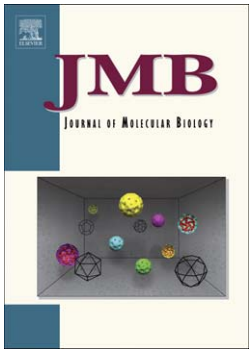


# Transcription and DNA damage: holding hands or crossing swords?

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# Transcription and DNA damage: holding hands or crossing swords?

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## Highlights

- Transcription is a potential source of genome instability.
- DNA double-strand breaks (DSBs) in actively transcribed genes are repaired faster and preferentially via homologous recombination.
- RNA binding proteins and transcription factors are recruited to DSBs.
- sncRNAs are generated in proximity of DSBs in several organisms (DDRNAs and diRNAs).
- DDRNAs and diRNAs participate in DDR signaling and repair via HR, respectively.

## Abstract

Transcription has classically been considered a potential threat to genome integrity. Collision between transcription and DNA replication machinery, and retention of DNA:RNA hybrids, may result in genome instability. On the other hand, it has been proposed that active genes repair faster and preferentially via homologous recombination. Moreover, while canonical transcription is inhibited in the proximity of DNA double-strand breaks (DSBs), a growing body of evidence supports active non-canonical transcription at DNA damage sites. Small non-coding RNAs (sncRNAs) accumulate at DSB sites in mammals and several other organisms and are involved in DNA damage signaling and repair. Furthermore, RNA binding proteins (RBPs) are recruited to DNA damage sites and participate in the DNA damage response. Here, we discuss the impact of transcription on genome stability, the role of RBPs at DNA damage sites, and the function of sncRNAs generated upon damage in the signaling and repair of DNA lesions.

## Introduction

The stability of our genome is continuously challenged by endogenous and exogenous factors [1]. A DNA lesion activates a cellular response, known as DNA damage response (DDR), that leads to the recruitment of repair proteins to sites of DNA damage and to the activation of checkpoint responses that slow down, or arrest, cell-cycle progression, until repair in fully carried out [2; 3]. Among different kinds of lesions, DNA double-strand breaks (DSBs) are recognized by the MRE11-RAD50-NBS1 (MRN) complex, which recruits the ataxia telangiectasia mutated (ATM) protein kinase, responsible for the phosphorylation of the histone variant H2AX (γH2AX). The consequent spreading of γH2AX along the chromosome [4] and the recruitment of additional DDR factors, such as the mediator of DNA-damage checkpoint (MDC1), and the p53-binding protein (53BP1), generate a microscopically detectable focus [2]. The repair of DSBs mainly relies on either non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ simply stitches back together the broken DNA ends, and functions throughout all cell cycle phases. During the S/G2 phase, resection of broken DNA ends commits repair toward additional mechanisms based on HR [5]. Upon DNA end resection, replication protein A (RPA)-coated ssDNA activates ataxia telangiectasia and Rad3-related protein (ATR)-dependent signaling and checkpoint kinase 1 (Chk1) phosphorylation, by recruiting ATR and ATR interacting protein (ATRIP) [6; 7]. Resected DNA ends can invade the homologous sequence from the undamaged sister or homologous chromatid through the RAD51 recombinase, resulting in error-free repair. Alternatively, the exposure of homologous sequences on the resected DNA ends may result in the error-prone DSB repair pathways known as alternative end joining (alt-EJ) and single-strand annealing (SSA) [8].

A new component has recently been integrated into the classical DDR cascade: the RNA. Upon damage, small non-coding RNAs (sncRNAs), named DNA damage response RNAs (DDRNAs) and DSB-induced RNA (diRNA), accumulate near DSBs in several organisms [9; 10; 11]. DDRNAs have been discovered in mammals, where they are generated by DROSHA and DICER cleavage of a presumably longer precursor RNA, and participate in DDR signaling [9]. diRNAs have been

discovered in plants where they are produced by DICER-mediated cleavage in an ATR-dependent fashion. Differently from DDRNAs, diRNAs have been proposed to participate in DNA repair and not in DDR activation [11; 12; 13]. The production of sncRNAs upon DSBs generation suggests the transcription of a longer precursor RNA that undergoes further processing. Conversely, however, upon DNA damage, inhibition of canonical transcription has been observed in yeast and mammals [14; 15; 16; 17; 18]. Moreover, a role of transcription as a source of DNA damage has also been extensively described [19]. In this review, the detrimental and beneficial effects of transcription on genome stability, the recruitment of RNA binding proteins (RBPs) to DNA damage sites, and the role of sncRNAs generated from DNA lesions are discussed.

**The dual role of transcription at DNA damage sites**

Transcription may be a harmful process for DNA integrity [20]. Collision between the transcription and replication machineries causes replication-fork stalling, which is often associated with DNA damage and recombination [21; 22]. During transcription, the pairing of the newly synthetized RNA with the template DNA generates a DNA:RNA hybrid which displaces the non-template ssDNA to form a three-stranded nucleic acid structure known as R loop [23] (Figure 1a). Short DNA:RNA hybrids form physiologically during DNA replication [19], during transcription, and have a role in transcription regulation [24; 25]. Unscheduled R loops formation may be facilitated by several factors. The negative DNA supercoiling associated with transcription favors DNA unwinding and increases DNA accessibly to RNA, with the consequent formation of DNA:RNA hybrids [26]. Negative supercoiling is counteracted by the action of the DNA topoisomerases; indeed, high levels of DNA:RNA hybrids are found in topoisomerase mutants in bacteria, yeast and human cells [27; 28; 29]. Moreover, high G-content [30], collision between transcription and replication machinery [31], and pausing of RNA Polymerase II (RNA Pol II) due to the presence of extended trinucleotide repeats [32; 33; 34] also promote unscheduled R loops formation.

