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2 **R-loops as Janus-faced modulators of DNA double-strand break repair**

3

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10

11

12 **Abstract**

13 **R-loops are non-B DNA structures with intriguing dual consequences for gene expression**
14 **and genome stability. In addition to their recognized roles in triggering DNA double-strand**
15 **breaks (DSBs), R-loops have recently been demonstrated to accumulate in response to DSBs,**
16 **especially when induced in transcriptionally active loci. In this review, we discuss whether R-**
17 **loops actively participate in DSB repair or are detrimental by-products that must be removed**
18 **to avoid genome instability.**

19

20

21 R-loops are dynamic non-B DNA structures that arise during transcription, when nascent RNA
22 forms Watson-Crick base pairs with the template DNA strand, generating an DNA:RNA hybrid
23 duplex and a displaced single DNA strand. Despite their discovery five decades ago, the prevalence
24 and the roles of R-loops remained mostly ambiguous until the advent of high-throughput
25 sequencing techniques which enabled their detection genome wide^{1,2}. R-loops mostly accumulate
26 on the 5' and 3' ends of GC-skewed, transcriptionally active loci^{3,4}. Their formation and removal
27 are regulated via several pathways, including mRNA processing (e.g. the THO/TREX complex⁵),
28 RNA modification⁶, negative supercoiling⁷, single-strand DNA binding proteins (e.g.⁸) and
29 specialized R-loop processing machineries such as RNA:DNA hybrid helicases, nucleases and,
30 more surprisingly, endonucleases involved in DNA damage repair⁹.

31
32 R-loops are usually considered as toxic by-products of ongoing transcription that generate
33 structural obstacles requiring removal to allow the processivity of RNA and DNA polymerases.
34 They are considered as sources of DNA damage that trigger genome instability^{1,10,11}, although the
35 mechanism(s) by which they cause such instability are still being characterized¹². Accordingly,
36 downregulation of R-loops removal pathways triggers a large range of detrimental outputs, at
37 molecular levels from replication and transcription defects to clinical levels as illustrated by the
38 dramatic phenotype of patients bearing mutations in the *senataxin* gene¹³. However, R-loops are
39 also now recognized to have beneficial roles through their ability to regulate various processes
40 including transcription, chromatin structure, telomere integrity and replication in physiological
41 contexts^{1,14}. A key recent study showed that they can act as topological sinks to release in a non-
42 enzymatic manner the negative supercoils imposed by processive activities on DNA^{7,15}. This
43 suggests that beyond being simple detrimental roadblocks that must be eliminated, R-loops also
44 represent active players in DNA-related processes.

45
46 While R-loops are potent inducers of DNA damage, they are also key to maintain telomere integrity
47 (for a recent review see ¹⁴) and recent findings surprisingly revealed that DNA double-strand breaks
48 (DSBs) elicit RNA:DNA hybrid formation^{11,16,17}. DSBs are harmful DNA lesions that, apart from
49 being generated by programmed endonuclease activity, can be provoked both by environmental
50 sources such as chemotherapeutic drugs and exposure to radiation, and by oncogene-induced
51 replication stress and topoisomerase-induced transcriptional activation¹⁶. Here, we review the
52 conflicting evidence regarding the mechanisms leading to DSB-induced R-loop accumulation, as
53 well as their potential to regulate resection, transcriptional arrest and ultimately DSB repair. We
54 will discuss whether RNA:DNA hybrids represent roadblocks with detrimental consequences for
55 DSB repair or whether they are beneficial structures that actively contribute to this process.

56
57 **Several models can account for RNA:DNA hybrid accumulation in *cis* to DSBs**
58 The first evidence for R-loop accumulation at DSBs came from immunofluorescence studies
59 showing that a catalytically-inactive mutant RNaseH protein used as a probe for R-loops is readily
60 detected at sites of laser-induced DSBs¹⁸. Many reports later confirmed the accumulation of R-
61 loops in *cis* to DSBs provoked by various DNA damaging agents (irradiation, targeted reactive
62 oxygen species, nuclease-based DSB induction) in several model systems using various R-loop
63 measurement methods¹⁹⁻³⁴. However, the mechanism by which these RNA:DNA hybrids
64 accumulate at DSBs is still unclear. This is probably in part due to the methods to induce DSBs,
65 the position of DSBs on the genome and the number of DSBs analyzed (hundreds versus a few)³⁵.
66 Yet the manner in which DSB-induced hybrids accumulate is not only critical in determining the
67 mechanistic details but may also inform us as to their “raison d’être”.

