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Break-induced RNA–DNA hybrids (BIRDHs) in homologous recombination: friend or foe?

Belén Gómez-González & Andrés Aguilera*

Abstract

Double-strand breaks (DSBs) are the most harmful DNA lesions, with a strong impact on cell proliferation and genome integrity. Depending on cell cycle stage, DSBs are preferentially repaired by non-homologous end joining or homologous recombination (HR). In recent years, numerous reports have revealed that DSBs enhance DNA–RNA hybrid formation around the break site. We call these hybrids "break-induced RNA–DNA hybrids" (BIRDHs) to differentiate them from sporadic R-loops consisting of DNA–RNA hybrids and a displaced single-strand DNA occurring co-transcriptionally in intact DNA. Here, we review and discuss the most relevant data about BIRDHs, with a focus on two main questions raised: (i) whether BIRDHs form by *de novo* transcription after a DSB or by a pre-existing nascent RNA in DNA regions undergoing transcription and (ii) whether they have a positive role in HR or are just obstacles to HR accidentally generated as an intrinsic risk of transcription. We aim to provide a comprehensive view of the exciting and yet unresolved questions about the source and impact of BIRDHs in the cell.

Keywords DNA damage; DNA–RNA hybrids; DSBs; recombination; repair
Subject Category DNA Replication, Recombination & Repair
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Introduction

Double-strand breaks (DSBs) are the most harmful form of DNA damage. Essential processes like replication and transcription rely on the continuity of the DNA strand used as the template, and DSBs impede their progression. When incorrectly processed, DSBs may generate chromosome rearrangements and changes in DNA sequence, thus constituting a potential source of genome instability (Al-Zain & Symington, 2021), which is a hallmark of cancer cells (Gaillard *et al*, 2015). DSB repair constitutes a major biological process in all organisms from bacteria to mammals to preserve genome integrity. It was likely born as a mechanism to fix accidental breaks occurring during vegetative growth of cells. However, DSB repair evolved into specific processes that acquired essential physiological roles, such as in meiotic recombination and immunoglobulin (Ig) class-switching, the goals of which are to generate the genetic diversity that sustains evolution and the immune response, respectively.

Cells employ two main types of DSB repair pathways, non-homologous end-joining (NHEJ) and homologous recombination (HR). HR is a homology-dependent pathway that relies on copying the intact information from a homologous template, among which the intact sister-chromatid is preferred to preserve the genetic information. NHEJ instead relies on the joining of the two DSB ends and can be mutagenic due to the insertion or deletion of nucleotides between them. Research over more than four decades at both *in vivo* and *in vitro* levels has led to detailed knowledge of the different steps of these DSB repair reactions relying on a varied number of specialized proteins (Heyer *et al*, 2010; Chang *et al*, 2017; Scully *et al*, 2019; Stinson & Loparo, 2021). Interestingly, an unexpected role for RNA has emerged in recent years, among which DNA–RNA hybrids have received particular attention as potential intermediates of HR.

R-loops and DNA–RNA hybrids are terms used indistinctly in the field, but R-loops contain the displaced ssDNA in addition to the DNA–RNA hybrid. In some studies, the enhanced vulnerability of the displaced ssDNA to targeted mutagenesis was used to confirm the formation of R-loops (Yu *et al*, 2003; Li & Manley, 2005; Gomez-Gonzalez & Aguilera, 2007; Garcia-Pichardo *et al*, 2017; Malig *et al*, 2020). However, most methods just detect DNA–RNA hybrids even though they are likely part of R-loops when forming in the context of an intact dsDNA molecule. R-loops were found to accumulate in *Escherichia coli top1* mutants at highly expressed genes having a negative impact on transcription (Drolet *et al*, 1995), and to form at Ig gene switch (Ig S) regions (Daniels & Lieber, 1995; Yu *et al*, 2003). A new role for DNA–RNA hybrids as a source of genome instability was revealed through the observation that the *Saccharomyces cerevisiae hpr1* mutant of the THO complex, involved in RNA metabolism, accumulated DNA–RNA hybrids that were responsible for a hyper-recombinant phenotype (Huertas & Aguilera, 2003; Gomez-Gonzalez & Aguilera, 2007). The role of R-loops in genetic instability was further supported by studies of other factors involved in RNA metabolism in yeast and mammals (Li & Manley, 2005; Paulsen *et al*, 2009; Wahba *et al*, 2011; Stirling *et al*, 2012). R-loops trigger genetic instability mainly by interfering with DNA replication, which ultimately leads to DSBs (reviewed in Aguilera &

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Garcia-Muse, 2012; Garcia-Muse & Aguilera, 2019; Brickner *et al*, 2022).

An intriguing twist on the relationship between DNA–RNA hybrids and genome integrity has emerged from the observation that not only hybrids (within R-loops) lead to DSBs but that DSBs also lead to hybrid accumulation. This has nourished an ongoing and enriching debate about novel exciting possibilities for the potential impact of hybrids on DSB repair (Aguilera & Gomez-Gonzalez, 2017; Paull, 2019; Puget *et al*, 2019; Brambati *et al*, 2020; Marnef & Legube, 2021). This phenomenon is unrelated to other putative functions of RNA molecules, such as their use as templates for DNA repair (Keskin *et al*, 2014) or in damage signaling; a role which has been attributed to small RNA molecules synthesized *de novo* at DSBs (Francia *et al*, 2012, 2016; Michelini *et al*, 2018; Burger *et al*, 2019; Pessina *et al*, 2019). Here we review and discuss this subject, with the aim of updating and rationalizing conclusions based on the key steps of HR on which DNA–RNA hybrids could have an impact, including protective roles at DNA ends and regulation of the choice of repair pathway. To put the discussion in the right context and for readers who are not familiar with the DSB repair field, we first briefly summarize the current knowledge of the key steps of the DSB repair pathways with a focus on HR, before discussing the most relevant literature connecting DNA–RNA hybrids with DSB repair via HR.