Transcription-dependent R loops have been extensively reported as a source of genome instability

[19]. The stimulatory effect of transcription on recombination-mediated DNA repair is well known from yeast [35] to mammalian cells [36; 37]. Highly transcribed regions are characterized by increased mutagenesis and recombination rate, respectively known as transcriptional-associated mutation (TAM), and transcription-associated recombination (TAR) phenotypes [38]. The observation that TAR depends on DNA replication [39] supports the idea that R loop-mediated genome instability may arise from replication fork stalling [28; 40; 41]. This notion has been further strengthened by the recent observation that DNA damage associated with R loops formation upon estrogen stimulation requires cells to undergo DNA replication [42]. Moreover, the ssDNA displaced upon DNA:RNA hybrid formation is exposed to several assaults that contribute to genome instability [38], like the spontaneous, or activation-induced, deaminase (AID)-dependent cytosine to thymidine deamination [43; 44] (Figure 1a).

Tight regulation of R loops formation is imperative to avoid the detrimental effects of their accumulation. Coating of the nascent RNA by RBPs prevents the annealing of RNA to DNA and the consequent R loops formation. For example, R loop accumulation is observed in cells depleted for the the splicing factor SRSF1 [45], as well as the THO/TREX-2 complexes, which mediates the proper packaging of newly synthetized RNA with RBPs [46; 47]. Once formed, DNA:RNA hybrids are removed by RNase H enzymes [48; 49] or by helicases like Senataxin [50; 51; 52] and Aquarius [53].

In human and yeast cells genome instability, monitored as γH2AX foci, YAC minichromosome instability, or Rad52 foci, is observed in cells deficient for several RBPs, as well as factors involved in RNA biogenesis, processing, and degradation. The decreased genome instability observed in those mutants upon RNase H overexpression indicates the direct contribution of DNA:RNA hybrids to DNA damage generation [49; 54; 55]. Recently, accumulation of DNA:RNA hybrids and genome instability in RNA processing mutants (defective for RNA elongation (*leo1Δ*), degradation (*kem1*Δ and *rrp6*Δ), and transcriptional repression (*med12Δ* and *sin3*Δ)), has been attributed to the recombination protein Rad51 [56], introducing a new and unexpected role for a protein usually considered a "guardian" of genome stability [57]. Interestingly, a role for RecA, the bacterial

orthologue of Rad51, in promoting DNA:RNA hybrids formation *in vitro* has already been reported in the past [58; 59].

Additional roles for R loops in several physiological processes have also emerged in the last few years [60]. An example is the involvement of R loops in immunoglobulin gene class-switch recombination [61]. Moreover, recently, a high-resolution strand specific profiling of R loops in mammalian cells has revealed R loops accumulation at gene promoters and terminators, corresponding, respectively, to open and close chromatin structures [62]. R loops were already observed at CpG islands promoters [63; 64], and the connection between R loops and open chromatin structure is further supported by their reported role in favoring a hyper-acetylated chromatin state [65]. Furthermore, R-loops accumulation at gene terminators [62; 64] is in line with the finding that R loop-induced antisense transcription facilitates the deposition of repressive chromatin marks at gene terminators [66]. The observation that R loops form throughout the genome in physiological conditions [62] supports their active role in several cellular processes and has challenged the classical view of R loops as undesired threats to genome instability.

The link between transcription and genome stability is, however, more complicated than described here so far. Actively transcribed genes are not only DNA damage hot-spots [38], but they are also repaired faster than non-transcribed genes [67; 68]. However, only the transcribed strand of the active genes is repaired with a faster kinetic, excluding increased accessibility of transcribed regions as a potential cause of this phenomenon, and instead suggesting a more specific mechanism [69]. Indeed, a specific mechanism exists in which lesions that block RNA Pol II translocation in the template strand of actively transcribed genes are removed by Transcription Coupled Repair (TCR), a specialized Nucleotide Excision Repair (NER) pathway well conserved in prokaryotes and eukaryotes [70]. Interestingly, also repair of DSBs is faster in actively transcribed genes compared to inactive genes, suggesting a role for transcription in the repair of this type of lesions [71]. Another interesting feature of DSBs located in actively transcribed genes is their preferential repair by the HR machinery [72]. Using a ChIP-Seq approach, Legube's group has recently classified

endonuclease-induced DSBs as HR- or NHEJ-prone, respectively, on the basis of either RAD51 or XRCC4 enrichment at the breaks [72]. Interestingly, the HR-prone DSBs are preferentially located in transcriptionally active chromatin, characterized by H3K36me3 or H3K9Ac chromatin marks and enrichment of the elongating form of RNA Pol II. According to the authors, the H3K36me3 chromatin modification is recognized by LEDGF (p75), a chromatin-binding protein already shown to facilitate DNA end resection, thus committing repair to HR [73]. Additionally, transcriptionally active chromatin may recruit the bromodomain containing protein ZMYND8 via TIP60-induced H4 acetylation. In turn, ZMYND8-mediated recruitment of the NurD complex could mediate transcriptional silencing and HR mediated repair [74]. In both scenarios, HR-mediated repair is promoted by the chromatin modifications associated with active transcription (already present before DNA damage induction). A role for RNA molecules in this process has not been demonstrated, as instead discussed later. However, it is worth mentioning that a recent paper has shown no preferential recruitment of HR proteins to transcriptionally active loci [75]. This observation is in line with studies showing that also pre-existing transcriptionally silent chromatin favors the recruitment of HR proteins [76]. Taken together, these contrasting observations may simply be two different mechanisms used by different chromatin regions (euchromatin versus heterochromatin) to recruit HR factors.

**DNA damage-induced transcriptional silencing**

Cell homeostasis can be altered by the expression of aberrant transcripts from actively transcribing damaged loci. For this reason, transcriptional silencing of damaged loci is a phenomenon well known and conserved. In Neurospora crassa, the expression of aberrant transcripts from a damaged sequence is suppressed by small RNAs generated from the damaged site, in a process known as the endo-siRNA response [10]. In mammals, physical blockage and consequent proteasome degradation of RNA Pol II upon UV and other bulky adduct-induced DNA damage [77; 78] is responsible for a strong decrease in RNA Pol II occupancy at actively transcribing damaged genomic loci. Similarly,

UV laser microirradiation causes an overall exclusion of the active form of RNA Pol II, accompanied by a drop in transcription [79; 80; 81; 82].

