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# RNA at DNA Double-Strand Breaks: The Challenge of Dealing with DNA:RNA Hybrids

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**RNA polymerase II is recruited to DNA double-strand breaks (DSBs), transcribes the sequences that flank the break and produces a novel RNA type that has been termed damage-induced long non-coding RNA (dilncRNA). DilncRNAs can be processed into short, miRNA-like molecules or degraded by different ribonucleases. They can also form double-stranded RNAs or DNA:RNA hybrids. The DNA:RNA hybrids formed at DSBs contribute to the recruitment of repair factors during the early steps of homologous recombination (HR) and, in this way, contribute to the accuracy of the DNA repair. However, if not resolved, the DNA:RNA hybrids are highly mutagenic and prevent the recruitment of later HR factors. Here recent discoveries about the synthesis, processing, and degradation of dilncRNAs are revised. The focus is on RNA clearance, a necessary step for the successful repair of DSBs and the aim is to reconcile contradictory findings on the effects of dilncRNAs and DNA:RNA hybrids in HR.**

## 1. Introduction: The Complexity of DNA Double-Strand Break (DSB) Repair

DSBs can be caused by external agents, either physical or chemical, or arise as a result of improperly managed DNA single-strand breaks. Furthermore, some cellular processes such as topoisomerase-mediated reactions and meiotic recombination, require DSB formation as functional intermediates.[1,2] Regardless of the mechanism by which they are produced, unrepaired DSBs can threaten the integrity of the genome and lead to potentially tumorigenic chromosomal rearrangements.

Eukaryotic cells have evolved sophisticated DNA damage responses (DDRs) to detect and repair DSBs, and to inhibit the proliferation of cells that harbor unrepaired damage. Most DDR factors discovered throughout the many years of intensive research are proteins, and their functions are relatively well understood. More recently, however, studies in plants, yeast, and animal cells have revealed that RNA is also involved in DSB repair. Here, we will first introduce the current understanding of the basic DSB repair, and will thereafter discuss the experimental evidence that supports the existence of an RNA component in the DDR.

Several DNA repair pathways are able to sense DSBs and orchestrate the recruitment of the DNA repair machinery. The two major DSB repair pathways, homologous recombination (HR) and non-homologous end joining (NHEJ), use different repair strategies and engage different sets of proteins (**Figure 1**). The MRE11-RAD50-NBS1 (MRN) complex functions as a major DSB sensor and activates ATM, a phosphatidylinositol 3-kinase related kinase (PIKK) that plays a central role in the assembly of the DNA repair machinery. Among other substrates, ATM phosphorylates the H2AX histone variant, and the phosphorylated H2AX ($\gamma$H2AX) acts as a platform to recruit DNA repair factors and chromatin remodelers that in turn facilitate the accessibility of the DNA repair factors to the DSB.[3–5] The assembly of the repair machinery is a very rapid process (within minutes) and gives rise to the formation of large DDR foci that can be easily visualized by immunofluorescence microscopy using antibodies against $\gamma$H2AX or DNA repair factors.

HR involves a synapsis between the damaged DNA and an undamaged, homologous DNA molecule, usually a sister chromatid synthesized during S phase. The nucleotide sequence of the homologous DNA strand serves as a replication template to restore the damaged strand, which results in faithful repair reaction. HR is mostly active in the mid-S and G2 phases of the cell cycle when sister chromatids are available. However, DSBs in the rDNA can be repaired by HR throughout the entire cell cycle thanks to the availability of paralogous sequences

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The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/bies.201900225



