing sequence by using the nascent complementary RNA as template. Their data further suggest that in post-mitotic cells such as neurons, NHEJ is the dominant DSBR pathway [146].

8. A potential role of RNA-containing triplexes in DNA repair?

In addition to forming RNA:DNA hybrids, RNA may also form triplexes through binding to the major groove of purine-rich stretches of DNA through Hoogsten or reverse Hoogsten hydrogen bonding (reviewed in [147]). A recent study revealed that such triplexes are far more abundant than previously thought and that the RNAs originate from both coding and non-coding loci [148]. Triplexes, especially when involving lncRNAs, affect chromatin state through recruitment of epigenetic modifiers, but little is known to what extent these structures affect DNA repair. One notable exception is the recent report of a cisplatin-sensitivity-associated lncRNA (CISAL), which binds to the BRCA1 promoter and forms an RNA-DNA triplex structure that inhibits BRCA1 expression. Knockdown of CISAL mediated increased tumor growth in

xenografts in the presence of cisplatin [149]. Clearly, further research is warranted to elucidate potential functions of RNA triplexes in the DDR, and to what extent such triplexes can mediate e.g. tethering of broken DNA ends.

9. RNA methylation in DNA repair

m^6A is the most common internal modification in eukaryotic mRNA and has been associated with a wide range of RNA transactions as well as in the response to cell stress [150–152]. Unexpectedly, m^6A in RNA was rapidly induced at DNA lesions in U2OS cells in response to UV, and depletion of the m^6A writer METTL3 mediated increased UV-sensitivity and delayed repair of CPDs [153]. METTL3-induced m^6A RNA methylation was found to depend on PARP and resulted in recruitment of DNA polymerase κ. METTL3 was not, however, required for recruitment of canonical NER or SSB factors and the authors propose that the response constitutes a distinct and early UV-induced DDR pathway. Very recently, two groups reported deposition of m^6A on the RNA moieties of R-loops. Whereas one group proposed that m^6A promoted R-loop formation to facilitate transcription termination [154], the other group found that the modification recruits the m^6A reader YTHDF2, which destabilizes RNA:DNA hybrids. Depletion of YTHDF2 resulted in elevated levels of DSBs [155], in agreement with a previous genome-wide siRNA screen that identified YTHDF2 as a factor contributing to genome stability [156]. The authors propose that m^6A contributes to genome stability by preventing accumulation of co-transcriptional R-loops. Given the above findings, a potential role of m^6A in DNA-damage induced R-loops warrants further investigation.

Very recently, m^5C modification of mRNA at DSB sites was demonstrated to promote HR, presumably by serving as a signal to aid recruitment of DNA repair factors to the damage sites. Specifically, m^5C-modified RNA accumulated in RNA:DNA hybrids and increased the localization of RAD51 and RAD52 to DSBs [157]. Since m^6A was induced at UV damage sites but not at DSBs [153], RNA modifications may serve as codes that signal different types of DNA damage.

mRNAs are also enzymatically modified by other types of methylation as well as by deamination, pseudouridylation and acetylation (reviewed in [158]). RNA ends can even be ADP-ribosylated by several PARPs [159]. These modifications may alter base-pairing properties of the RNA and/or recruit specific “reader” proteins that dictate functional outcomes. Although aberrant deposition of such modifications has been associated with a broad range of human cancers [160] it remains virtually unexplored to what degree they contribute directly to DNA repair.

10. Is RNA-mediated phase separation involved in DNA repair?

Formation of distinct cellular compartments and membraneless organelles (MLOs) by liquid-liquid phase separation (LLPS) plays a crucial role in cellular organization. MLOs form through dynamic self-organizing processes involving condensation of RNA and proteins (reviewed in [161]) and recent studies suggest that this also occurs in the response to genotoxic stress [162–164] including DNA repair [165–167]. Here, one obvious function could be to concentrate proteins involved in a specific DNA repair pathway while keeping proteins involved in other DNA transactions at a distance. LLPS could also provide protective insulation of DNA repair intermediates, which may be even more toxic than the original lesion, or delay repair until the appropriate cell cycle phase. Increased macromolecular crowding offered by phase demixing may even modulate the physicochemical properties of the DNA repair proteins themselves [168].