The study of the impact of DSBs on transcription has led to several, not always consistent, conclusions. A DSB occurring within a gene body leads to the inhibition of its transcription, but not of the transcription of adjacent genes, by a mechanism mediated by DNA-PK which results in the exclusion of RNA Pol II from the gene body and its promoter [18]. Interestingly, if DNA-PK activity is inhibited, RNA Pol II is no longer excluded, and seems to bypass the lesion [18]. A transient DDR-dependent decrease of transcription of damaged genes, and not of neighboring ones, has also been observed in a mice model expressing the homing nuclease I-PpoI [83]. Differently, it has been reported that when multiple DSBs are induced upstream of a reporter gene, transcriptional silencing relies on the spread of chromatin condensation for several kilobases [17]. DNA damage-induced chromatin condensation is ATM-dependent and is associated with the ubiquitination of histones H2A and H2AX by the E3 Ubiquitin Ligase Ring Finger Proteins RNF8, and RNF168. H2A ubiquitination can additionally be favored by recruitment of the Polycomb Repressive Complex 1 (PRC1), through ATM-dependent phosphorylation of either the transcriptional elongation factor ENL [84] or the chromatin remodeling complex Polybromo BRG1 (Brahma Related Gene 1) Associated Factor (PBAF) [85]. The link between ubiquitination and transcriptional repression is consistent with the observed reduced *de novo* mRNA synthesis in ubiquitin-enriched chromatin domains resulting from spontaneous DNA lesions [86]. Repression of canonical transcription *in cis* to DNA damage is also supported by the recruitment of repressive chromatin complexes to DNA damage sites, like PBAF, Polycomb, and the Nucleosome Remodeling Deacetylase NuRD complex [79; 85]. Differently from mammalian cells, in Saccharomyces cerevisiae transcriptional silencing *in cis* to DSBs [14] is not mediated by the phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKKs) activity, but instead relies on nucleolytic resection of both the template and the non-template sequence [15].

ATM-dependent transcription inhibition is not only restricted to RNA Pol II, but is also observed

for RNA Pol I mediated transcription of rDNA upon nucleolar DSBs generation [16; 87]. Interestingly, ATM-dependent silencing of RNA Pol I transcription has been described not only upon nucleolar DSBs, but also when DNA damage does not fall inside the nucleoli [88]. This *in trans* silencing of Pol I transcription associates with NBS1 relocation to nucleoli, an event that is controlled by the ATM-dependent interaction of NBS1 with Treacle-TOCF1 [88; 89].

In addition to the above-described DSB-induced transcriptional repression of nearby actively transcribing genes, active transcription of non-coding RNAs from broken DNA ends [9; 10; 11] (Figure 1b), together with RBPs and transcription factors recruitment [90], has been observed. Even if seemingly in contrast, transcriptional activation and transcriptional repression upon DNA damage may rather be two intimately connected events. Upon DSB generation, chromatin undergoes profound changes in order to allow the signaling and repair of the DNA lesions. It has been proposed that upon DNA damage a first chromatin condensation phase is followed by prolonged relaxation and recruitment of DNA repair factors [91; 92]. However, other reports suggest that a first PARP-dependent wave of chromatin modifications creates an open and easily accessible chromatin structure, and is followed by an ATM-dependent condensation phase required for further amplification of DDR signaling [93; 94]. It is intriguing to speculate that the PARP-dependent chromatin relaxation, crucial for the accessibility of DDR sensors at DNA damage sites, may facilitate the recruitment of transcription factors, either due to their affinity for open DNA structures or to a more regulated mechanism. One possibility is that PARylation itself not only favors chromatin relaxation, but also facilitates the recruitment of transcription factors to DNA ends. This, in turn, might enhance transcription by RNA Pol II, an intriguing hypothesis, consistent with the already described affinity of RNA Pol II for DNA ends [95; 96]. Small RNA transcripts generated from broken DNA ends would favor DDR activation [9] (Figure 1b) that, in turn, would contribute to ATM-dependent RNA Pol II exclusion and/or chromatin condensation resulting in DNA damage-induced transcriptional silencing. However, while transcription factors recruitment to DNA lesions has already been shown to be PARP-dependent [90], no data describing the effect of PARP inhibition

on damage-induced sncRNAs production are yet available. A similar scenario is observed at the pericentromeric locus in Schizosaccharomyces pombe, where small RNAs generated by Dicer contribute to heterocromatin gene silencing [97; 98].

**Transcription factors and RNA binding proteins recruitment to DNA lesions**

An unexpected number of RBPs and transcription factors have been reported to localize to DNA damage sites [90]. While the role of transcription factors at DNA lesions has not been fully addressed, several experimental results support a direct role for RBPs in the DNA damage response [99]. Indeed, unsurprisingly, RNA metabolism has emerged as one of the most enriched categories in proteomic and functional DDR screens [54; 100; 101; 102]. Proteins involved in several aspects of RNA metabolism, such as transcription termination, splicing, and degradation, have been reported as ATM or ATR substrates [100] and their depletion have been linked to DNA damage, as monitored by γH2AX foci [54]. In several cases the involvement of RBPs in DDR is not direct and may be explained by their role in modulating the expression of DDR signaling and repair factors. This is the case, for example, of hnRNPC and hnRNPG (RBMX), that modulate HR by facilitating the expression of key HR factors [103; 104]. However, RBPs may also directly contribute to DDR signaling and DNA repair in several ways, such as modulation of the chromatin landscape, or direct interaction with DNA repair proteins. The first mechanism has been reported for the chromatin associated scaffold attachment factor B1 (SAFB1), an RBPs that is transiently recruited to DNA lesions where it facilitates the formation of a a permissive chromatin structure, that in turn promotes DNA damage signaling [105]. Similarly, the splicing regulator FUS localizes to DNA lesions [106; 107; 108], interacts with the chromatin modifiers HDAC1 [109], and seems to contribute to repair by both HR and NHEJ [106]. Interestingly, amyotrophic lateral sclerosis (ALS) patients harboring familial mutations in the FUS gene have increased genome instability and express FUS variants defective for DDR and DNA repair that also interact less efficiently with HDAC1, underlining a possible role of FUS mediated chromatin changes in DDR modulation [109].