68

69 *The “DSB as promoter” model*

70 Several studies led to the proposal that DSB-induced hybrids are generated from *de novo*
71 bidirectional transcription in an unconventional manner through DSB ends acting as promoters
72 (Fig.1a)^{23,24,36-39}. Indeed, DNA ends can directly recruit RNA Polymerase II (RNAPII) *in vitro* in
73 a process that depends on the MRN complex^{38,39}. Moreover, *in vivo* data show that DSBs induced
74 by the homing endonuclease I-PpoI recruit RNAPII and factors of the pre-initiation complex
75 (CDK7 and CDK9)^{24,25,37}. CDK7 and CDK9 are key regulators of the regular transcription cycle.
76 They phosphorylate RNAPII on its carboxy-terminal domain (CTD) heptad repeats, first at serine
77 5 (CDK7) to promote initiation, then at serine 2 (CDK9) to induce elongation. The recruitment of
78 these kinases³⁷ suggests that the transcription initiated at DSBs follows the canonical mode.
79 Accordingly, live and super-resolution imaging studies show both nascent transcription and
80 RNAPII colocalizing with DNA breaks^{37,38,40,41}. Of interest, DNA-PK, a key mediator of DSB
81 signaling, can enhance transcriptional activity outside the DSB context⁴², which suggests a role
82 for this kinase in transcription activation at DSBs. Since resection was found to be required for
83 hybrid formation, it was proposed that these newly synthesized transcripts (called dilncRNAs for
84 DSB-induced long non-coding RNAs) can form RNA:DNA hybrids at the site of DSBs, rather than
85 actual R-loops (which are composed of the RNA:DNA hybrid and the displaced ssDNA)²³. In
86 conclusion, such a model proposes that *de novo* bidirectional transcription, initiated from DSBs,
87 triggers RNA:DNA hybrid formation and is a necessary and active process that systematically
88 occurs at all DSBs to ensure correct repair (Fig. 1a).

89 However, this model is hard to reconcile with the transcriptional repression known to occur *in cis*
90 to DSBs and with genome-wide studies revealing that damage-induced R-loops mostly form *in cis*
91 to DSBs arising at loci that display prior RNAPII occupancy (see below).

92

93 *The “transcription regulation” model*

94 As an alternative model, DSB-induced hybrids may form as a consequence of transcriptional
95 repression occurring *in cis* to DSBs rather than as a consequence of *de novo* transcription (Fig. 1b).
96 Accordingly, outside the DSB context, R-loops form in response to impaired transition from
97 initiating to elongating RNAPII and to decreased RNAPII processivity within genes. For instance,
98 R-loops accumulate at the 5' end or near the 3' transcription termination site⁴ where RNAPII
99 elongation rate is low⁴³. Moreover, increased RNAPII idling at promoter proximal pause sites is
100 correlated with increased R-loop levels in many cases^{44–47}. Importantly, when induced within or
101 close to an active locus, DSBs trigger transcriptional repression *in cis* (reviewed in^{16,48}). This local
102 transcriptional shut down involves alterations of RNAPII and its associated factors, as well as
103 changes in chromatin structure^{16,48}. Of particular interest, DSBs trigger (i) the recruitment of
104 NELF-E⁴⁹, involved in promoter proximal pausing, (ii) a decrease of RNAPII CTD-Ser2
105 phosphorylation, the elongating form of RNAPII^{50,51} and (iii) an increase of RNAPII CTD-Tyr1
106 phosphorylation⁴¹, a mark associated with pausing at the 5' and 3' ends of genes^{52,53}. Such
107 alterations probably trigger modifications in RNAPII distribution across the damaged gene, with a
108 shift from elongating RNAPII within the gene to RNAPII accumulation at promoters and 5' pause
109 sites. These changes could account for the previously reported increase of RNAPII at DSBs in
110 some instances (e.g.: when induced in promoters). It is also interesting to note that H2BK120
111 ubiquitination and H3K79me2 levels, previously associated with high RNAPII elongation rate^{54–}
112 ⁵⁶, decrease around DSBs⁵⁷, in agreement with a DSB-induced slowing down or pausing of
113 RNAPII. Finally, DSBs also recruit multiple transcription termination factors and RNA
114 degradation complexes^{21,22,26,28,32,58–61} (e.g. DROSHA, SETX and the nuclear exosome). Taken
115 together, these studies suggest that the transcription cycle (*i.e.* initiation, elongation, and
116 termination) is heavily affected on genes *in cis* to DSBs with a decreased elongation rate, increased

117 promoter-proximal pausing and premature termination (Fig. 1b), all of which are features
118 associated with R-loop formation. This raises the possibility that DSB-induced R-loops may occur
119 as a consequence of transcription shut down *in cis* to DSB.