Studies of the phase separation process initially focused on protein-driven mechanisms where the role of RNA was merely being scaffolds for multivalent RNA-binding proteins. Many RNA-binding proteins harbor internal disordered regions (IDRs) with multiple interaction motifs that can dynamically recruit a large number of other proteins [169], eventually leading to MLO formation. However, phase separation may also be triggered by RNA alone through self-driven RNA-RNA interactions and the formation of specific RNA structures such as RNA G-quadruplexes [170].

In addition to RNA, the RNA-like polymer poly(ADP-ribose) (PAR) can also initiate phase separation. PAR is synthesized as linear or branched structures by a family of poly(ADP-ribose) polymerases (PARPs) and the substrates include the PARPs themselves, histones, chromatin-associated proteins and even DNA [171]. Within seconds after induction of a single- or double-strand break, PARP1 is recruited to the lesion and immediately starts PARylation. This leads to recruitment of several core DDR proteins as well as phase separating proteins such as FUS, EWSR1 and TAF15 to sites of DNA damage [166].

To what degree RNA structures formed at DNA lesions cooperate with PAR in phase separation at DNA lesions, has received little attention. To shed some light on this, we compared proteins reported to bind RNA:DNA hybrids [128], with PAR-binding proteins identified after genotoxic treatment [166,172,173] and LLPS-inducing proteins (phasepro.elte.hu, bio-comp.ucas.ac.cn/llpsdb). We found that more than one third of the PAR-binding proteins also bind RNA:DNA hybrids. Among the RNA:DNA-binding proteins, a considerably larger fraction is LLPS-inducing, than among the PAR-binding proteins. Eight proteins were common to all three categories (Fig. 4). These findings may suggest that R-loops, dilncRNAs and PAR all contribute to LLPS at sites of DNA damage. This is also supported by the recent finding that R-loops bind PARP1 [174]. Since PARylation is a very early and transient event at DNA breaks, it is possible that PAR seeds liquid demixing by rapidly recruiting LLPS-inducing proteins such as FUS [175], which is required for both NHEJ and HR [176,177] and binds RNAPII [178]. Potentially, lncRNAs may fine-tune the physicochemical properties of the demixed phase and contribute to regulating access of factors that determine the choice of pathway, such as 53BP1 and BRCA1. Indeed, 53BP1 was recently shown to be directly involved in phase separation at DSBs, and conditions that impair phase separation impair 53BP1-dependent activation of p53 [164]. Further research along these lines may answer key questions regarding how DSBs are funneled into end-joining or recombinatorial pathways and how this is coordinated with cell cycle stage and in different genomic contexts.

11. Could RNA damage constitute an early warning system in DDR?

RNA is subject to many of the same lesions that occur in DNA [179–181]. RNA would normally be subject to many types of damage before DNA since it is the quantitatively dominating nucleic acid, lacks protection by histones and is closer to the sites of ROS generation and incoming genotoxic agents. Moreover, since RNA is largely single-stranded and the bases less protected by hydrogen bonds, RNA is more vulnerable to both oxidative and alkylating damage than double-stranded DNA [182,183]. This may potentially offer some buffering towards DNA damage by RNA acting as a damage “sponge”, at least at low doses of exposure. It is also tempting to speculate that RNA damage may constitute an early indicator of some types of genotoxic stress and that mechanisms exist to convey this signal to the nucleus as part of the DDR. Experimental evidence suggests such mechanisms. For example, genotoxic stress promotes cleavage of tRNA by ANG into two halves [184] to form regulatory tiRNAs [38]. This tRNA cleavage is conserved from bacteria to mammals, indicating a fundamental role in stress response [184,185]. Importantly, the cleavage is thought to be triggered by damage-induced disruption of the tRNA tertiary structure, enabling ANG to gain access to its tRNA cleaving site. Such structural tRNA disruptions were observed much earlier than any detectable DNA damage induced by cisplatin or ionizing radiation [186], substantiating the notion of RNA as an early genotoxic stress indicator. Interestingly, tRNA demethylation by the DNA repair enzyme ALKBH3 was shown to promote cleavage by ANG, demonstrating an overlap between tiRNA