Another mechanism by which RBPs may contribute to DDR signaling and DNA repair is their direct interaction with DNA damage proteins. This is the case of the splicing factor SFPQ/PSF which is rapidly and transiently recruited to DNA lesions [110; 111] where it interacts with RAD51D, one of the RAD51 paralogs with a role in HR, and is necessary for HR [112]. The role of PSF in HR has been further supported by *in vitro* studies [113; 114], although an involvement of PSF in NHEJ has also been proposed when in a complex with p54(nrb) [115]. Similarly, the exosome component EXOSC10 (and its orthologue RRP6 in Drosophila), an RNA surveillance factor usually involved in RNA processing and degradation, has been shown to interact with RAD51 and facilitate repair by HR[116]. Another example is PRP19, an E3 ubiquitin ligase with a role in splicing regulation, recently identified as an interactor of RPA coated ssDNA [117]. Upon DNA damage, PRP19 co-localizes with RPA within laser-induced γH2AX stripes where it ubiquitylates RPA and favors the accumulation of the ATR-ATRIP complex, further amplifying DDR signaling. Similarly, the PRP19 partner CDC5L is recruited to DNA lesions via RPA [117], interacts with ATR and participates in ATR-mediated checkpoint activation [118]. The ability to modulate DNA end resection has also been described for hnRNPUL1/2 (heterogeneous nuclear ribonucleoprotein U-like), hnRNPs (heterogeneous nuclear ribonucleoproteins) involved in several aspects of RNA maturation, from alternative splicing to mRNA stabilization and transcriptional regulation [119]. In particular, hnRNPUL1/2 localize to DNA lesions in an MRN-dependent fashion, promote DNA end resection by recruiting the helicase BLM, and contribute to the ATR-dependent signaling and DSB repair by HR [80]. In another report, a PARylation-dependent recruitment of hnRNPUL1 has also been reported [120]. DNA damage-dependent PARP1-mediated PARylation is one of the earliest events in DDR. It facilitates the initial recruitment of DDR proteins to DNA lesions and orchestrates a wave of chromatin remodeling events that first generate open chromatin, easily accessible to DDR proteins, and then resets the chromatin structure to the initial state [3]. The localization of several transcription factors and RBPs to DNA lesions relies on PARylation. For example, this is the case of FUS, TAF15 and EWS [106; 107; 108], the splicing factors NONO [121], SAF-A/hnRNPU [107], RBMX [103], and

SAFB1 [105]. Generally, RBPs interaction with PAR chains is mediated by their RNA binding motifs [80; 106; 121], suggesting a possible competition between the RNA- and the DNA damage-related function, although this is presently unclear. Once recruited to DNA lesions, RBPs may modulate DNA damage signaling and repair through the above-described functions, such as reorganization of chromatin structure and interaction with DNA repair proteins. However, although speculative, it cannot be excluded that RBPs recruitment to DNA lesions contributes to the biogenesis and processing of RNA, such as damage-induced sncRNAs.

The early and rapid PARP-dependent recruitment of RBPs to DNA lesions is generally followed by their ATM-, ATR- or DNA-PK-dependent exclusion [107]. For example, the exclusion of the RNA processing factor THRAP3 from laser stripes is mediated by all the three PIKKs (ATM, ATR and DNA-PK) and reflects the drop in local transcription level, as monitored by the incorporation of the nucleoside analogue 5-ethynyl uridine (EU), and loss of the active form of RNA Pol II [82]. Interestingly, inhibition of H2A ubiquitination, already known to rescue transcription upon DNA damage [17], also abrogates THRAP3 exclusion, further supporting a link between transcription and RBPs recruitment to DNA damage sites [82]. The PIKKs-dependent exclusion of RBPs from DNA lesions fits well with the previously proposed scenario in which PARP-dependent chromatin relaxation favors transcription of sncRNA from broken DNA ends, thus favoring DDR activation, in turn followed by transcriptional silencing and the concomitant exclusion of RBPs from the damaged loci.

**DNA damage-induced sncRNAs: from gene silencing to DNA damage signaling and repair**

The generation of small ncRNAs upon damage has been well documented in the last few years in several organisms [9; 10; 11].

In Drosophila cells, sncRNAs are generated upon transfection of a linearized plasmid only when the linearization interrupts a transcribed region [10]. These small RNAs contain the sequence of the plasmid DNA ends and may act as siRNA in post-translational silencing of transcripts generated

nearby the lesion, a process known as endo-siRNA response. The use of RNA molecules like siRNA in gene silencing is referred as RNA interference (RNAi) and is a well-known and evolutionary-conserved mechanism [122]. siRNAs are generated by the cleavage of a double-stranded precursor transcript by Dicer proteins and are subsequently loaded on Argonaute proteins in the RNA-Induced Silencing Complex (RISC) to inhibit the expression of target transcripts. A similar function, but different biogenesis, characterizes another class of sncRNAs, known as microRNAs (miRNAs). In this case, the dsRNA substrate for Dicer proteins is generated by the prior processing of a hairpin precursor by the Drosha complex. The miRNAs generated by Drosha and Dicer proteins are then loaded in the RISC complex and generally inhibit mRNAs translation through the action of GW family proteins. In Drosophila, the siRNA and miRNA biogenesis pathways do not share the same components. Reflecting the role of siRNA in gene silencing, the endo-siRNA response in Drosophila is coupled with a reduced expression of the gene interrupted by a DSB in the plasmid expressing it and it is dependent on siRNA biogenesis factors [10]. Moreover, no effect on HR-mediated repair of the DNA lesions is observed in Drosophila cells defective for siRNA biogenesis [123]. Depletion of miRNA factors only impairs a specific subtype of HR-mediated repair, the SSA, and is probably due to the accompanying cell cycle alterations [123].

Similarly to the endo-siRNA response in Drosophila, Quelling is a mechanism used by Neurospora crassa to inhibit the expression of genes with a sequence homologous to multiple transgene copies [124]. The transcript encoded by the transgene is converted to dsRNA by an RNA-dependent RNA Polymerase and is cleaved by Dicer-like proteins to generate siRNAs that are loaded on the RISC complex. Recently, DNA damage-dependent induction of the Argonaute protein QDE-2 and small RNA named qiRNAs (QDE-2 interacting RNAs) has been demonstrated [125; 126; 127]. The biogenesis of qiRNAs requires components of the Quelling pathway and HR proteins, leading to the hypothesis that HR mediates the recognition of repetitive DNA and initiates the transcription of qiRNA precursors upon DNA damage [128; 129]. Similarly to Quelling-mediated gene silencing, qiRNAs, which are mainly transcribed from ribosomal DNA, inhibit ribosomal biogenesis and protein

translation upon damage [125].