120
121 In contrast to the “DSB promoter” model, the “transcription regulation” model posits that formation
122 of DSB-induced hybrids is not a ubiquitous feature but rather is restricted to DSBs falling within
123 regions that were transcriptionally active prior to DNA damage. This proposal is supported by
124 genome-wide DRIP-seq studies, which show specific accumulation of hybrids *in cis* to DSB
125 induced at loci occupied by RNAPII prior to damage but not around DSB induced in intergenic
126 loci, despite equivalent DSB induction levels^{21,62}. R-loops induced by reactive oxygen species were
127 also specifically observed at transcribed damaged loci¹⁹. Furthermore, use of NET-seq, which
128 captures nascent RNA embedded in RNAPII, enabled the identification of bidirectional transcripts
129 around only those DSBs that were induced in RNAPII pre-bound loci⁴¹. Finally, the presence of
130 DDRNAs (small double stranded RNA produced by diRNA processing^{38,63}), was shown to be
131 restricted to I-PpoI-induced DSBs falling within highly transcribed and repetitive regions⁶⁴.
132 Importantly, the *de novo* bidirectional transcription observed by live imaging⁴⁰ and NET-seq⁴¹ may
133 arise from such R-loop accumulation induced by transcriptional repression, since R-loops function
134 as intrinsic promoters⁶⁵.

135
136 In conclusion, the “transcription regulation” model implies that R-loop production is not a
137 generalizable feature occurring at all DSBs but occurs only at DSBs harboring prior RNAPII
138 occupancy, as a consequence of the DSB-induced transcriptional repression (Fig. 1b). Unlike the
139 “DSB as promoter” model, in which *de novo* transcription at DSBs allows to generate the RNA
140 moiety of the R-loop and hence precedes the RNA:DNA hybrid formation, the “transcription

141 regulation" model posits that R-loops form due to DSB-induced transcriptional repression, and that
142 *de novo* transcription occurs subsequent to R-loop formation as a result of R-loops acting as
143 promoters.

144

145 *Potential contributions of RNAPII backtracking, pre-mRNA and trans RNAs*

146 Besides the two models just described, other mechanisms could contribute to R-loop formation at
147 DSBs in transcribed loci (Fig. 1c). First, one can envision a contribution of *cis*-pre-mRNA to R-
148 loop formation^{33,66}. Indeed, the extensive histone modification changes observed following DSB
149 induction⁵⁷ could offer the opportunity for pre-existing pre-mRNA to anneal back to the template
150 DNA^{7,67}. Moreover, the availability of ssDNA produced by resection may also contribute to
151 stabilize RNA:DNA hybrids with pre-mRNA transcripts, consistent with increased R-loop
152 formation in *cis* to DSB in G2, a cell cycle phase in which resection is particularly efficient²³.
153 Second, RNAPII pausing that occurs when RNAPII encounters obstacles^{68,69} can trigger RNAPII
154 backtracking^{68,69}. This was proposed to produce anterior R-loops, *i.e.* R-loops that form in front of
155 the backtracked RNAPII⁶⁹. Hence, collision of RNAPII with factors accumulated on DSBs could
156 cause formation of nearby R-loops. Finally, *trans* RNA produced from a remote homologous locus
157 or following processing could also contribute to R-loop accumulation. Hybrids generated by *trans*
158 RNA have been seen at the *IgH* locus⁷⁰, during CRISPR/Cas9-mediated DSB induction⁷¹, TERRA
159 ncRNA assembly at telomeric sequences⁷², and during lncRNA *trans* action on distant loci (e.g.
160 APOLO in *Arabidopsis thaliana*⁷³). For *trans* RNAs to promote R-loop formation they must invade
161 duplex DNA. This strand invasion was proposed to be mediated by RecA/RAD51, the ssDNA-
162 binding protein usually mediating classical strand invasion during homologous recombination
163 (HR)^{72,74–76}, although this has been recently debated^{77,78}. RPA, another key ssDNA-binding
164 protein, known for its function in ssDNA stabilisation and RAD51 filament assembly, also displays

165 the potential to bind RNA and catalyse R-loop formation⁷⁸, supporting a role for this ssDNA
166 binding-protein in inverse strand invasion. In all these models, nascent *de novo* transcription at
167 DSBs would also occur as a consequence of R-loop formation (Fig. 1c), since R-loops are
168 themselves able to initiate *de novo* transcription⁶⁵.

