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► **To cite this version:**

Aline Marnef, Gaëlle Legube. R-loops as Janus-faced modulators of DNA double-strand break repair. Nature Cell Biology, 2021, 23 (4), pp.305-313. 10.1038/s41556-021-00663-4 . hal-04125277

**HAL Id: hal-04125277**

**<https://ut3-toulouseinp.hal.science/hal-04125277>**

Submitted on 12 Jun 2023

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

3

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5

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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<sup>1,2</sup>. R-loops mostly accumulate  
26 on the 5' and 3' ends of GC-skewed, transcriptionally active loci<sup>3,4</sup>. Their formation and removal  
27 are regulated via several pathways, including mRNA processing (e.g. the THO/TREX complex<sup>5</sup>),  
28 RNA modification<sup>6</sup>, negative supercoiling<sup>7</sup>, single-strand DNA binding proteins (e.g.<sup>8</sup>) and  
29 specialized R-loop processing machineries such as RNA:DNA hybrid helicases, nucleases and,  
30 more surprisingly, endonucleases involved in DNA damage repair<sup>9</sup>.

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<sup>1,10,11</sup>, although the  
35 mechanism(s) by which they cause such instability are still being characterized<sup>12</sup>. 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* gene<sup>13</sup>. 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<sup>1,14</sup>. 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<sup>7,15</sup>. 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 <sup>14</sup>) and recent findings surprisingly revealed that DNA double-strand breaks  
48 (DSBs) elicit RNA:DNA hybrid formation<sup>11,16,17</sup>. 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 activation<sup>16</sup>. 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 DSBs<sup>18</sup>. 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 methods<sup>19-34</sup>. 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)<sup>35</sup>.  
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)<sup>23,24,36-39</sup>. Indeed, DNA ends can directly recruit RNA Polymerase II (RNAPII) *in vitro* in  
73 a process that depends on the MRN complex<sup>38,39</sup>. 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)<sup>24,25,37</sup>. 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 kinases<sup>37</sup> 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<sup>37,38,40,41</sup>. Of interest, DNA-PK, a key mediator of DSB  
81 signaling, can enhance transcriptional activity outside the DSB context<sup>42</sup>, 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)<sup>23</sup>. 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 site<sup>4</sup> where RNAPII  
99 elongation rate is low<sup>43</sup>. Moreover, increased RNAPII idling at promoter proximal pause sites is  
100 correlated with increased R-loop levels in many cases<sup>44–47</sup>. Importantly, when induced within or  
101 close to an active locus, DSBs trigger transcriptional repression *in cis* (reviewed in<sup>16,48</sup>). This local  
102 transcriptional shut down involves alterations of RNAPII and its associated factors, as well as  
103 changes in chromatin structure<sup>16,48</sup>. Of particular interest, DSBs trigger (i) the recruitment of  
104 NELF-E<sup>49</sup>, involved in promoter proximal pausing, (ii) a decrease of RNAPII CTD-Ser2  
105 phosphorylation, the elongating form of RNAPII<sup>50,51</sup> and (iii) an increase of RNAPII CTD-Tyr1  
106 phosphorylation<sup>41</sup>, a mark associated with pausing at the 5' and 3' ends of genes<sup>52,53</sup>. 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<sup>54–</sup>  
112 <sup>56</sup>, decrease around DSBs<sup>57</sup>, 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<sup>21,22,26,28,32,58–61</sup> (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<sup>21,62</sup>. R-loops induced by reactive oxygen species were  
127 also specifically observed at transcribed damaged loci<sup>19</sup>. 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 loci<sup>41</sup>. Finally, the presence of  
130 DDRNAs (small double stranded RNA produced by diRNA processing<sup>38,63</sup>), was shown to be  
131 restricted to I-PpoI-induced DSBs falling within highly transcribed and repetitive regions<sup>64</sup>.  
132 Importantly, the *de novo* bidirectional transcription observed by live imaging<sup>40</sup> and NET-seq<sup>41</sup> may  
133 arise from such R-loop accumulation induced by transcriptional repression, since R-loops function  
134 as intrinsic promoters<sup>65</sup>.