**DOI: 10.1002/bies.201900225**

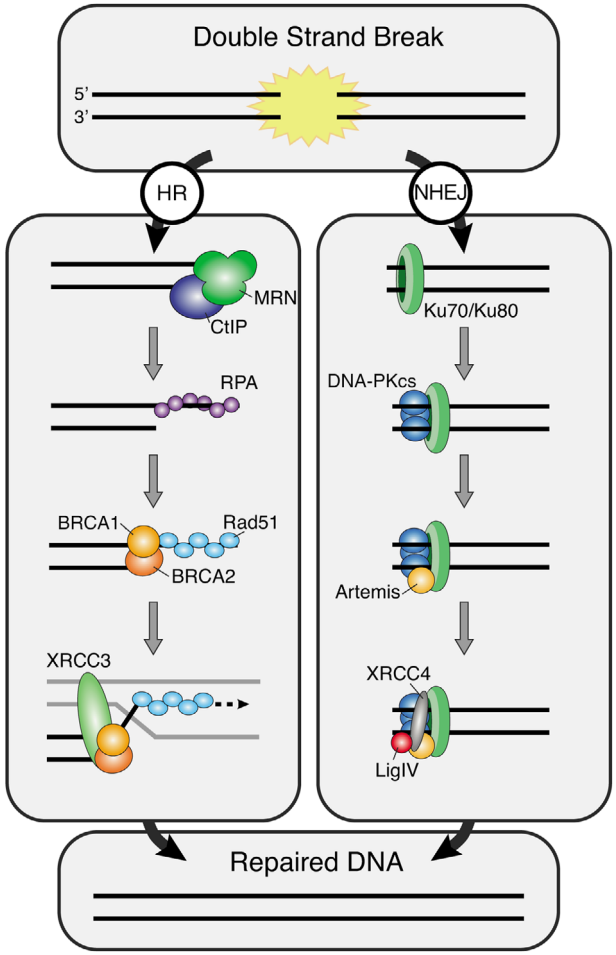


**Figure 1.** Homologous recombination and non-homologous end joining. The figure presents a simplified description of the two major DSB repair pathways. Left: In the HR pathway, CtIP and the MRN complex produce a nick in the DNA next to the DSB and initiate DNA end resection, which results in the formation of ssDNA tails at both sides of the break. The ssDNA tails are immediately coated with RPA and subsequently by RAD51 through a complex exchange process that involves additional DNA repair factors, including the tumor suppressors BRCA1 and BRCA2. RAD51 drives homology search and strand invasion, which eventually leads to the formation of Holliday junctions and to the resolution of recombination intermediates with the help of multiple repair proteins including the ATPase XRCC3. Right: In the NHEJ pathway, the Ku70/80 heterodimer senses the damage and induces the activation of DNA-PKcs. After minor processing of the damaged DNA ends by enzymes such as Artemis, the junction is eventually ligated by the XRCC4/DNA ligase IV complex.

in the repetitive rDNA locus.[6] Whether other types of repeats in metazoan genomes also use HR in a cell cycle independent manner remains to be investigated.

The NHEJ pathway is a fast DSB repair mechanism that does not require sequence homology, is error prone, and is active through the entire cell cycle. In the NHEJ pathway, the Ku70/Ku80 dimer acts as a DSB sensor and rapidly binds the broken DNA ends. Binding of Ku70/Ku80 to the DSB triggers the recruitment of another PIKK, DNA-PKcs, and facilitates the assembly of DNA processing enzymes that modify the DNA ends before the specialized DNA ligase complex XRCC4/DNA ligase IV catalyzes the ligation reaction and restores the continuity of the DNA (reviewed in refs. [7, 8]).

The choice between NHEJ and HR is influenced by multiple parameters, including the presence of the Ku70/Ku80 complex bound to the DNA ends, the stage of the cell cycle in which the damaged is produced[9] and the initiation of DNA end resection. DNA end resection is a process by which one of the DNA strands at each side of the DSB is degraded in the 5′ to 3′ direction, which prevents NHEJ and commits the DSB toward the HR pathway. In S and G2, the ssDNA endonuclease CtIP is phosphorylated by cyclin dependent kinases and is recruited to DSBs by the MRN complex.[10] CtIP and MRE11 produce a nick in the dsDNA that is used by the long-range exonucleases EXO1 or DNA2 to catalyze the resection reaction, which results in the formation of long ssDNA tracks that become rapidly coated by the abundant ssDNA-binding protein RPA.[11,12] RPA not only protects and stabilizes the ssDNA tracks, but also controls long-range resection.[13] RPA is subsequently replaced by the ATPase RAD51, a conserved exchange factor that directs homology searches and sister-chromatid strand invasion.[14]

## 2. De Novo RNA Synthesis at DSBs: An Unexpected Discovery

The study of DNA repair in transcribed chromatin has received particular attention and it is well documented that DSBs have an inhibitory effect on transcription at nearby promoters. The process of DSB-induced inhibition of transcription by RNA polymerase II (RNAPII) requires ATM and/or DNA-PKc[15–17] as well as the chromatin remodeler BRG1.[18] This transcriptional arrest is characterized by reduced levels of elongating RNAPII along the gene body as indicated by loss of phosphorylation of the RNAPII carboxy terminal domain at Serine 2 and Serine 5 (CTD Ser2/5P). The inhibitory effects of DSBs on genic transcription correlates with the distance between the DSB and the gene, and inhibitory effects have been observed as far as 1 Mb away from the break.