Source of DSBs

It has been estimated that human cells undergo around 25–50 DSBs per day. Half of these are generated from single stranded breaks (SSBs), which are much more common lesions estimated to occur more than 50,000 times per day (Lindahl & Barnes, 2000; Vilenchik & Knudson, 2003; Tubbs & Nussenzweig, 2017). SSB conversion into DSBs can be due to their close proximity in the two DNA strands (staggered DSBs) or their replication (replication-induced DSBs; Fig 1). In vegetatively growing microbial cells and in somatic cells, DSBs are generated by internal agents such as reactive oxygen species (ROS) and external agents, such as ionizing irradiation (IR; Ciccia & Elledge, 2010). Moreover, DSBs can vary in nature depending on their source, being “clean” if they have 5′-phosphate and 3′-OH ends, or “dirty” if not (Reginato & Cejka, 2020). Whether such variations might imply differences in the initial steps of the DSB repair reaction is unknown.

To study their repair, DSBs are induced in the laboratory in multiple ways, such as by IR, UV laser microirradiation, topoisomerase inhibitors, alkylating agents, or nucleases. In most of the cases, these agents do not trigger DSBs directly but, as is the case with endogenous DSBs, through the generation of SSBs. Thus, IR has been estimated to induce the formation of radicals that attack the sugar phosphate backbone of the DNA strands, implying that over 80% of DNA breaks caused by IR may be SSBs (Ward, 1988). Similarly, DSBs induced by the topoisomerase 1 (Top1) inhibitor camptothecin (CPT) can be the product of a two-step process in which SSBs are initially generated and then converted into DSBs in a second step during replication (Pommier, 2006). In contrast, DSBs induced by endonucleases (including HO, I-SceI, PpoI, AsiSI, Flp, and Cas9; Jasin, 1996; Potts *et al*, 2006; Nielsen *et al*, 2009; Aymard *et al*, 2014; Haber, 2016; Jasin & Haber, 2016; Ortega *et al*, 2019) occur most likely in one step. However, since most endonucleases are dimeric proteins in which each monomer does not always cleave its strand with the same efficiency as the other, a proportion of these DSBs might also be caused by an SSB followed by replication.

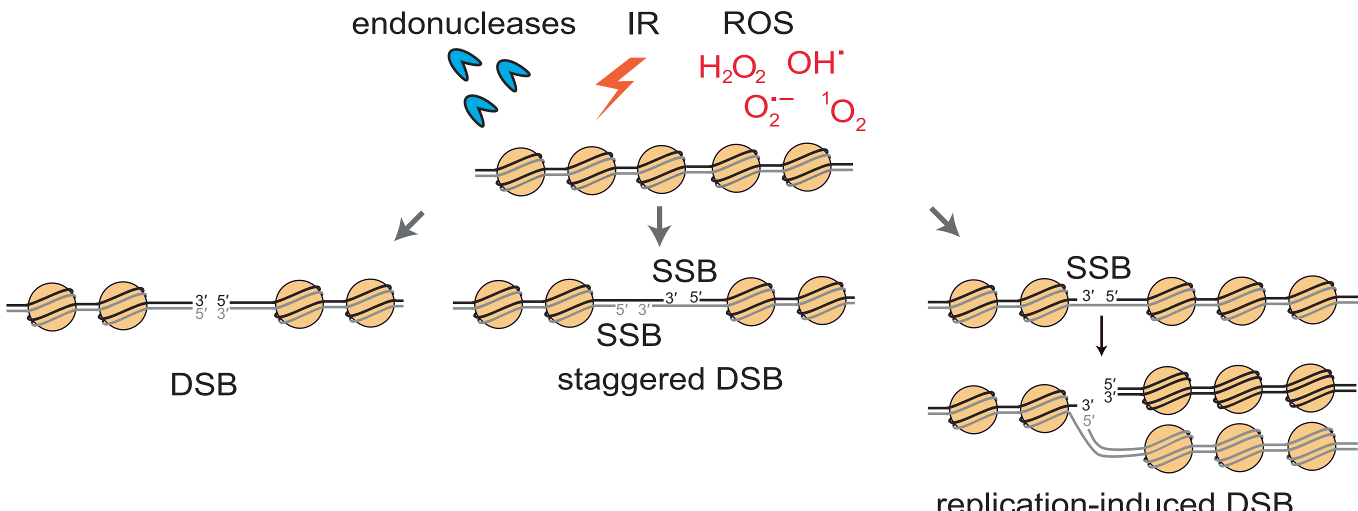

Figure 1. Different ways in which double-strand breaks (DSBs) can be generated in the cell.

DNA breaks can be chemically generated in the cell by the electrophilic attack of the phosphodiester bond by reactive oxygen species (ROS) or hydroxyls generated by X- or γ-rays (IR). In addition, DNA breaks can occur naturally from the action of endonucleases, such as XPF or XPG involved in nucleotide excision repair (NER), or artificially by the induction of different endonucleases (HO, I-SceI, PpoI, AsiSI, etc.), as used in many different studies. Endonucleases can either cleave both DNA strands or act as nickases, such as a mutated form of Flp recombinase or the yeast HO endonuclease when targeting an HO site reduced in size. Single-strand breaks (SSBs) may be converted into DSBs by independent cleavage of the complementary strand (staggered DSB), or, likely the more common way, after the replication fork hits the SSB (replication-induced DSB). The two parental DNA strands are indicated in different colors (black and gray) to differentiate the sister chromatids.

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Endonuclease-driven SSBs can be induced by reducing the endonuclease target site (Cortes-Ledesma & Aguilera, 2006) or with a mutated version of the enzyme (Nielsen *et al*, 2009; Ortega *et al*, 2019). Replication converts these SSBs into DSBs (Fig 1). Concordantly, chemical induction of DSBs often occurs in a replication-dependent manner. Many such chemicals are used in cancer therapies due to the ability of DSBs to block replication progression. Thus, it is possible that many spontaneous and a good number of induced DSBs occur during replication, meaning that in an experimental context, the method used to induce DNA breaks should be considered when interpreting results.