169
170 The ever expanding range of genome-wide methods being developed to map RNA:DNA hybrids
171 to specific strands at single nucleotide resolution (e.g. DRIPc-seq⁷⁹, or qDRIP⁸⁰) should enable
172 discrimination between these models, particularly when combined with annotated DSB induction
173 systems³⁵.

174

175 **Regulation of R-loop levels by nucleases and RNA modifications**

176 Beyond the crosstalk between R-loops and transcription, R-loops also possess the potential to alter
177 a large range of dsDNA and ssDNA templated processes, such as helicase-mediated unwinding,
178 exo- and endonuclease activities, nucleofilament assembly, strand invasion and DNA synthesis, all
179 of which are critical steps in the repair process. Moreover, R-loops may act as recruitment platforms
180 recognized by several *bona fide* repair proteins, such as RPA^{78,81}, BRCA1^{23,82}, CSB^{19,27} and
181 Rad52^{20,27,83}, and are directly or indirectly required for loading of MDC1⁴¹, BRCA2^{23,47,84} and
182 53BP1^{22,41} at DSB sites. This suggests that R-loops are an integral component of the repair process.
183 In agreement with their strong potential to regulate the repair process, the R-loop level is subjected
184 to intense regulation at the site of damage. First, numerous R-loop removal factors are recruited at
185 DSBs, including SETX²¹, RNaseH1/2²³, DDX1³³, DDX5²⁶, XPG²⁰ and EXOSC10^{32,60}. Such
186 recruitment of several, potentially redundant, R-loop removal activities suggest that R-loops act as
187 roadblocks that require elimination. Second, recent studies indicate that chemical modifications
188 can take place on the RNA moiety of R-loops with consequences for their stability across the

189 genome^{6,85-89}. These RNA modifications, termed “epitranscriptomic” marks, which include 5-
190 methylcytidine (m5C) and N6-methyladenosine (m6A), regulate many aspects of RNA life, e.g.:
191 export and stability⁹⁰ and many cellular processes, including DNA damage^{30,91,92}. Notably, the
192 m6A-writer, METTL3 is required for efficient UV-damage repair⁹¹, and m6A-modified
193 RNA:DNA hybrids were found to accumulate post-DSB induction in an ATM-dependent
194 manner^{30,89}. In *cis* to DSB, m6A-RNA modification is required to stabilize nascent modified RNAs
195 that form hybrids, by a mechanism that involves the YTHDC1 m6A reader^{30,93}. Of interest, m⁵C
196 also accumulates on mRNAs *in cis* to DSB, and the methylation was proposed to take place on
197 RNA:DNA hybrids⁹². Despite the lack of recruitment of the classical m5C reader, ALYREF, to
198 m5C-modified DSB-induced hybrids in human cells⁹², its yeast homologue Yra1 (mostly known
199 for its role in mRNA export and in stabilizing DNA:RNA hybrids⁹⁴) has been implicated in DSB
200 repair⁹⁵. Altogether, these epitranscriptomic marks could act as a combinatorial code to allow
201 dynamic and rapid modulation of R-loop levels at DSBs and could also act as a recruitment
202 platform, given their ability to be recognized by reader proteins.