In contrast with the well documented inhibition of transcription reported above, recent studies have revealed that de novo transcription occurs at free DNA ends in a process that generates non-polyadenylated damage-induced long non-coding RNAs (dilncRNAs).[19–23] DilncRNAs were identified using strand specific RT-qPCR (ssRT-qPCR) and were postulated to be the precursors of damage-induced small RNAs (diRNAs).[19,20,22,24] Production of dilncRNAs is sensitive to RNAPII inhibitors, but not to inhibitors specific to other RNA polymerases. Their RNAPII dependency has been further supported by the observation of a DSB-dependent increase of RNAPII occupancy in a 4 kb window around break sites and a corresponding increase of transcripts mapping to sequences around the break.[16,25] Moreover, dilncRNAs have been detected in native RNA pull-down experiments after the induction of a sequence specific DSB.[25] Together, these results strongly imply that dilncRNAs are transcribed by RNAPII and are produced de novo after a DSB event. Investigations on how it is mechanistically possible to actively inhibit transcription at promoters and at the same time initiate transcription locally have just begun. Similar to promoter-associated transcription, DSB-dependent transcription is sensitive to the inhibition of the

general transcription factor TFIIH by Triptolide as well as inhibition of the CDK9 kinase by DRB and is accompanied by phosphorylation of the RNAPII CTD Ser2/5, which is associated with the elongating form of RNAPII.[22,24,25] Since the DSB-dependent inhibition of promoter-associated transcription is regulated at the initiation level[16] and DSBs are promoter-less, it is conceivable that DSB-induced transcription is initiated via a novel mechanism, while elongation follows a canonical process.

One possibility is that the transcriptional initiation is an unregulated event resulting from the suddenly accessible DNA ends generated at the break. The intrinsic affinity of RNAPII to free DNA is well documented and is suggested to cause pervasive transcription at nucleosome free regions such as DSBs.[16,21,26] However, there is also evidence for a regulated recruitment of RNAPII to the DSB. The RNAPII located at DSBs is characterized by a CTD phosphorylated at Tyrosine 1 (Y1P). This phosphorylation mark is placed by the tyrosine kinase c-Abl, which in turn is required for dilncRNA transcription.[25] Moreover, the MRN complex is involved in the assembly of a transcription pre-initiation complex at DSBs.[23] Despite these recent observations, the specifics of the initiation mechanism are not well understood. Interestingly, a detailed RNA analysis using deep sequencing revealed that dilncRNAs emerge only a few nucleotides away from the break site.[22,24] This finding argues against transcription being initiated by a canonical pre-initiation complex, which would place the polymerase upstream of the transcriptional start site and would leave an untranscribed sequence near the DSB.[27]

Whether DSB-dependent transcription is a general phenomenon and whether it is dependent of the chromatin context or transcriptional status at the DSB are still open questions. Transcription at DSBs close to transcriptionally active regions has been unambiguously demonstrated in a variety of experimental systems, but studies of transcription at intergenic or transcriptionally inactive DSBs are less unanimous. Single-gene studies and a genome wide transcriptional analysis of DSBs induced at AsiSI sites could not identify dilncRNAs originating from transcriptionally inactive regions.[28,29] However, case studies of specific, unique intergenic DSBs as well as an in vitro model of linearized transcriptionally inactive plasmids that mimics DSBs, could detect dilncRNAs even from silent loci.[22]