Pathway choice and the initiation of DSB repair

When DSBs occur, they are signaled by the DNA damage response (DDR), which triggers a complete series of events including the regulation of cell cycle progression (Ciccia & Elledge, 2010). DSBs are signaled by ATM kinase, which triggers the subsequent set of events necessary to process the DNA break. One of the first events is the phosphorylation of the histone variant H2AX (γ-H2AX; H2A in yeast), which spreads around the break site and triggers a signaling cascade that leads to changes in chromatin to facilitate the recruitment and action of subsequent DSB repair factors for each pathway (Fig 2).

NHEJ and HR DSB repair pathways differ in two key initial features (Fig 2). First, HR is preferentially limited to the S/G2 phase of the cell cycle since it uses the sister chromatid as a template for repair (Kadyk & Hartwell, 1992; Gonzalez-Barrera *et al*, 2003). On the other hand, NHEJ can be used throughout the cell cycle and is mainly employed during G1 or in non-dividing cells. This is channeled in one direction or another by the differential ATM phosphorylation-mediated activation of the specific factors triggering NHEJ or HR (Fig 2). Second, and most importantly, HR relies on the resection of the 5′-ended strands of the DSB, whereas NHEJ requires both ends to be protected for subsequent ligation. The decision between resection and no resection is thus a key step in pathway choice (Huertas, 2010; Cejka & Symington, 2021). 5′-end DNA resection occurs in a fast and processive manner to cover up to several kilobases, thereby releasing a 3′-end ssDNA that is quickly covered by the ssDNA binding protein complex RPA. RPA is then removed in mammalian cells via the mediator factor BRCA2 to load the strand exchange factor RAD51, which forms a nucleofilament with the 3′-ended DNA strand. This RAD51 nucleoprotein filament engages into the homology search to finally catalyze strand exchange with the sister chromatid (Heyer *et al*, 2010; Fig 2). If the homology is found in an ectopic DNA sequence located in the same (intramolecular) or a different (intermolecular) chromosome instead of the sister chromatid, it can lead to chromosomal rearrangements and genome instability. Importantly, however, RAD51 and other repair factors including MRE11 or BRCA2, not only function in DSB repair but also in the degradation or protection of stalled replication forks (Feng & Jasin, 2017). This may render the inference of mechanisms difficult if the results are only based on the recruitment of specific factors.

DSB repair pathway choice is not only affected by the cell cycle phase in which it occurs, but also by the genomic context. This is likely related to the pre-existing chromatin state, as recently reviewed (Ortega *et al*, 2021a). For instance, transcription can impair the spreading of γ-H2AX, as shown in budding yeast (Lee *et al*, 2014) and human cells (Iacovoni *et al*, 2010). However, it seems that there are mechanisms to ensure proper repair in transcribed regions and to direct the repair towards HR. Genome-wide analysis of endonuclease-induced DSBs at euchromatin regions revealed that there is a preference for the detection of RAD51 in regions with histone marks generally associated with transcriptionally active chromatin and elongating RNAPII (Aymard *et al*, 2014). This and other results led to the proposal that histone marks of actively transcribed euchromatin channel DSB repair towards HR and gave rise to the concept of transcription-coupled HR (Clouaire & Legube, 2015). However, a different study, involving DNA damage induction by IR or bleomycin, reported a preferential binding of NHEJ factors in RNAPII transcribed regions and no differences in RAD51 or RAD52 (Chakraborty *et al*, 2016). Although RAD51 recruitment might also reflect the frequent fork stalling on transcribed chromatin in the different damaging conditions tested, preferential repair of transcribed regions by HR has been confirmed in other reports (Yasuhara *et al*, 2018; Ouyang *et al*, 2021).

Changes in chromatin and transcription in the region around a DSB

Two relevant features occur in the chromatin around the DSB concomitantly with the induction of DNA damage and recruitment of

Figure 2. Scheme of the most relevant initial steps of the two pathways of DSB repair, NHEJ, and HR.

DSBs are signaled by the protein kinase Ataxia Telangectasia Mutated (ATM in mammals, Tel1 in *S. cerevisiae*). The MRN complex (MRX in *S. cerevisiae*) comprising the MRE11 nuclease, RAD50 and NBS1 (Xrs1 in *S. cerevisiae*), localizes to DSBs very quickly and associates with ATM, which phosphorylates the histone variant H2AX (H2A in yeast). This phosphorylation (γ-H2AX) spreads around the break site and triggers a signaling cascade that leads to changes in chromatin to facilitate the recruitment and action of DSB repair factors. In G1 cells, DSB-bound MRN associates with the 53BP1 protein that exerts an antagonistic role to BRCA1 in DNA resection. 53BP1 protects from 5′-end resection together with RIF1 and Shieldin, thus channeling DSB repair towards NHEJ which requires the KU70/80 complex, among other proteins. In S/G2 cells, DNA-bound MRN activates phosphorylation by ATM of the three MRN subunits as well as BRCA1 and CtIP (Sae2 in yeast) that promote 5′ DNA-end resection. Since 5′-end DNA resection occurs in the S/G2 phase to channel repair to HR with the sister chromatid, this step is regulated by cell cycle-dependent kinases (CDKs) that phosphorylate CtIP, among other factors. Resection is a two-step process. First, a short-range resection catalyzed by MRN and its cofactor CtIP occurs in the vicinity of the DSB. Second, a long-range resection of the 5′ end takes place by the action of the EXO1 exonuclease and the DNA2 nuclease, which act together with a DNA helicase, likely either BLM or WRN in human cells, or Sgs1 in yeast. Regardless of the nuclease used, resection occurs in a fast and processive manner up to over several kilobases. 5′-end resection releases thus a 3′-end ssDNA that is then covered by the ssDNA binding protein complex RPA. For HR to occur, RPA is removed in mammalian cells via BRCA2, in order to upload the strand exchange factor RAD51, which then forms a nucleofilament with the 3′-ended DNA strand. This RAD51 nucleoprotein filament engages into the homology search to finally catalyze strand exchange with the homologous template of the sister, generating a D-loop that will then allow DNA synthesis to proceed with HR. DNA strands are indicated in different colors (black or gray) to differentiate the homologous sequences.