203

204 **Functions of DSB-induced R-loops**

205 *Control of transcriptional activity near DSBs*

206 As mentioned earlier, DSB induction results in transient repression of transcription of nearby loci,
207 which can lead to RNA:DNA hybrid formation. As well as being a consequence of transcriptional
208 silencing, DSB-induced hybrids may also hold the capacity to mediate transcriptional shut down.
209 This would create a positive feedback loop whereby hybrid formation is further required to
210 reinforce repression. This hypothesis is supported by the fact that outside the DSB context R-loops
211 ensure the recruitment of the PRC1 complex⁹⁶, a complex also involved in DSB-induced
212 transcriptional silencing⁹⁷⁻⁹⁹. DSB-induced hybrids could also contribute to premature

213 transcription termination via the recruitment of histone methyltransferase G9a, local H3K9me3/2
214 deposition and HP1 γ binding, which occur in *cis* to DSBs^{100–103}, since all these chromatin changes
215 were reported to arise in an R-loop-dependent manner and to promote transcription
216 termination^{104,105}. Moreover, several RNA:DNA helicases that resolve DSB-induced hybrids, such
217 as SETX/Sen1^{21,31}, DHX9²⁸, DDX1³³ or DDX5²⁶, also mediate premature or normal transcription
218 termination on undamaged DNA^{104,106–109} (Fig. 2).

219

220 *Control of resection*

221 As mentioned above, the RNA moiety of the R-loop displays an extraordinary potential to regulate
222 multiple steps of DSB repair, not only through its acting as a recruitment platform, but also by
223 substituting for the dsDNA substrate on which DSB repair processes naturally occur. A conserved
224 and essential step of HR repair is the generation of a long 3' ssDNA on either side of the break, a
225 process known as resection^{110,111}. Resection is believed to be initiated by the concerted action of
226 MRN and CtIP, which produce ssDNA nicks near both sides of the DSB. While the 3' end is
227 processed towards the DSB by the MRN complex, further long-range resection is ensured through
228 the action of 5' to 3' exonucleases Exo1, DNA2 and the BLM helicase. ssDNA is further coated
229 by the ssDNA binding protein RPA, which is often used as a proxy to measure resection *in vivo*.
230 RPA exchange and RAD51 nucleofilament formation on ssDNA are subsequently required for
231 homology search and HR completion^{110,111}.

232

233 Many reports suggest that R-loops act as roadblocks which modulate resection (Fig. 3a). Indeed,
234 elevated R-loop levels caused by the depletion of several removal factors (HNRNPD²⁹, DDX5²⁶,
235 USP42²⁸, RNase H1/2²⁴) coincides with decreased resection. Conversely, overexpression of RNase

236 H fosters resection²⁴. Consistent with these findings, *in vitro* data showed that long RNA:DNA
237 hybrids prevent the action of BLM and Exo1¹¹². However, the impact of RNA:DNA hybrids on
238 resection is proving to be more complex than first thought. Indeed, DSB-induced hybrids may, in
239 contrast to the above observations, trigger non-canonical and extensive resection (Fig. 3a). First,
240 the key resection factor CtIP is still recruited at DSBs in the presence of persistent R-loops, after
241 depletion of EXOSC10/RRP6 or DDX1^{32,33}, and it is capable of R-loop removal in other
242 contexts¹¹³. Second, a number of studies reported that accumulation of RNA:DNA hybrids, by
243 SETX, DDX1, EXOSC10/RRP6 or XRN2 depletion, did not dramatically decrease resection in
244 *vivo*, and even increased it in a number of cases^{21,22,31–33,114}. Resection analysis using Single
245 Molecule Analysis of Resection Tracks (SMART) revealed that EXOSC10 depletion, associated
246 with increased R-loops, significantly increased the length of resection tracks at irradiation-induced
247 DSBs³². Notably, in yeast, the absence of Sen1 (SETX homologue), which promotes R-loop
248 persistence at DSBs, activates a non-canonical resection pathway at an HO-induced break, that
249 involves Mre11, Sae2 (CtIP) and DNA2, but does not require Exo1 and Sgs1 (BLM homologue)³¹.
250 Moreover, an XPG-dependent, non-canonical resection pathway might prevail at transcriptionally
251 active loci that accumulate hybrids in human cells²⁰, where this structure-specific flap
252 endonuclease (reported to resolve R-loops outside the DSB context^{108,115,116}) would simultaneously
253 promote hybrid resolution and resection²⁰. In addition, DSB-induced large deletions are more
254 frequent in cells depleted for SETX, further suggesting that resection can occur despite excessive
255 accumulation of R-loops³⁴. Interestingly, these large-scale deletions depend on XPF³⁴, another flap
256 endonuclease able to process R-loops¹¹⁵, supporting a broad involvement of flap endonucleases in
257 non-canonical resection. Such a role for R-loops in promoting long-range non-canonical resection
258 could also account for the extensive resection observed at breaks induced in ribosomal DNA, a
259 locus naturally prone to R-loop formation¹¹⁷. It might also explain why the RNase III