## 3. Double-Stranded RNAs are Formed at DSBs as a Result of Directional Transcription

DilncRNAs can participate in the formation of double-stranded RNA structures (dsRNAs).[22,25] Immunofluorescence experiments utilizing a dsRNA specific antibody revealed an increase of nuclear and cytoplasmic dsRNA in DNA damaged cells, and experiments that coupled RNA immunoprecipitation (RIP) to quantitative PCR confirmed the DSB-site specificity of the detected dsRNAs.[30] The formation of dsRNA requires the presence of not only a transcript directed away from the break site, but also a complementary RNA transcribed in the opposite direction (toward the break). There are two possibilities on how dsRNA could be generated at DSBs (**Figure 2**). In one scenario, dsRNAs could be the result of two separate de novo transcription events: one initiating at the break site and elongating away from the break, and a second simultaneous event initiating away from the break and transcribing the opposite DNA strand toward the break site (Figure 2a). Conflicting results have been reported on the existence of transcripts elongating toward the break. They have been identified by ssRT-qPCR in genic and intergenic regions as well as in CTD Y1P RIP experiments following DSB induction in genic regions,[22,25] but could not be confirmed in another study of sequence specific DSBs.[29] It is possible that transcription toward the break occurs, but that the resulting transcripts are short lived and their abundance is near the detection limit of standard RT-qPCR methods. On the other hand, dilncRNAs synthesized by RNAPIIs that initiate transcription at the DSB and elongate away from the break could anneal with an already existing transcript (Figure 2b). This mechanism would exclude the generation of DSB-induced dsRNAs from regions without pre-existing transcription, such as intergenic regions, or from sequences located downstream of the DSB where a pre-existing genic transcript would have identical sequence to that of the dilncRNA. Such a model is supported by the finding that diRNAs at I-PpoI induced DSBs in ribosomal DNA loci were only found between the promoter and the break site, but not further downstream of the DSB.[24]

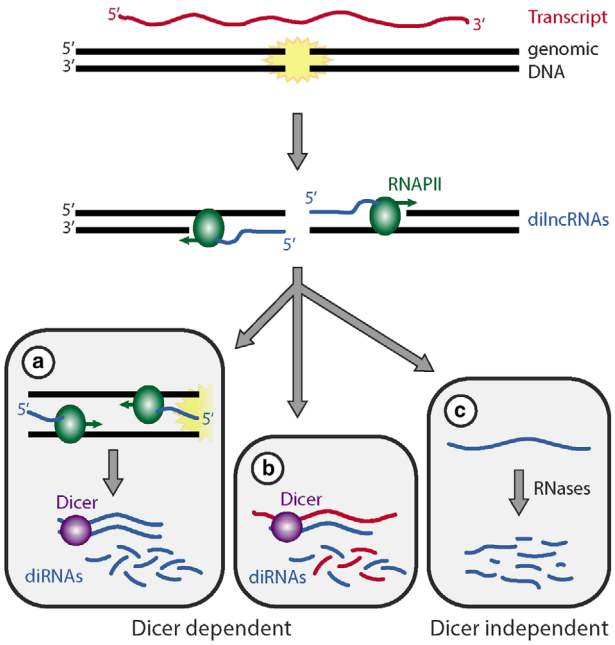


**Figure 2.** Biogenesis of Dicer-dependent and Dicer-independent diRNAs. RNAPII transcribes the free DNA ends exposed at the DSB and produces dilncRNAs that extend bidirectionally away from the break. Multiple pathways can be envisioned for the cleavage of dilncRNAs into diRNAs. a) RNAPII may also initiate a second transcription event elongating toward the DSB. The dilncRNA transcripts produced will be perfectly complementary and can form a dsRNA that is cleaved by Dicer into short diRNAs. b) The dilncRNAs can base-pair with a pre-existing, promoter-dependent transcript. The resulting dsRNA will also be a Dicer substrate. c) Other RNases acting on ssRNA can cleave dilncRNAs producing a heterogeneous population of diRNAs.

In summary, it is clear that dsRNAs are produced at DSBs, regardless of the mechanisms that are responsible for their formation. An important question that is still largely unresolved is the functional significance of these dsRNAs, as discussed below.

## 4. Processing and Degradation of DilncRNAs

The presence and functional importance of RNA-related proteins at DSBs has become well documented in recent years and has established a link between RNA processing and DNA repair.[31] Not surprisingly, dilncRNAs have multiple fates through the action of different ribonucleases and RNA processing enzymes. Some of these enzymes act to produce stable and potentially functional transcripts, as described below, while others catalyze the degradation of the dilncRNAs.