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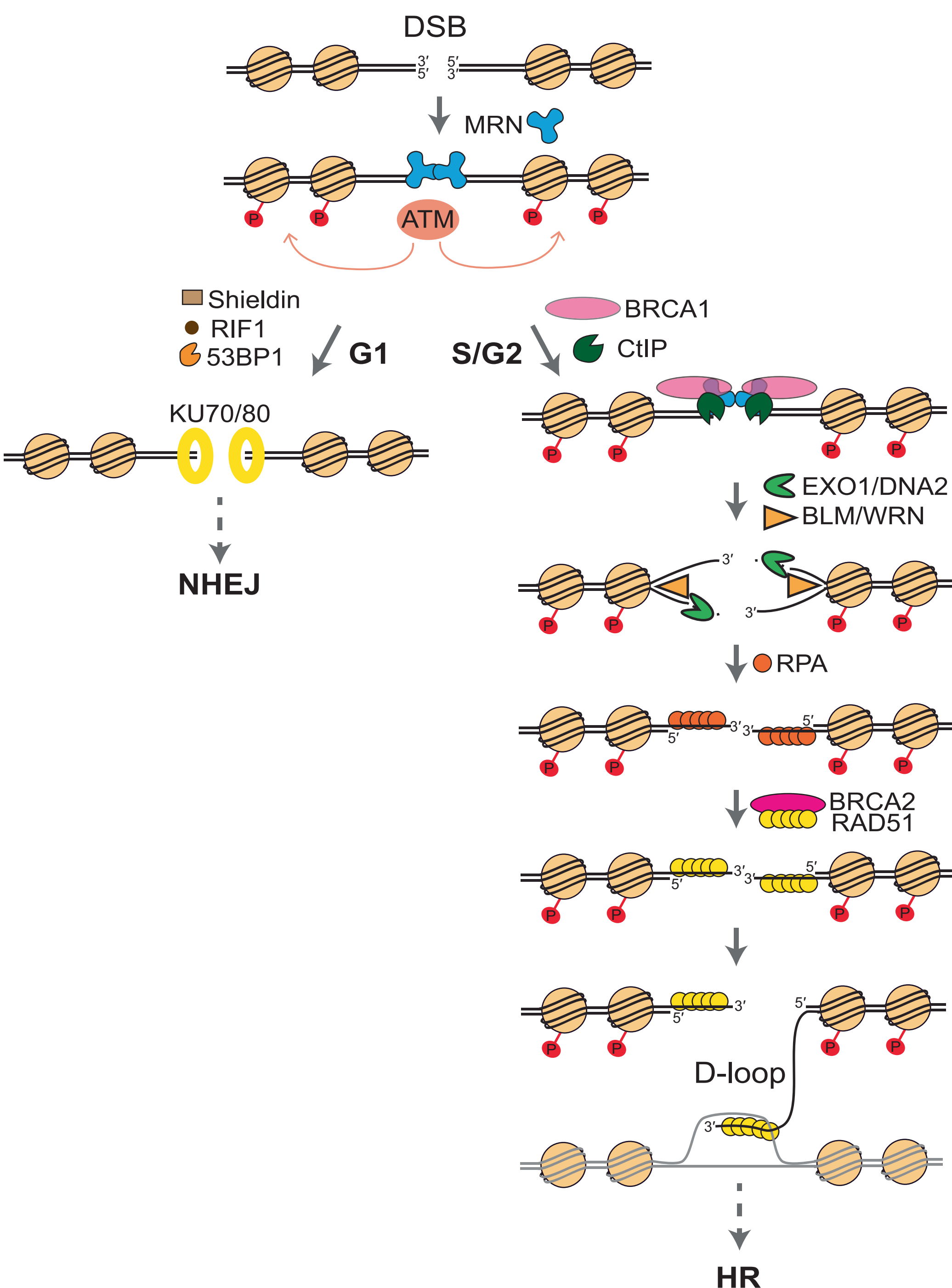

Figure 2.

repair factors: chromatin changes and transcription shutdown. Among the chromatin changes, DNA damage leads to the transient destabilization of chromatin organization. Using the HO endonuclease in budding yeast, it was shown that a first event is the eviction of nucleosomes around the break (Tripuraneni *et al*, 2021). In principle, this could rely on a passive response, since any break in the DNA will impact on the torsional stress associated with the natural negative superhelicity of DNA. Nevertheless, DSB-induced chromatin alteration seems to be a more complex and enzymatically catalyzed process. Thus, the human DDR factor 53BP1 was found by super-resolution microscopy to reshape chromatin topology around DSBs (Ochs *et al*, 2019). In addition, analysis of histone H2AB dynamics *in vivo* revealed a rapid reduction in condensation of chromatin fibers around the break 20 s after microirradiation (Kruhlak *et al*, 2006). Indeed, chromatin decondensation requires energy and occurs concomitantly with the recruitment of the first

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DSB repair factors (Kruhlak *et al*, 2006). However, the dynamics of chromatin changes around the break differ depending on the repair pathway employed, since decondensation or recompaction of chromatin around DSBs occur at different steps of NHEJ or HR (see Caron & Polo, 2020; Mohan *et al*, 2021). In any case, it is clear that destabilization of nucleosomes at DSBs by chromatin remodelers and histone modifiers is needed for the recruitment and action of either NHEJ or HR factors, although full mechanistic details are lacking (for reviews, see Papamichos-Chronakis & Peterson, 2013; Clouaire & Legube, 2015; Seeber *et al*, 2018; Ortega *et al*, 2021a).