260 endoribonuclease Drosha which promotes R-loop formation in *cis* to DSB, potentiates resection²².
261 Taken together, these studies suggest that persistence of R-loops around DSBs strongly affects
262 DSB processing, which could activate a non-canonical resection pathway to form an unusually
263 long resected strand (Fig. 3a). Such R-loop-driven large-scale resection may occur when canonical
264 R-loop removal pathways are inefficient, as in a SETX deficient background, but could also take
265 place at specific genomic locations that are more prone to form stable hybrids. However, several
266 lines of evidence suggest that the extended ssDNA produced may not be competent for
267 nucleofilament assembly and later steps in homologous recombination (HR) (see below).

268

269 *Regulation of RAD51 nucleofilament assembly and HR repair*

270 A few reports have suggested that R-loop formation is required for RAD51 focus assembly. Indeed,
271 depletion of Drosha, involved in forming DSB-induced R-loops, or METTL3 , involved in
272 stabilizing them, reduced RAD51 focus formation and HR^{22,30}. Interestingly, R-loops may promote
273 RAD51 nucleofilament assembly through a non-canonical mechanism that relies on CSB and
274 RAD52, but not BRCA1 and BRCA2, as proposed for DNA damage induced by reactive oxygen
275 species¹⁹. Such non-canonical RAD51 assembly also requires the TRDMT1-dependent m5C
276 modification of DSB-induced R-loops⁹².

277 In contrast, most studies highlight the need to remove R-loops, since persistent R-loops prevent
278 RAD51 filament assembly and downstream HR^{21,24,26,28,32,33,60,114}. For example, the R-loop removal
279 factors SETX, EXOSC10, DDX5, and DDX1 are all required for correct RAD51 focus formation
280 and ‘error-free’ HR repair^{21,26,32,33,60}. Consequently, SETX depletion increases translocation
281 frequency (*i.e.*: illegitimate rejoining of two independent DSBs) and strongly impairs cell survival
282 following DSB production in active loci, which are prone to form DSB-induced R-loops²¹ (Fig.
283 3b).

284 Overall, RNA:DNA hybrid accumulation and subsequent removal appear to be essential steps for
285 resection, nucleofilament assembly and HR. Conflicting data about the role of DSB-induced
286 hybrids likely arises from difficulties in experimentally recapitulating the dynamic and transient
287 nature of DSB-induced hybrids. Indeed, tipping the balance towards their absence or stabilization
288 may both result in detrimental outcomes. Another likely source of discrepancy is the variety of
289 methods used to measure resection and HR. Among the most striking examples is the possibility
290 that RNA:DNA hybrids actually form on ssDNA thereby precluding RPA binding. In this scenario,
291 resection would be detected by quantitative biochemical assay but not by using RPA as a proxy of
292 resection. This clearly highlights the need to use several methods for measuring resection, as well
293 as for detecting nucleofilament assembly and HR.

294

295 **Roles of R-loops in alternative repair pathway usage**

296 As an additional layer of complexity, R-loops and in particular their RNA moiety, may also be
297 substrates for alternative DSB repair pathways.

298 *RNA-templated repair*

299 Mounting evidence now supports the existence of RNA-templated DSB repair pathways, where *cis*
300 mRNAs support repair of their own DNA by two distinct mechanisms. cDNA-templated DSB
301 repair (also called c-TDR) uses a DNA copy of the transcript for repair; in yeast it relies on Ty
302 reverse transcriptase activity¹¹⁸. The second mechanism, called R-TDR, directly uses the RNA
303 transcript to mediate DNA synthesis by DNA polymerase ζ (Fig. 3c)¹¹⁸⁻¹²⁰. In yeast and human,
304 R-TDR is RAD51-independent and requires RAD52 for the *cis*-RNA to invade minimally resected
305 dsDNA, thereby forming an RNA:DNA hybrid by inverse strand invasion⁶⁶. Interestingly,
306 RAD51/RecA can also mediate inverse strand exchange with a *trans* RNA^{72,74-76} raising the