In mammalian cells, sncRNAs with the sequence of the damaged locus, known as DDRNAs, are generated upon DSB induction [9]. Differently from DSB-induced siRNA or qiRNA generation, in Drosophila and in Neurospora crassa, respectively, DDRNAs production in mammals does not require preexisting transcription, and is observed also when DNA damage does not fall in a transcriptionally active unit [9]. DDRNAs are processed (most likely from a longer precursor) by DROSHA and DICER, two components of the RNA interference pathway (RNAi). According to the canonical RNAi view, DROSHA and DICER mediate the processing of long precursor RNAs, in the nucleus and in the cytoplasm, respectively, to generate miRNAs that inhibit mRNAs translation through GW-182 proteins. DDRNAs are clearly different from miRNA, as their function is independent of GW-182 proteins [9]. Presently, it is still unclear where DDRNAs processing takes place. While some reports support an exclusively cytoplasmic localization of DICER [130; 131; 132], a large body of evidence suggests multiple roles for and the presence of DICER in the nucleus [66; 133; 134; 135; 136; 137; 138; 139; 140; 141]. This suggests the possibility that DDRNAs are directly processed at DNA damage sites, where they are needed for DDR activation and DDR foci formation [142] (Figure 2b). The role of DICER in genome stability maintenance has been further supported by its reported role in facilitating the release of RNA Pol II from regions prone to transcription-replication collisions, such as highly transcribed loci, thus avoiding DNA damage accumulation [143].

Impairment of DDRNAs biogenesis by DROSHA and DICER depletion strongly compromises DDR activation, as monitored by 53BP1, pATM and MDC1 foci formation, checkpoints activation, and maintenance of oncogene-induced cellular senescence [9]. The effect of DICER depletion on pATM foci formation has been supported by other reports [85], while the effect on 53BP1 foci formation, being restricted to the earliest time points (less than 10 minutes) after irradiation [9], was missed when analyzed at later times [12]. Moreover, RNA depletion by RNAse A treatment coupled with transcriptional inhibition leads to the disassembly of DDR foci (53BP1 and MDC1 among others) that reform upon transcription reactivation or addition of small RNAs carrying the sequence

of the damage locus, indicating a direct role for RNA in DDR [9]. Consistently, MDC1 has been recently identified as an RBP in a nuclear RNA interactome study [144], and MOF, a chromatin modifier important for MDC1 recruitment to DNA lesions, has been reported to bind to ncRNAs [145].

During DDR activation, the direct recognition of DNA ends by DDR sensor proteins, such as NBS1, is known as "primary recruitment" [146] and leads to the phosphorylation of the histone variant H2AX (γH2AX). This, in turn, prompts the "secondary recruitment" of the DDR mediators MDC1 and 53BP1 [147; 148; 149], that further amplify the DDR signaling and spread for several kilobases generating cytologically detectable foci [150]. Recently, a role for DDRNAs in the secondary recruitment of DDR factors has been reported [151]. Indeed, DROSHA or DICER depletion, as well as RNase A treatment, does not affect the recruitment of the DDR sensor NBS1 to DNA lesions, while impairing 53BP1 and MDC1 recruitment [9].

While the generation of damage-induced sncRNAs has not been reported yet at endogenous loci, an essential function of DICER and the DROSHA partner DCGR8 in DNA damage resolution has been shown in the rapidly proliferating cells (embryonic stem cells and in the developing cerebellum of DICER knock-out mice), that are known to accumulate high level of endogenous DNA damage [152].

Generation of sncRNAs from the sequence flanking a DSB has been also reported in plants [11]. diRNAs production in Arabidopsis thaliana requires the classical small RNA pathway factors: Dicer-like proteins (DCL), DNA-dependent RNA Pol IV (Pol IV), and RNA-dependent RNA Pol (RDR2 and RDR6), as well as the PI3 kinase ATR. Upon DNA damage, a precursor transcript generated by RNA Pol IV is presumably converted by RNA-dependent RNA Pol (RdRP) into small dsRNA that are involved in DNA repair [11]. Although a comparative analysis of DDRNAs and diRNAs is not yet available, these sncRNAs, although sharing some common features, differ for others. In particular, DDRNAs have been discovered in mammals and are produced by DROSHA and DICER most likely from a precursor RNA generated from the broken DNA ends. The fact that

they are DICER products is further supported by the observation that they have a nucleotide bias at their ends [9]. Differently from DDRNAs, diRNAs appear to be produced from the vicinity of the DSB, but not immediately around it. Moreover, diRNAs biogenesis is ATR dependent, suggesting that their production requires DNA resection or the presence of single strand DNA [11]. Furthermore, diRNAs do not seem to participate in DDR activation but only in HR-mediated repair of DNA lesions [11; 12; 13]. No impact of diRNAs on NHEJ has been observed [12]. Similarly, no effect on NHEJ-mediated repair of the V(D)J locus during B-cell development has been reported in DICER conditional knock-out mice [153]. However, it is important to note that V(D)J recombination is clearly different from canonical NHEJ: firstly, DSBs generated by the RAG1 and RAG2 nucleases are closed hairpin-capped DSBs, that are then cleaved by Artemis before NHEJ-mediated repair [154]; secondly, V(D)J recombination does not require ATM [76], indeed unaltered V(D)J recombination is observed in cells from AT patients [155].

In human cells, reduced HR efficiency is observed upon DICER and AGO2 depletion [11; 12]. The ability of AGO2 to bind to diRNA [11] and RAD51 [12] supports a model in which the AGO2-RAD51 complex, through diRNAs, mediates the loading of RAD51 to DSBs, facilitating HR-mediated DNA repair. Furthermore, the observation that AGO2 interacts with the two chromatin modifiers MMSET and TIP60 and it is required for their proper localization to DSBs suggests a role for chromatin modifications in diRNA-mediated HR repair [12]. diRNA, through AGO2, may facilitate TIP60 and MMSET recruitment and mediate chromatin relaxation and the subsequent recruitment of the HR proteins BRCA1 and RAD51. Indeed, both TIP60 and MMSET recruitment and their associated chromatin modifications are impaired when diRNA biogenesis is compromised by DROSHA and DICER depletion (Figure 2a). Interestingly, AGO2 uses two different domains for MMSET/Tip60 and RAD51 binding: the mutated form of AGO2 unable to bind MMSET/Tip60 does not rescue RAD51 recruitment to DSBs in AGO2-depleted cells; differently, a form of AGO2 carrying a mutation in the RAD51 binding site, is still able to rescue RAD51 foci formation, suggesting a stronger role for chromatin modification in mediating RAD51 recruitment and HR.