The second event occurring around a DNA break, transcription shutdown, is likely needed to prevent any kind of interference between transcription and the DDR machinery. This is indeed well-known for damage induced by UV-irradiation, which causes global transcription shutdown in eukaryotes (Gregersen & Svejstrup, 2018). For DSBs, transcriptional silencing was first shown by monitoring RNA polymerase I (RNAPI) activity in mouse embryonic fibroblasts subjected to irradiation and photobleaching, which revealed that chromosome breakage impedes RNAPI initiation complex assembly and promotes displacement of elongating RNAPI from ribosomal DNA (Kruhlak *et al*, 2007). For RNAPII, transcription silencing was shown in human cells, based on the fact that histone H2A ubiquitylation by RNF8 and RNF168 was associated with DSBs and transcription silencing (Shanbhag *et al*, 2010). In this case, it was reported that a DSB generated by the FokI endonuclease caused silencing of a transcriptional reporter several kilobases away in cis from the DSB in an ATM-dependent manner. Similarly, IR-induced DSBs silenced endogenous genes as determined by run-on experiments as a direct way to measure transcriptional activity (Shanbhag *et al*, 2010). Importantly, such RNAP losses are not a passive consequence of facing the break, but actively induced by DDR signaling kinases as shown for ATM in the case of RNAPI (Kruhlak *et al*, 2007) and RNAPII, which also depends on DNAPK (Pankotai *et al*, 2012). In addition, transcriptional silencing relies on chromatin remodeling and modifications that are known to occur concomitantly with the different DSB repair steps (Caron *et al*, 2019; Long *et al*, 2021). In agreement with a general transcription shutdown, a novel assay based on the MS2 RNA sequence to visualize nascent RNA by live super-resolution microscopy experiments has revealed that induction of DSBs results in a rapid suppression of pre-existing transcription, regardless of the genomic location (Vitor *et al*, 2019). However, these experiments have also reported pervasive bi-directional transcription at intragenic DSBs (Vitor *et al*, 2019), suggesting that RNA polymerases are highly promiscuous and efficient in loading at DNA breaks and initiating transcription. In agreement, transcription has been shown at DNA breaks *in vitro* (Sharma *et al*, 2021). Whether this occurs at all DSBs or only at a small fraction, and either randomly or depending on particular structural, chromatin, functional, or cell cycle features of such breaks is unknown.

DNA–RNA hybrids in genome integrity

Research on the mechanisms and factors that control R-loop homeostasis and their relevance in cell physiology has grown exponentially (Li *et al*, 2023). RNA hybridization back to the DNA template from which it was transcribed is generally prevented by either the co-transcriptional processes of RNA translation in prokaryotes, or RNA-protein assembly and export to the cytoplasm in eukaryotes. However, the negatively supercoiled milieu created behind the advancing RNA polymerase, particular RNA processing mutants or elevated gene expression conditions can promote the induction of R-loops, which have the potential to interfere with DNA and RNA metabolic pathways such as DNA replication, transcription or RNA processing (Aguilera & Gomez-Gonzalez, 2008). Nowadays it is established that unscheduled R-loops are a natural source of DNA breaks and that transcription–replication conflicts are the main cause of R-loop-mediated genome instability (Fig 3A). Accordingly, multiple factors can help remove R-loops, including not only specific RNases (type H) that degrade the RNA moiety of hybrids and helicases that unwind hybrids, but also replication-associated repair factors and chromatin modifiers. This has been reviewed extensively (Garcia-Muse & Aguilera, 2019; Niehrs & Luke, 2020; Brickner *et al*, 2022; Cerritelli *et al*, 2022; Petermann *et al*, 2022) and we will not get into the details here. However, the relatively recent findings that DNA–RNA hybrids form at DSBs (Fig 3B) and impact their repair, whether positively or negatively, raises important questions that need to be specifically approached to understand whether these structures constitute undesired or desired companions in DSB repair.

The first hint of hybrid accumulation after DNA damage dates back to more than a decade ago, when *in vitro* studies revealed that an SSB in an Ig S fragment transcribed by T7-RNA polymerase induces DNA–RNA hybrid formation (Roy *et al*, 2010). Since this study was carried out *in vitro*, it suggests that SSBs induce the formation of hybrids without the need of any catalytic factor. Later, it was reported in cultured human cells that laser microirradiation, which leads to SSBs and DSBs, induces the accumulation of a catalytically inactive version of *E. coli* RNase H1 in a transcription-dependent manner (Britton *et al*, 2014). Other studies in cultured human cells and fission yeast detected DNA–RNA hybrids after DSB induction either directly by immunoprecipitation with the anti-DNA–RNA hybrid antibody (DRIP), or indirectly by ChIP with inactive RNase H (Li *et al*, 2016; Ohle *et al*, 2016). Since then, multiple laboratories have been able to detect hybrid accumulation associated with DNA breaks in different cells by either DRIP, immunofluorescence or slot-blots (Li *et al*, 2016; Ohle *et al*, 2016; Brustel *et al*, 2018; Cohen *et al*, 2018; D'Alessandro *et al*, 2018; Lu *et al*, 2018; Teng *et al*, 2018; Yasuhara *et al*, 2018; Alfano *et al*, 2019; Domingo-Prim *et al*, 2019; Jang *et al*, 2020; Matsui *et al*, 2020; Rawal *et al*, 2020; Tan *et al*, 2020; Yu *et al*, 2020; Zhang *et al*, 2020; Sessa *et al*, 2021; Ortega *et al*, 2021b). Additionally, the hybrid-binding domain of human RNase H1 fused to GFP allows for the detection of hybrids by live-cell microscopy after damage induction by laser microirradiation (Liu *et al*, 2021; Silva *et al*, 2022).