### 4.1. Processing of DilncRNAs into DiRNAs by Dicer

The micro RNA (miRNA) pathway is governed by two main RNase III enzymes, Drosha and Dicer, that cleave dsRNA substrates. It has been shown that a wide variety of Drosha and Dicer deficient cells display an impaired DDR response,[20,28,32–35] although the mechanisms behind this phenomenon are not well understood. Even though miRNAs do regulate cell cycle checkpoints and the abundance of some DDR effectors (reviewed in ref. [36]), the direct effects of Drosha or Dicer depletion on DSB repair seem to be largely independent of canonical miRNA biogenesis.[20,28]

One proposed mechanism by which DSB repair is dependent on Drosha and Dicer is via the processing of dilncRNAs into diRNAs. This model assumes that dilncRNAs form dsRNAs which are cut into 20–25 nt long diRNAs by Drosha and Dicer. These diRNAs are then incorporated into an Argonaute (Ago) containing complex and are able to act as guides to direct DDR effectors to the break site to facilitate chromatin changes and DSB repair.[19,20,32,37] This model is supported by a number of indirect observations. First, defects in DDR foci formation caused by RNase A treatment can be rescued by supplementing short RNAs extracted from irradiated wild-type cells but not from Dicer mutant cells.[20] Second, the 20–25 nt long diRNAs formed at sequence-specific DSBs in the rDNA locus in human and mouse cells were strongly reduced in Dicer null mutants,[24] which supports the role of Dicer in the biogenesis of at least a subset of diRNAs. Third, diRNAs can be incorporated into Ago complexes and Ago is enriched at DSB sites. Moreover, Ago can directly bind Rad51—an interaction required for the timely Rad51 enrichment at the DSB.[19,37,38] However, the role of Drosha in diRNA production has generated conflicting observation. Generally, Drosha requires a hairpin structure with flanking ssRNA overhangs in order to bind and cleave double stranded RNAs.[39] The precursors of diRNAs, however, are believed to be perfectly matching, long dsRNAs, and would therefore not be optimal Drosha substrates. Also, diRNA production at the rDNA locus was unaffected in Drosha null mutants.[24] Altogether, these observations suggest that dilncRNAs form dsRNAs that are cleaved by Dicer without Drosha involvement, and that the resulting diRNAs are incorporated into Ago-containing complexes.

Despite the solid evidence for de novo transcription at DSBs and diRNA biogenesis, the pervasiveness and functionality of diRNAs still meet strong reservation. In *Drosophila*, where diRNAs have been readily detected, it was demonstrated that neither Drosha nor Dicer are required for DSB repair.[38] In mammalian systems, the production of diRNAs at DSBs in unique sequences located outside of reporter constructs in naïve chromatin has not been demonstrated. Two large scale studies investigated diRNAs at DSBs induced by the sequence-specific endonucleases AsiSI or I-PpoI could not detect diRNA production at unique genic or intergenic DSBs.[24,28] Only DSBs in the highly repetitive and strongly transcribed ribosomal locus could produce measurable diRNA levels in mammalian cells.[24] Interestingly, diRNAs were first described and measured also in a highly transcribed and repetitive DSB model system in plants,[19] and it has been suggested that also in plants diRNAs production is a feature of highly expressed, repetitive loci.[40] In summary, there is strong experimental support for the production of diRNAs at DSBs in repetitive and transcribed sequences, but it has not been possible to demonstrate a diRNA response at unique loci. DilncRNAs have instead been consistently detected at DSBs produced in both unique and repetitive sequences.[22,24]

Even if diRNAs were not to play a role in the DDR, Drosha and Dicer might have non-canonical functions to aid in DSB repair. There is compelling evidence that Drosha depletion causes DNA repair defects[20,28] and it has been suggested that Drosha contributes to DSB repair by promoting the formation of DNA:RNA hybrids at DSBs, which in turn would have a positive effect on HR.[28]

The contribution of Dicer to DSB repair is also still unclear. Dicer is phosphorylated in response to DNA damage, which leads to its relocation into the nucleus.[30] It might regulate transcription termination at DSBs via its interaction with RNAPII[41] through a mechanism that could be similar to that described at sites of replication stress in highly transcribed genes in *S. pombe*.[42]