307 possibility that RAD51 could also direct such RNA-templated repair. R-TDR is further stimulated
308 by RPA⁶⁶, which acts as an R-loop sensor when associated with transcribed genes, and whose
309 presence at R-loops fosters DNA synthesis by human DNA polymerases *in vitro*^{78,81}. Accordingly,
310 R-TDR is mediated by the translesion DNA polymerase ζ that uses the *cis* mRNA hybrids as a
311 template to initiate DNA synthesis at sites of DNA damage¹²⁰. R-TDR has been detected in the
312 absence of RNaseH, but it has been argued that R-TDR is used as a fall back mechanism for
313 BRCA1-dependent HR^{66,118,120}. R-TDR would be ideal for mediating repair in G0/G1 phase, in
314 which canonical HR repair is strongly inhibited. Accordingly, an RNA-templated recombination
315 necessitating CSB, RAD52 and hybrid formation was proposed to occur at transcriptionally active
316 loci in G0/G1 cells^{83,121}.

317

318 *A role in triggering Pol α - and Pol δ - dependent synthesis?*

319 Landmark work from the de Lange lab indicated that the CST-Pol α complex is mobilized to
320 counteract resection at DSBs, by initiating a fill-in reaction¹²². During replication, Pol α normally
321 initiates DNA synthesis using a small RNA primer, synthesized by the primase. Yet there are
322 precedents for non-canonical replicative DNA synthesis using R-loops as a primer: origins of *E.*
323 *coli* multicopy plasmids and the yeast rDNA locus are examples^{123,124}. One could thus envisage the
324 RNA moiety of DSB-induced R-loop priming Pol α -dependent fill-in (Fig. 3c).

325 Moreover, compelling evidence indicates that in the absence of Sen1 and RNaseh1/2 in yeast, DSB
326 are repaired by a Pol32-dependent Break-Induced Replication (BIR) pathway^{125,126}. In human cells,
327 BIR (which involves POLD3/POLD4, Pol32 orthologs) has been reported to occur mainly on
328 telomeric sequences in ALT (Alternative Lengthening of Telomeres) positive cells^{127,128}. Indeed,
329 BIR-ALT increases in conditions where R-loop removal is impaired¹²⁹⁻¹³², and telomeric DSB

330 repair was recently shown to depend on an R-loop-mediated CSB-RAD52-POLD3 pathway²⁷.
331 Whether a similar pathway is followed at intra-chromosomal DSBs in the absence of R-loop
332 removal needs further clarification, but our recent work suggests that a POLD3-dependent pathway
333 may indeed be initiated at DSBs induced in active loci in absence of SETX (Fig.4)¹³³.

334

335 **Outlook**

336 Recent work hence brought to light the unanticipated presence and function of RNA:DNA hybrids
337 at DSBs. However, a large number of questions remain regarding their accumulation and their
338 exact roles.

339 For instance, the involvement of numerous R-loop removal factors raises several important
340 questions that deserve future investigation: are these removal factors redundant or do they
341 cooperate? do they deal with a homogenous or heterogeneous population of hybrids that current R-
342 loop mapping methods are unable to distinguish? Moreover, transcription termination pause sites
343 prone to form R-loops have been recently reported to trigger the synthesis of a new class of RNA
344 (DNA damage associated small RNAs, or sdRNAs) that are required for PALB2/Rad52
345 recruitment and repair of single strand breaks arising at those genomic locations¹³⁴. Can such
346 sdRNAs also arise from DSB-induced R-loops and can they also contribute to DSB repair?.

347

348 Another exciting future avenue of research is the function of R-loops in large-scale DSB
349 movement. Indeed, use of alternative repair pathways has been associated with relocalization of
350 DSBs to the nuclear periphery and nuclear pores, which act as docking sites for initiating error-
351 prone non-canonical repair pathways such as BIR and Microhomology-Mediated End joining
352 (reviewed in¹³⁵⁻¹³⁷). Interestingly, many factors involved in mRNA export and associated with
353 nuclear pores prevent R-loop formation outside the DSB context^{138,139}. It is thus tempting to