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<sup>33,66</sup>. Indeed, the extensive histone modification changes observed following DSB  
149 induction<sup>57</sup> could offer the opportunity for pre-existing pre-mRNA to anneal back to the template  
150 DNA<sup>7,67</sup>. 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 efficient<sup>23</sup>.  
153 Second, RNAPII pausing that occurs when RNAPII encounters obstacles<sup>68,69</sup> can trigger RNAPII  
154 backtracking<sup>68,69</sup>. This was proposed to produce anterior R-loops, *i.e.* R-loops that form in front of  
155 the backtracked RNAPII<sup>69</sup>. 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* locus<sup>70</sup>, during CRISPR/Cas9-mediated DSB induction<sup>71</sup>, TERRA  
159 ncRNA assembly at telomeric sequences<sup>72</sup>, and during lncRNA *trans* action on distant loci (e.g.  
160 APOLO in *Arabidopsis thaliana*<sup>73</sup>). 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)<sup>72,74–76</sup>, although this has been recently debated<sup>77,78</sup>. 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 formation<sup>78</sup>, 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* transcription<sup>65</sup>.

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-seq<sup>79</sup>, or qDRIP<sup>80</sup>) should enable  
172 discrimination between these models, particularly when combined with annotated DSB induction  
173 systems<sup>35</sup>.

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<sup>78,81</sup>, BRCA1<sup>23,82</sup>, CSB<sup>19,27</sup> and  
181 Rad52<sup>20,27,83</sup>, and are directly or indirectly required for loading of MDC1<sup>41</sup>, BRCA2<sup>23,47,84</sup> and  
182 53BP1<sup>22,41</sup> 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 SETX<sup>21</sup>, RNaseH1/2<sup>23</sup>, DDX1<sup>33</sup>, DDX5<sup>26</sup>, XPG<sup>20</sup> and EXOSC10<sup>32,60</sup>. 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<sup>6,85-89</sup>. 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 stability<sup>90</sup> and many cellular processes, including DNA damage<sup>30,91,92</sup>. Notably, the  
192 m6A-writer, METTL3 is required for efficient UV-damage repair<sup>91</sup>, and m6A-modified  
193 RNA:DNA hybrids were found to accumulate post-DSB induction in an ATM-dependent  
194 manner<sup>30,89</sup>. 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<sup>30,93</sup>. Of interest, m<sup>5</sup>C  
196 also accumulates on mRNAs *in cis* to DSB, and the methylation was proposed to take place on  
197 RNA:DNA hybrids<sup>92</sup>. Despite the lack of recruitment of the classical m5C reader, ALYREF, to  
198 m5C-modified DSB-induced hybrids in human cells<sup>92</sup>, its yeast homologue Yra1 (mostly known  
199 for its role in mRNA export and in stabilizing DNA:RNA hybrids<sup>94</sup>) has been implicated in DSB  
200 repair<sup>95</sup>. 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 complex<sup>96</sup>, a complex also involved in DSB-induced  
212 transcriptional silencing<sup>97-99</sup>. 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 $\gamma$  binding, which occur in *cis* to DSBs<sup>100–103</sup>, since all these chromatin changes  
215 were reported to arise in an R-loop-dependent manner and to promote transcription  
216 termination<sup>104,105</sup>. Moreover, several RNA:DNA helicases that resolve DSB-induced hybrids, such  
217 as SETX/Sen1<sup>21,31</sup>, DHX9<sup>28</sup>, DDX1<sup>33</sup> or DDX5<sup>26</sup>, also mediate premature or normal transcription  
218 termination on undamaged DNA<sup>104,106–109</sup> (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<sup>110,111</sup>. 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<sup>110,111</sup>.

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 (HNRNPD<sup>29</sup>, DDX5<sup>26</sup>,  
235 USP42<sup>28</sup>, RNase H1/2<sup>24</sup>) coincides with decreased resection. Conversely, overexpression of RNase

236 H fosters resection<sup>24</sup>. Consistent with these findings, *in vitro* data showed that long RNA:DNA  
237 hybrids prevent the action of BLM and Exo1<sup>112</sup>. 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<sup>32,33</sup>, and it is capable of R-loop removal in other  
242 contexts<sup>113</sup>. 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<sup>21,22,31–33,114</sup>. 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 DSBs<sup>32</sup>. 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)<sup>31</sup>.  
250 Moreover, an XPG-dependent, non-canonical resection pathway might prevail at transcriptionally  
251 active loci that accumulate hybrids in human cells<sup>20</sup>, where this structure-specific flap  
252 endonuclease (reported to resolve R-loops outside the DSB context<sup>108,115,116</sup>) would simultaneously  
253 promote hybrid resolution and resection<sup>20</sup>. 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-loops<sup>34</sup>. Interestingly, these large-scale deletions depend on XPF<sup>34</sup>, another flap  
256 endonuclease able to process R-loops<sup>115</sup>, 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 formation<sup>117</sup>. It might also explain why the RNase III