Hence, a wealth of evidence from studies in several organisms using different DNA damaging agents and tools to measure DNA–RNA hybrids supports that hybrids accumulate at broken DNA. However, conclusions vary with respect to the source of the RNA molecule that form hybrids at the DNA breaks, whether produced *in cis* or *in trans* and *de novo* or present before the break (Fig 4). To simplify from now on, we will refer to Break-Induced RNA–DNA

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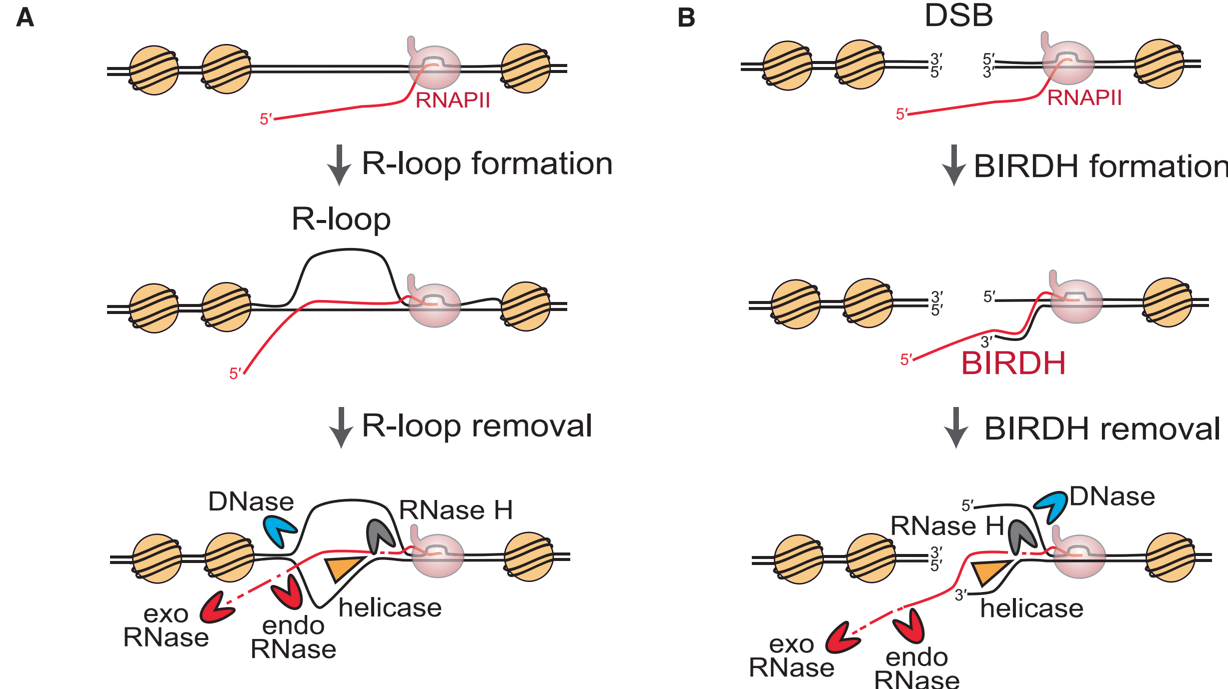

Figure 3. Co-transcriptional formation of DNA–RNA hybrids and BIRDHs, and factors involved in their removal.
(A) Co-transcriptional formation of R-loops in intact DNA. R-loops can be targeted by NER DNases, such as XPG and XPF, that can target the DNA flanking the DNA–RNA hybrid; RNases H, that degrade the RNA moiety of the DNA–RNA hybrid; and helicases, that unwind DNA–RNA hybrids. In addition, endo and exoRNases might degrade the RNA extruding from the DNA–RNA hybrid. (B) Induction of DNA–RNA hybrids after a DSB. These hybrids would not form R-loops, and we name them here BIRDHs (Break-Induced RNA–DNA hybrids). The release of the topological constraint imposed by a closed DNA molecule allows rotation and free separation of the DNA ends, facilitating the hybridization of the nascent DNA back with its DNA template. Any factor found to remove harmful R-loops is potentially capable of removing BIRDHs. Thus, BIRDHs can be targeted by R-loop resolvases such as DNases, RNases H and helicases, endo- and exo-RNases. It is unknown whether there are DNA–RNA hybrid resolvases with a preferential and/or specific role on BIRDHs versus R-loops and *vice versa*.

Hybrids as BIRDHs in contrast to R-loops, which are not associated with breaks (Fig 3).

Cis versus trans origin of RNAs forming hybrids and requirement of Rad51

The genomic source of the RNA that forms BIRDHs is a question of debate. In the case of spontaneous R-loops causing genome instability, hybrids are believed to form with RNA produced *in cis*, since they arise co-transcriptionally and involve the hybridization of nascent transcripts with the template DNA from which they were copied (Drolet *et al*, 1995; Huertas & Aguilera, 2003). This reaction could be enabled by the negative supercoil accumulated behind the elongating RNAP (Ma & Wang, 2016) that facilitates the transient opening of the two DNA strands, allowing R-loop formation, consistent with early results in *topA* mutants of *E. coli* and *top1* of *S. cerevisiae* (Drolet *et al*, 1995; El Hage *et al*, 2010). However, inspired by the fact that the bacterial strand-exchange protein RecA can catalyze hybrid formation *in vitro* (Kasahara *et al*, 2000; Zaitsev & Kowalczykowski, 2000), a study in budding yeast with an artificial chromosome showed that recombination was induced by transcription of a distant homologous locus and was sensitive to RNase H1 overexpression (Wahba *et al*, 2013). This suggested that hybrids could also arise from RNAs generated *in trans* with the help of the Rad51 strand-exchange activity. This study relied on a genetic system that infers an action of a nascent RNA *in trans* by determining intermolecular ectopic recombination, known to require Rad51. Thus, it seems also possible for nascent RNA to form an R-loop in *cis* with its DNA template, leading subsequently to a DSB and enabling the 3′-end of this DSB to act in *trans* to invade and induce the recombination event in a tri-parental recombination event, as previously shown (Ray *et al*, 1989; Ruiz *et al*, 2009; Piazza *et al*, 2017) and discussed (Gomez-Gonzalez & Aguilera, 2021). Indeed, a second study that measured intramolecular ectopic recombination between direct-repeats, which is Rad51-independent, showed that hyper-recombination caused by harmful R-loops was unaffected by transcription of a homologous sequence located in a different locus, thus favoring the hypothesis that harmful R-loops form *in cis* (Lafuente-Barquero *et al*, 2020).