### 4.2. Multiple RNA Processing and Degradation Enzymes Relocate to Sites of DNA Damage

The presence of multiple RNases at sites of DNA damage suggests that ribonucleolysis is a common phenomenon at DSBs and predicts the existence of different types of RNA intermediates that result from the cleavage of damage-induced transcripts. Indeed, next generation sequencing experiments in genetically unperturbed human and mouse cells have revealed sequence specific short RNAs, distinct from the 20–25 nt long Dicer-dependent diRNAs, that appear in response to DSBs (Figure 2c). This more heterogeneous diRNA population probably represents dilncRNA cleavage products generated by different endo- and exoribonucleases.[24]

Many RNases and RNA processing enzymes are recruited to sites of DNA damage. The precise nature of their substrates and the specific mechanisms by which they contribute to the DDR are not fully understood. One of them is XRN2, a 5′ to 3′ exo-RNase that degrades nascent RNA downstream of the poly(A) cleavage site to promote RNAPII transcription termination.[43] XRN2 interacts with several DNA repair factors, is targeted to DSBs and its loss results in increased genomic instability.[44] Remarkably, several RNases detected at DSBs affect DNA end resection. For example, XRN1, another 5′ to 3′ exo-RNase involved in mRNA decay, promotes end resection in *S. cerevisiae*, probably by degrading RNAs in the vicinity of the DSB.[45] The pre-mRNA splicing

complex SF3B has also been found to play a role in end resection by regulating the expression of CtIP and controlling its recruitment to DSBs.[46] Another protein that relocates to DSBs is EDC4, a component of the mRNA decapping complex. EDC4 interacts with BRCA1, a tumor suppressor that regulates DNA repair by HR, and promotes end resection through a mechanism that is independent of its role in mRNA decapping.[47] The RNA exosome is also rapidly recruited to DSBs through a mechanism that requires the DNA:RNA helicase Senataxin (SETX).[48,49] Cells depleted of Exosome Component 10 (EXOSC10), one of the catalytic subunits of the exosome, show increased levels of dilncRNAs produced at sequence-specific DSBs, which suggests that EXOSC10 is involved in the degradation of damage-induced transcripts.[29]

## 5. DilncRNAs Produced at DSBs Engage in DNA:RNA Hybrid Formation

If not degraded by RNases, dilncRNAs can engage in DNA:RNA hybrid formation. DNA:RNA hybrids have been mostly studied in the context of R-loops and have been generally associated with genomic loci with high transcriptional activity. R-loops are nucleic acid structures composed of three strands that are generated when an RNA molecule displaces one of the DNA strands in a double-stranded DNA and forms a DNA:RNA hybrid. R-loops have been ascribed important physiological roles such as initiation of antisense transcription by RNAPII[50] and transcription termination through a mechanism that involves hybrid resolution by SETX.[51] However, R-loops can cause genome instability and, during transcription, RNA binding proteins and processing factors assemble with the nascent RNA and prevent R-loop formation.[44,52–55]

A variety of experimental strategies have been applied in recent years to study the occurrence of DNA:RNA hybrids at sites of DNA damage.[28,29,52,56,57] These studies support the conclusion that DNA lesions in active transcription units cause RNAPII arrest and promote R-loop formation, and DNA:RNA hybrids have been proposed to play a key role in promoting accurate DSB repair by contributing to the recruitment of DNA repair factors such as RAD52.[58] Interestingly, not only promoter-dependent transcripts but also dilncRNAs can engage in DNA:RNA hybrid formation. In S-phase and G2, when CtIP is phosphorylated and DNA end resection is favored, dilncRNAs can base-pair to resected ssDNA tracks and form DNA:RNA hybrids.[29,57] In fission yeast, the DNA:RNA hybrids have been shown to regulate DNA end resection and recruitment of the ssDNA-binding protein RPA.[59] In mammalian cells, they have been proposed to play an important role in promoting HR by directly recruiting BRCA1, a protein that regulates HR by binding RAD51 and enhancing its recombinase activity.[57,60]

In spite of the positive effects that DNA:RNA hybrids exert on HR repair, recent studies have also revealed the need of resolving DNA:RNA hybrids at DSBs,[29,56,57] which adds an additional level of complexity to the mechanistic relationships between dilncRNAs, DNA:RNA hybrids and DNA repair. Depletion of the exosome subunit EXOSC10 in human cells results in increased dilncRNAs and increased DNA-RNA hybrid levels at DSBs, prevents the recruitment of RPA to the damaged sites and inhibits