In the scenario emerging from the results described above, damage-induced sncRNA, in the form of DDRNAs and diRNAs, favors, respectively, 53BP1 recruitment, as results of DDR activation, and HR. This may appear contradictory in light of the inhibitory effect of 53BP1 on DNA end resection and HR [156]. However, a more careful analysis of the available data indicates that only 53BP1 and BRCA1 recruitment to DSBs, but not DNA end resection, are inhibited when biogenesis of damage-induced sncRNAs is impaired. This is consistent with the observation that BRCA1 is required to counteract the inhibitory role of 53BP1 on DNA end resection [157]. In other words, in the absence of 53BP1, cells may no longer require BRCA1 to undergo DNA end resection and HR. However, although not necessary for DNA end resection, diRNAs allow RAD51 loading on RPA-coated resected DNA ends. This may explain why diRNAs, while impacting on both 53BP1 and BRCA1 recruitment to DNA lesions, but not on DNA end resection, are nevertheless necessary for efficient HR-mediated repair.

**The role of lncRNAs and transcript RNAs in the DNA damage response**

Similar to sncRNAs, several lncRNAs are induced upon damage and affect genome stability [158]. Generally, lncRNAs control the expression of factors involved in DNA damage signaling and repair. For example, this is the case of NORAD (non-coding RNA activated by DNA damage), which modulates the expression of DNA repair and replication genes by sequestering factors negatively regulating their expression [159]. Another example is the lncRNA DINO (Damage Induced Non-coding), which is induced upon DNA damage in a p53-dependent fashion. Once expressed, DINO binds and stabilizes p53, and modulates the expression of p53 target genes, contributing to the establishment of a feedback loop mechanism that amplifies the cellular response to DNA damage [160]. Differently from DINO, the p53-dependent induction of lincRNAp21 upon damage is required for the repression of p53 target genes. In particular, lincRNAp21 interacts with the RBP hnRNP-K, thus controlling its proper localization to promoters of p53 target genes [161]. Interestingly, recent reports have also shown a direct involvement of lncRNA in DNA repair. For example, it has

been reported that the lncRNA DDSR1 not only controls the expression of DNA damage related-genes, but it also contributes to HR-mediated repair by directly binding to hnRPUL1, a factor already known to control DNA end resection [80]. The DDSR1-hnRNPUL1 complex modulates BRCA1 recruitment to DNA breaks and HR [162]. Similarly, the lncRNA LINP1 controls NHEJ mediated-repair of DSBs by providing a scaffold linking the two key NHEJ players Ku80 and DNA-PKcs [163]. The role of lncRNAs in DNA damage modulation has also been described at physiological DSB, the telomeres, where TERRA transcripts participate in several telomere functions, as well as telomere stability [164].

The RNAi pathway is not present in the model yeast Saccharomyces cerevisiae. However, a role for RNA in HR has been well documented also in this system [165; 166; 167]. Exogenous RNA oligonucleotides have been reported to be used as templates for DSBs repair in yeast and human cells [165; 166]. More recently, Keskin and colleagues have demonstrated that endogenous RNA transcripts can be used as templates for HR-mediated DSBs repair [167]. The endogenous transcript does not only template the synthesis of cDNA molecules displaying homology to the damaged locus, but can also be used directly as a template for HR without the formation of cDNA intermediates. The repair by homologous RNA sequence is stimulated by the absence of RNase H functions and implies the formation of DNA:RNA hybrids intermediates [167]. Interestingly, RNase H2 deficiency in Aicardi-Gautiers (AGS) patients is associated with the accumulation of "non-canonical" DNA:RNA hybrids in intergenic regions, that are usually not prone to accumulate DNA:RNA hybrids. An intriguing possibility is that these DNA:RNA hybrids may be used as intermediates for the repair of some of the DNA breaks in AGS patients [168]. However, conversely, DNA:RNA hybrids accumulation at transcribed loci has been recently reported to be an obstacle for HR-mediated repair [169].

The formation of DNA:RNA hybrids during the RNA-templated HR repair may be facilitated by Rad52, an HR protein that mediates the annealing of RNA to DNA substrates *in vitro* [167]. In line

with these observations, in mammalian cells, RAD52 has been shown to interact with RNA Pol II and with endogenous transcripts at damaged genes [75]. A similar interaction is observed also for NHEJ factors, suggesting that RNA-templated repair could be extended also to NHEJ [75].

**Conclusions**

The negative impact of transcription on genome stability has been extensively studied for many years [19]. Moreover, transcriptional silencing has been observed upon DNA damage induction [17 18]. However, recently, active transcription, in the form of damage-induced sncRNAs, has been detected at DSBs and it has been proposed to have a role in maintaining genome stability [9; 10; 11]. Although several studies from different groups have contributed to our understanding of such events, a clear picture of damage-induced sncRNAs biogenesis and mechanism of action remains unavailable. A major unanswered question is how these damage-induced RNAs with the sequence of the damaged loci are generated. We know that their biogenesis requires the RNAi pathway, but the nature of the precursor transcript from which they are generated is still unknown. The observation of *de novo* transcription from DNA breaks fits well with the observations of transcription factors and RBPs recruitment to DSBs [90]. However, whether recruitment of RBPs and transcription activation at DSBs involves a specialized mechanism or it is only the result of the increased chromatin accessibility in the early steps of DDR is unknown. Moreover, it is presently untested whether the recruitment of transcription factors and RBPs to DNA lesions actually contributes to DNA damage-induced transcription. At present, damage-induced sncRNAs have been widely studied at non endogenous loci, mainly composed of repetitive sequences. This feature and the low abundance of sncRNAs makes their detection, analysis, and characterization very challenging, and may be addressed by the availability of novel and more sensitive detection and sequencing techniques. Importantly, generation of sncRNAs from damaged endogenous sites awaits to be characterized. Another unanswered question is how DDRNAs mediate DDR factors recruitment and DDR activation. The importance of these findings becomes clear when considering that genomic

instability is a hallmark of cancer [170] and unrepaired DNA lesions may result in permanent inhibition of cell proliferation [2]. Recently, the inability of cells deficient for some DDR pathways to repair DNA lesions is being successfully used in cancer therapy [171]. For this reason, the identification of RBPs as one of the most enriched categories in screens for DDR factors [54; 100; 101; 102] and the involvement of RNA as a new player in DNA signaling and repair [2; 172] offers additional opportunities for cancer therapy.