The situation might be different at telomeres, where telomeric TERRA R-loops seem to have acquired a function promoting the recombination process responsible for the alternative lengthening of telomeres (ALT; Graf *et al*, 2017). In human cells, it has recently been shown that TERRA RNAs can form hybrids *in trans* in a RAD51-dependent manner (Feretzaki *et al*, 2020). This conclusion is supported by the observation that RAD51 can catalyze the invasion of RNA, in this case TERRA, into a circular dsDNA *in vitro* (Feretzaki *et al*, 2020). However, the need for both RAD51 and BRCA2 for TERRA RNAs to be recruited in *trans* to form DNA–RNA hybrids

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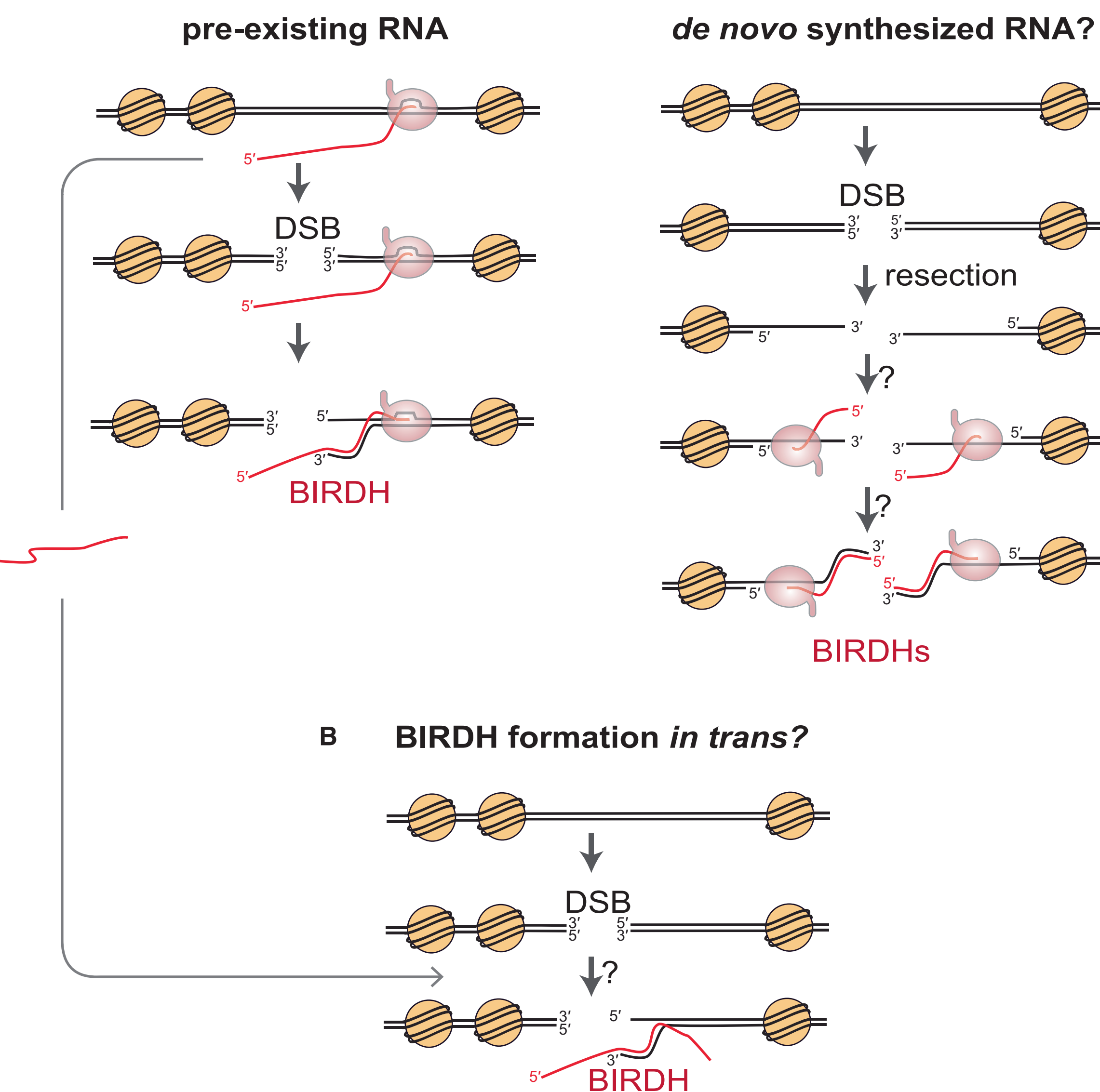

Figure 4. Source of RNAs forming BIRDHs.
(A) BIRDHs formed *in cis* can either involve pre-existing RNA molecules or maybe new small RNA molecules synthesized *de novo* at the DSB site. (B) BIRDHs could be formed *in trans* via annealing with RNA molecules originating from transcription at a distant homologous site.

plus the telomere fragility opens the possibility that this recruitment could also be mediated by a prior DSB. Given that TERRA R-loops form at multiple repeats, it would be interesting to see whether RAD51-mediated invasion of a broken telomeric DNA end during the ALT recombinogenic process could include a TERRA DNA–RNA hybrid formed in *cis* at repeats distal from the DNA 3′ end. Either way, telomeric repeats constitute a particular structure from which conclusions may not be easy to extend to R-loops formed at internal chromosomal regions not involving repetitive DNA sequences. Whether BIRDHs can be formed by RNAs generated *in trans* at DSBs occurring at unique or repetitive regions remains to be clarified (Fig 4).