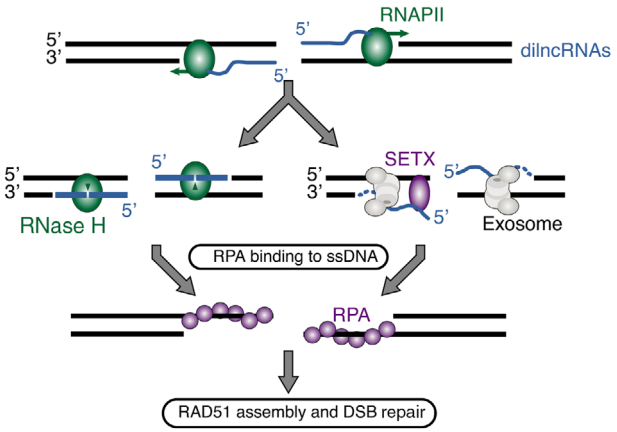


**Figure 3.** Resolution of DNA:RNA hybrids at DSBs. The DNA:RNA hybrids formed at DSBs are resolved through the action of different RNases that degrade dilncRNAs. RNase H degrades the dilncRNA moiety of the DNA:RNA hybrids. The exosome degrades the dilncRNAs released from DNA:RNA hybrids through the action of SETX. The exosome might also degrade single-stranded dilncRNAs and thereby prevent DNA:RNA hybrid formation. DilncRNA clearance renders the ssDNA accessible, which is a pre-requisite for the assembly of the HR machinery at the DSB.

HR repair. Interestingly, experimental overexpression of RNase H1 restores the RPA recruitment defect caused by EXOSC10 depletion, which shows that DNA:RNA hybrids counteract RPA-ssDNA assembly.[29] Altogether, these observations suggest that while the formation of DNA:RNA hybrids contributes to the assembly of the HR machinery during the early stages of the DDR, their stabilization has to be prevented to allow the subsequent recruitment of RPA. Moreover, these observations imply that the formation and resolution of DNA:RNA hybrids is tightly controlled.

## 6. Resolution of DNA:RNA Hybrids at DSBs

### 6.1. Degradation of DilncRNAs by RNase H

Different strategies contribute to the resolution of DNA:RNA hybrids formed at DSBs (**Figure 3**). One of them relies on the activity of RNase H enzymes, a family of endonucleases that hydrolyze the RNA moiety in DNA:RNA hybrids. Eukaryotic RNase H enzymes are classified into two main types, RNase H1 and RNase H2, that are differentially regulated during the cell cycle and differ in the range of substrates they act on.[61] Both types can resolve DNA:RNA hybrids in R-loops and, in addition, RNase H2 plays a specialized function in the cleavage of single ribonucleotides embedded in the genome.[62] RNase H enzymes have been directly linked to the degradation of DNA:RNA hybrids formed at DSBs and several observations support their functional contribution to DNA repair. First, RNase H2 associates with sites of DNA damage and can be co-immunoprecipitated with several DNA repair factors, including BRCA1 and BRCA2.[52,57] Second, depletion of RNase H2 results in increased DNA damage and increased persistence of DNA:RNA hybrids in the DNA.[63,64] Third, the DNA repair factor BRCA2 recruits RNase H2 to DSBs, and the impaired localization of RNase H2 to DSBs upon BRCA2

inactivation results in increased DNA:RNA levels at DSBs.[57] In summary, these observations show that RNase H2 plays an active role in HR repair by resolving DNA:RNA hybrids at DSBs.

### 6.2. Degradation of DilncRNAs by Senataxin and EXOSC10

DilncRNAs in DNA:RNA hybrids formed at DSBs can also be resolved by the exosome. Depletion of the exosome subunit EXOSC10 in human cells results in increased DNA:RNA hybrid levels at DSBs, prevents the recruitment of RPA and RAD51 to DSBs and inhibits DNA repair.[29] Interestingly, the catalytic activity of EXOSC10 is required for efficient DNA repair, and the RPA recruitment defect caused by EXOSC10 depletion can be rescued by experimental overexpression of RNase H1.[29] Altogether, these observations suggest that the RPA recruitment defect observed in EXOSC10-depleted cells is due to the presence of DNA:RNA hybrids at DSBs, and that these hybrids need to be degraded to allow the binding of RPA to the resected ssDNA, which in turn is needed for the assembly of the HR machinery.