354 speculate that localizing persistent DSBs to the nuclear pore/envelope would favor DSB-induced
355 hybrid removal and repair. Future lines of investigation should also aim at understanding the
356 implication of R-loops in DSB clustering, which also involves large-scale movements^{140–142}. While
357 the function of such clustering is as yet unknown, it mainly involves persistent DSBs induced in
358 transcribed loci that accumulate RNA:DNA hybrids¹⁴¹. Moreover, DSB clustering relies on phase
359 separation^{37,143–145}, thanks to the liquid-liquid demixing properties of 53BP1 that can be fostered
360 by RNA molecules³⁷. Of interest, RNA molecules as well as the m6A RNA modification (present
361 on DSB-induced hybrids) contribute to the assembly of many phase-separated bodies^{146–148}. The
362 *E. coli* SSB protein, the homologue of RPA which binds the displaced R-loop ssDNA strand⁸¹, also
363 displays liquid-liquid phase separation properties¹⁴⁹. Thus, whether R-loops promote phase
364 separation clearly deserves to be investigated. Such process may be particularly relevant for
365 neurological diseases-associated with aberrant R-loop formation and increased genomic instability,
366 given that one of the main hallmarks of many neurodegenerative diseases is the accumulation of
367 pathological protein aggregates, caused by aberrant phase separation behavior¹⁵⁰.

368
369 In conclusion, much remains to be understood regarding the functions of DSB-induced RNA:DNA
370 hybrids during repair, including their mode of accumulation at DSB, their relationship to
371 transcription, their role in non-canonical resection as well as their influence on the use of non-
372 canonical repair pathways. The apparent need of R-loops to recruit repair proteins underscores their
373 potential beneficial roles in the repair process. However, these functions must be regulated to be
374 compatible with the need for their ultimate removal to ensure safe repair and maintenance of
375 genome integrity. This duality of function is not restricted to DSB-induced hybrids, and advances
376 in the R-loop field will sooner or later reveal the true importance of DSB-induced hybrids during
377 the repair process.

378

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386

387 **Competing interest statement**

388 The authors declare no competing interests.

389

390

391 **Figure Legends**

392

393 **Figure 1: Current models for RNA:DNA hybrid accumulation in *cis* to DSB.**

394 (a) The “DSB as promoter” model postulates that DNA ends act as promoter, where the Pre
395 Initiation Complex (PIC), the Mediator and the RNAPII are loaded. *de novo* synthesized RNA
396 (dilncRNA) hybridizes back to the resected strand forming an RNA:DNA hybrid. dilncRNAs are
397 further processed and give rise to DDRNAs, that amplify the DDR response. (b) The “transcription
398 regulation” model posits that R-loops arise as a consequence of local transcription inhibition
399 elicited in *cis* to DSBs. The DSB induces changes in the transcription cycle of near-by genes, which
400 entails decreased elongation rate on genes bodies, increased promoter-proximal pausing and
401 premature termination. R-loops form as a consequence of these changes, as shown in other
402 contexts. These R-loops that accumulate in *cis* to DSB further act themselves as promoters,
403 triggering bidirectional *de novo* transcription not initiated from the DNA ends themselves. (c) R-
404 loop accumulation could also occur following the hybridization of prior pre-mRNA with the
405 resected strand, from RNAPII backtracking when it encounters a DSB on the gene body, or from
406 an RNA produced in *trans* (from a remote locus) that invades the dsDNA. In these conditions, as
407 in (b), R-loops then act as promoters leading to *de novo* transcription in the vicinity of DSBs.

408

409 **Figure 2: A function of R-loops in local transcriptional repression**

410 R-loops were shown in other contexts to display the potential to repress transcription by triggering
411 repressive histone modifications. R-loops can foster H3K9me2 by G9a and promote termination.
412 R-loops also trigger the PRC1 Polycomb complex recruitment, leading to H3K27me3. R-loops
413 induced at DSB could therefore contribute to terminate transcription locally around DSBs.

414

415 **Figure 3: Function of R-loops in the successive repair steps**

416 (a) R-loops regulate resection. When R-loops removal pathways are inefficient, R-loops block the
417 ability of exonuclease to act on the dsDNA. However, in such case, the XPF/XPG endonucleases
418 and potentially CtIP may excise the hybrids, as shown in other context, hence leading to extended
419 resection. (b) R-loops regulate Rad51 filament assembly. The presence of a RNA strand hybridized
420 to the resected strand inhibits Rad51 loading, and shall be removed to ensure HR repair. Defective
421 removal triggers alternative, error prone repair. (c) R-loops can also initiate alternative repair
422 pathways. Top panel: RNA can act as a template for pol ζ DNA synthesis. Middle panel: RNA can
423 prime Pol α DNA synthesis for fill in. Bottom panel: A persistent R-loop can transform a two
424 ended DSB into a one-ended DSB, further repaired by a Pol δ dependent pathway, such as Break
425 Induced Replication (BIR).

426

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