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## Figure legends

**Figure 1: The dual role of transcription in DNA damage generation, signaling and repair.**
a) Collision between the transcription and replication machineries and retention of DNA:RNA hybrids may result in DNA damage and recombination. Moreover, the ssDNA displaced in the R-loop is exposed to several assaults that may cause genome instability.
b) Upon damage, transcription of a precursor RNA and its processing by the RNAi nucleases DROSHA and DICER generates sncRNAs known as DDRNAs that participate in DDR signaling and repair.

**Figure 2: The role of damage-induced sncRNAs in DDR signaling and repair by Homologous Recombination.**

Upon damage, sncRNAs (diRNAs and DDRNAs) are generated from a precursor transcript by DROSHA and DICER mediated processing. diRNAs may facilitate Homologous Recombination by mediating the recruitment of the AGO2-RAD51 complex to DNA lesions. Another possibility is that diRNAs loaded on AGO2 could recruit AGO2-MMSET and AGO2-TIP60 complexes to DNA lesions. The TIP60- and MMSET-mediated chromatin remodelling would facilitate BRCA1 and RAD51 loading and repair by HR (a). DDRNAs play a role in DDR activation by facilitating the "secondary recruitment" of DDR proteins like pATM, 53BP1 and MDC1 to DSBs. The "primary recruitment" of DDR proteins, such as the MRN complex, is DDRNA independent, as well as ATM and H2AX phosphorylation.