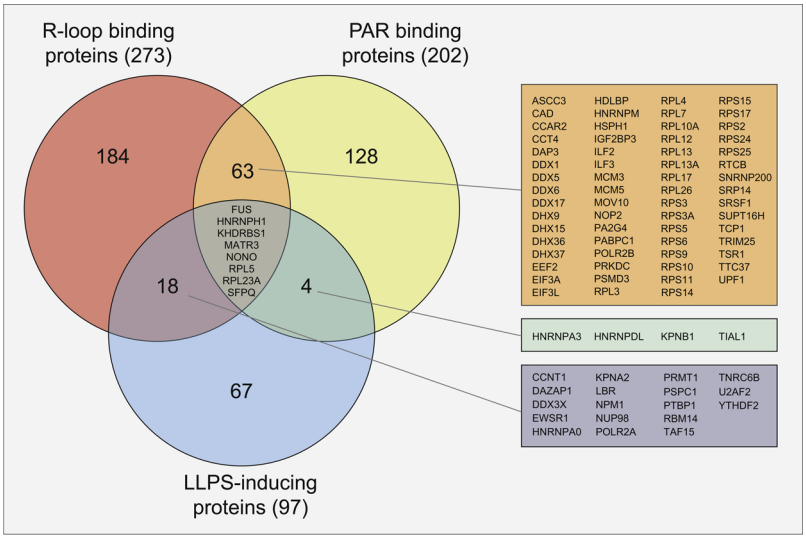

Fig. 4. Overlap of proteins binding to RNA:DNA hybrids from known R-loops [128], nuclear PAR-binding proteins identified after genotoxic treatment [166,172,173] and nuclear LLPS-inducing proteins as reported in the databases PhaSePro (https://phasepro.elte.hu) and LLPSDB (http://bio-comp.org.cn/llpsdb/home.aspx).

generation and DNA repair [187,188]. The functional significance of this ALKBH3 moonlighting is unclear. Both cytoprotective and cytotoxic effects of tiRNAs are reported, suggesting divergent roles in stress response [38,186,189,190].

Cells have also evolved several mechanisms to sense and respond to chemically damaged mRNA. The main sensor is the ribosome, which stalls when encountering lesions that disrupt base-pairing, like 8-oxoG and N1-purine alkylations [191,192]. Ribosome stalling is obviously detrimental to proteostasis and triggers no-go decay (NGD) and ribosome-quality control (RQC) pathways to degrade the damaged mRNA and incomplete peptide product, respectively (reviewed in [193,194]). Evidence supports extensive cytoplasmic-to-nuclear communication from RNA degradation pathways to the transcription machinery, in which the 5′-3′-endoribonuclease Xrn1 acts as a signaling hub [195]. Interestingly, a role of Xrn1 has also been shown in the DDR by its ability to promote formation of RPA-coated ssDNA at DSB ends [196]. Although the mechanistic details remain elusive, this supports that cytoplasmic RNA-processing proteins contribute to DNA repair.

Finally, several proteins that bind 8-oxoG–containing mRNAs have been discovered. Among these, proteins that are able to bind a single 8-oxoG seem to promote degradation of the bound mRNAs, while PCBP1, which recognizes only heavily oxidized mRNA, induces apoptosis [197–199]. This represents a means of adjusting the cellular response according to the severity of the oxidative insult. Although a direct link to DNA repair remains to be established, this further substantiates the ability of cells to use RNA damage as genotoxic stress indicator.

12. Concluding remarks

A large body of experimental data now demonstrated that RNA and RNA-metabolizing proteins play crucial roles in several DNA repair pathways. Nevertheless, many mechanisms remain poorly understood. This especially holds true for the roles of damage-induced non-coding RNAs and RNA:DNA hybrids formed at DSBs and their dependence on genomic context and cell cycle stage. To unravel their molecular choreography may prove to be a daunting task but holds promise to open entirely new avenues that can be exploited in the treatment of human disease.

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