The ability of EXOSC10 to degrade RNAs engaged in DNA:RNA hybrids requires the action of specialized RNA helicases. One such helicase is MTR4, a nuclear factor that is part of several exosome targeting complexes and facilitates the degradation of structured RNA substrates (reviewed in ref. [65]). At DSBs, the exosome collaborates primarily with the DNA:RNA helicase SETX. As described above, SETX is recruited to sites of DNA damage and is required to manage DNA:RNA hybrids at DSBs.[49,56] Depletion of either SETX or EXOSC10 result in DNA repair defects, which suggests that both activities are required for the resolution of hybrids at sites of DNA damage.

The involvement of the exosome in DNA repair has been also reported in insects and yeast. The RRP6 protein of *Drosophila melanogaster* is recruited sites of DNA damage and is needed for HR.[48] In *Saccharomyces cerevisiae*, RRP6 promotes the formation of RPA coated ssDNA.[45] These observations suggest that RNA clearance at sites of DNA damage is an evolutionarily conserved phenomenon.

Depletion of either RNase H, SETX or EXOSC10 results in increased levels of DNA:RNA hybrids and genomic instability, as described above,[29,49,54,63,64] which suggests that both RNase H and the SETX-exosome system are required to prevent the accumulation of DNA:RNA hybrids in the DNA. However, the reason why both nucleolytic systems are needed is not understood yet.

## 7. Conclusions and Outlook

The fact that transcription takes place at DSBs is now convincingly documented and accepted in the DNA repair field. However, the function of the RNAs produced at DSBs is still controversial. A general role for diRNAs in the early assembly of the DNA repair machinery is in conflict with the very fast DDR and with observations on the low abundance of diRNAs.[24,28] The dilncRNAs have instead been consistently detected and are regarded as functional RNAs.[22,29] An attractive hypothesis is that their function is to form DNA:RNA hybrids that act as a platform to recruit HR factors.[57] On the other hand, DNA:RNA hybrids have to be resolved to allow the subsequent assembly of RPA onto the ssDNA.[29] DNA:RNA hybrid resolution is achieved through the action of at least two dilncRNA clearance systems: RNase H2 and the exosome. Interestingly, both systems are required for efficient DSB repair, which indicates that their functions are not redundant.

Another interesting possibility is that DSB-induced transcription is functional in itself and contributes to HR by promoting DNA end resection, as initially shown in fission yeast.[59] However, a similar relationship between transcription and DNA resection has not been established in mammalian cells, where different studies have provided conflicting observations.[29,57]

Many central questions concerning DSB-induced transcription are still pending. One of them is understanding the timing of dilncRNA biogenesis, DNA:RNA hybrid formation and dilncRNA clearance. Another is how cells regulate whether dilncRNAs engage in DNA:RNA hybrid formation, are processed into diRNAs or are targeted for degradation. A third relevant issue is whether dilncRNAs are general or restricted to transcriptionally active chromatin. Transcribed genes are preferentially repaired by HR[56,66] and RNA-related factors are linked to HR (reviewed in ref. [31]). It is thus tempting to speculate that DSB-induced transcription and DNA:RNA hybrid formation occur at DSBs in transcriptionally active genes and constitute a mechanism of repair pathway choice. Such a mechanism would promote HR and avoid mutagenic repair in genic sequences.

## Acknowledgements

This work was supported by grants from The Swedish Research Council (grant 2015-04553 to N.V.) and The Swedish Cancer Society (grant CAN 2016/460 to N.V.). J.D.P. was supported by the Department of Molecular Biosciences, the Wenner-Gren Institute at Stockholm University. F.B. was supported by SFO funding from the Faculty of Science at Stockholm University.

## Conflict of Interest

The authors declare no conflict of interest.

## Keywords

DNA repair, EXOSC10, exosome, homologous recombination, RNA clearance, RNAse H, transcription

Received: November 17, 2019
Revised: February 2, 2020
Published online:

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