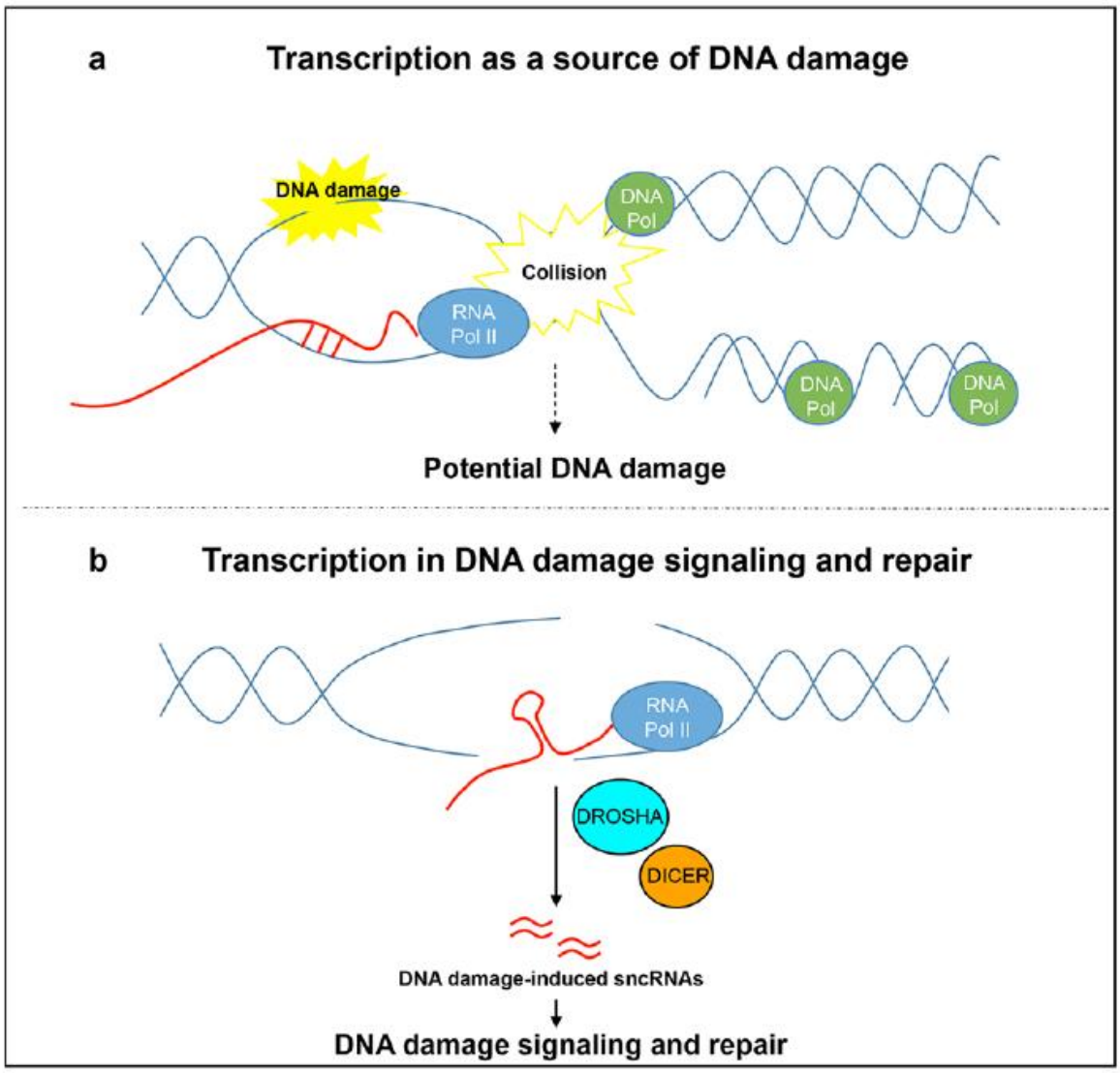



Figure 1

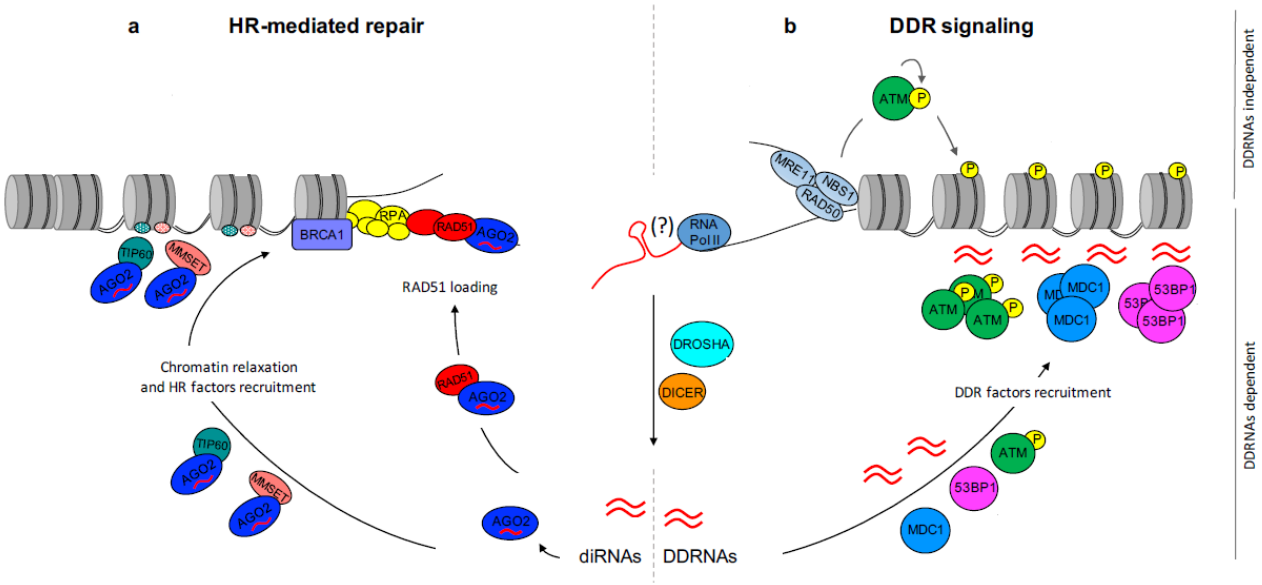
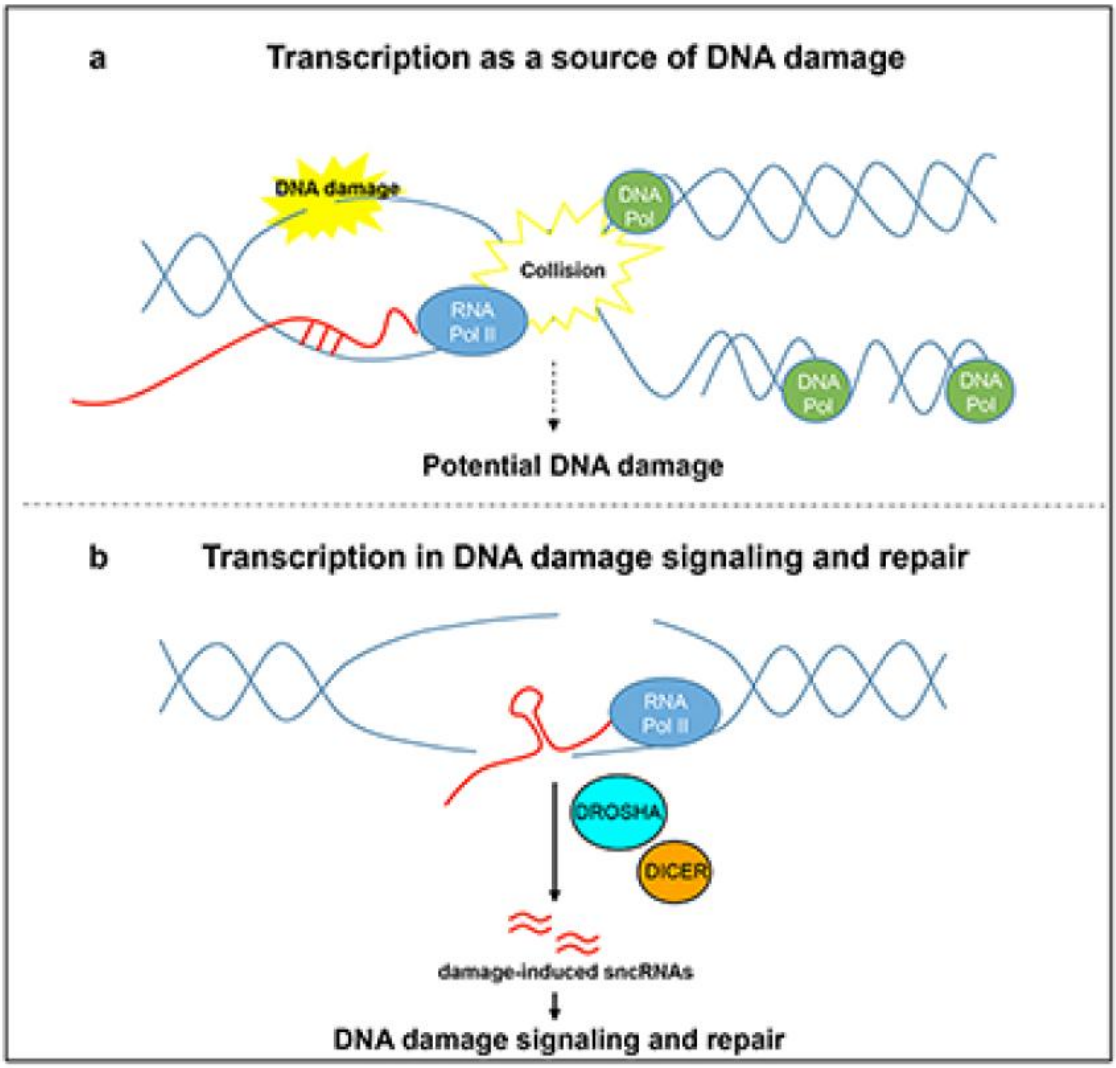



Figure 2




Graphical abstract