260 endoribonuclease Drosha which promotes R-loop formation in *cis* to DSB, potentiates resection<sup>22</sup>.  
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<sup>22,30</sup>. 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 species<sup>19</sup>. Such non-canonical RAD51 assembly also requires the TRDMT1-dependent m5C  
276 modification of DSB-induced R-loops<sup>92</sup>.

277 In contrast, most studies highlight the need to remove R-loops, since persistent R-loops prevent  
278 RAD51 filament assembly and downstream HR<sup>21,24,26,28,32,33,60,114</sup>. 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<sup>21,26,32,33,60</sup>. 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-loops<sup>21</sup> (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 activity<sup>118</sup>. The second mechanism, called R-TDR, directly uses the RNA  
303 transcript to mediate DNA synthesis by DNA polymerase  $\zeta$  (Fig. 3c)<sup>118-120</sup>. 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 invasion<sup>66</sup>. Interestingly,  
306 RAD51/RecA can also mediate inverse strand exchange with a *trans* RNA<sup>72,74-76</sup> raising the

307 possibility that RAD51 could also direct such RNA-templated repair. R-TDR is further stimulated  
308 by RPA<sup>66</sup>, 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*<sup>78,81</sup>. Accordingly,  
310 R-TDR is mediated by the translesion DNA polymerase  $\zeta$  that uses the *cis* mRNA hybrids as a  
311 template to initiate DNA synthesis at sites of DNA damage<sup>120</sup>. 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<sup>66,118,120</sup>. 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<sup>83,121</sup>.

317

#### 318 *A role in triggering Pol $\alpha$ - and Pol $\delta$ - dependent synthesis?*

319 Landmark work from the de Lange lab indicated that the CST-Pol $\alpha$  complex is mobilized to  
320 counteract resection at DSBs, by initiating a fill-in reaction<sup>122</sup>. During replication, Pol $\alpha$  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<sup>123,124</sup>. One could thus envisage the  
324 RNA moiety of DSB-induced R-loop priming Pol $\alpha$ -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<sup>125,126</sup>. 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<sup>127,128</sup>. Indeed,  
329 BIR-ALT increases in conditions where R-loop removal is impaired<sup>129-132</sup>, and telomeric DSB



330 repair was recently shown to depend on an R-loop-mediated CSB-RAD52-POLD3 pathway<sup>27</sup>.  
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)<sup>133</sup>.

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 locations<sup>134</sup>. 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 in<sup>135-137</sup>). Interestingly, many factors involved in mRNA export and associated with  
353 nuclear pores prevent R-loop formation outside the DSB context<sup>138,139</sup>. 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<sup>140–142</sup>. 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 hybrids<sup>141</sup>. Moreover, DSB clustering relies on phase  
359 separation<sup>37,143–145</sup>, thanks to the liquid-liquid demixing properties of 53BP1 that can be fostered  
360 by RNA molecules<sup>37</sup>. 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<sup>146–148</sup>. The  
362 *E. coli* SSB protein, the homologue of RPA which binds the displaced R-loop ssDNA strand<sup>81</sup>, also  
363 displays liquid-liquid phase separation properties<sup>149</sup>. 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 behavior<sup>150</sup>.

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

379 **Acknowledgments**

380

381 Funding in G.L.'s laboratory was provided by grants from the European Research Council (ERC-  
382 2014-CoG 647344), the Agence Nationale pour la Recherche (ANR-14-CE10-0002-01), the  
383 Institut National Contre le Cancer (INCA), and the Ligue Nationale Contre le Cancer (LNCC). We  
384 thank Dr Sylvain Egloff (CBI, Toulouse) and Dr David Lane (CBI, Toulouse) for critical reading  
385 of the manuscript and apologize to colleagues whose work was not cited due to space constraints.

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 $\zeta$  DNA synthesis. Middle panel: RNA can  
423 prime Pol $\alpha$  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 $\delta$  dependent pathway, such as Break  
425 Induced Replication (BIR).

426

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