De novo or pre-existing RNA at BIRDHs

Two possible scenarios for the source of RNA in BIRDHs can be considered: RNA molecules that are synthesized *de novo* at the break sites or, alternatively, RNA molecules that were already present in the region around the break (Fig 4). Although *de novo* RNAs have been found to be involved mainly in DDR signaling (Michelini *et al*, 2018), damage-induced long non-coding RNAs (dilncRNAs) have also been proposed to form BIRDHs at resected DNA ends and to help recruit recombination factors like BRCA2 or BRCA1 (D'Alessandro *et al*, 2018). In addition, exosome degradation of such BIRDHs has been reported to be required for both HR and

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NHEJ (Domingo-Prim *et al*, 2019). Another study reported that resection requires *de novo* transcription based on the observations that, upon treatment with the transcription inhibitor THZ1, recruitment of the CtIP resection factor at microirradiated sites was slowed down, whereas recruitment of the 53BP1 factor, which antagonizes resection, was faster (Gomez-Cabello *et al*, 2022). However, RNAPII retention could also explain these phenotypes, given that THZ1 not only reduces transcript levels but also causes the accumulation of RNAPII along gene bodies (Sampathi *et al*, 2019).

The detection of RNAPII at break sites has been used to claim the existence of *de novo* synthesized RNA molecules at breaks in several studies in *Schizosaccharomyces pombe* (Ohle *et al*, 2016) and human cells (Michelini *et al*, 2017; Burger *et al*, 2019; Jang *et al*, 2020). In agreement, MRN-driven melting of the DNA ends was shown to allow RNAPII transcription (Sharma *et al*, 2021), but another study reported the accumulation of RNAPIII, and not RNAPII at break sites (Liu *et al*, 2021). Strikingly, this accumulation was reduced upon MRE11 depletion, arguing that RNAPIII recruitment to DSBs is also dependent on MRE11 (Liu *et al*, 2021). Recently, these authors have discussed that only RNAPIII has the ability to initiate transcription at breaks, but not RNAPI and RNAPII (Liu *et al*, 2022). However, accumulation of a factor at a site may be explained by either *de novo* recruitment or retention. Therefore, RNAP accumulation could also correspond to pre-existing proteins that might be retained at the break site once this is signaled by the DDR. Indeed, no changes in transcript levels were observed at endonuclease-induced DSB sites in *S. pombe* (Ohle *et al*, 2016). Moreover, extensive small RNA sequencing allowed detection of damage-induced RNAs only at DSBs within highly transcribed repetitive regions (Bonath *et al*, 2018). Thus, further evidence is required to support the concept of *de novo* recruitment of RNAP and RNA synthesis at DSBs to significant levels such that is necessary to have a key physiological role. A different and attractive possibility is that the *de novo* transcription detected at breaks could be initiated by pre-existing or newly formed R-loops, given that different reports have shown that DNA–RNA hybrids can act as transcription initiators (Tan-Wong *et al*, 2019). In that case, BIRDHs could have a role in the initiation of transcription of small RNAs at break sites, and thus, contribute to signaling.

However, the fact that small RNAs were not detected in other studies and that pre-existing transcriptional activity predisposed to BIRDH formation does not favor the model that *de novo* small RNAs are involved in BIRDH formation (Lu *et al*, 2018). Further supporting that BIRDHs involve RNA molecules that were already present in the broken region, genome-wide analysis of human cell cultures revealed that pre-existing transcription is required for the formation of BIRDHs (Cohen *et al*, 2018; Bader & Bushell, 2020). Moreover, BIRDHs were strongly enhanced by transcription when measured at a GFP reporter in human cells (Ouyang *et al*, 2021) and BIRDH accumulation in *S. cerevisiae* occurred specifically when the cleavage region was transcribed (Ortega *et al*, 2021b). Therefore, regardless of whether *de novo* synthesis of any type of RNA occurs at breaks or not, so far, most evidence points to pre-existing RNA molecules as the most likely source of BIRDHs.

Cellular factors that modulate BIRDH levels

BIRDHs can passively or actively interfere with the repair of the DSB at different stages. Initial reports suggested that BIRDHs could be deleterious, since the inhibition of the RNA-binding proteins (RBPs) FUS or SAF-A prolonged the time that hybrids accumulated at breaks and inhibited both HR and NHEJ repair pathways (Britton *et al*, 2014). FUS belongs to nuclear bodies of unknown function called paraspeckles, which are induced upon stress. Interestingly, other paraspeckle proteins have also been related to DSB repair, such as NONO (Krietsch *et al*, 2012) or RBM14 (Simon *et al*, 2017). However, RBPs with a direct role at BIRDHs will likely be limited to those that can resolve DNA–RNA hybrids. Thus, we now introduce the subset of R-loop resolvases that have been described to impact BIRDH levels from those factors that are generally involved in the removal of harmful R-loops.

Control of BIRDHs by RNases

Thus far, type H RNases, which are present in all branches of life, are the only enzymes described as able to cleave the RNA moiety of DNA–RNA hybrids (Cerritelli & Crouch, 2009; Hyjek *et al*, 2019; Fig 3). A role for type H RNases in DNA damage repair was suggested in a study using *S. cerevisiae* mutants (*rnh1*Δ *rnh201*Δ), which led to persistent Rad52 foci and cell cycle arrest (Amon & Koshland, 2016), although the presence of BIRDHs was not directly studied. BIRDHs were reported in *rnh1*Δ *rnh201*Δ mutants in a study using *S. pombe* (Ohle *et al*, 2016), which analyzed the appearance of hybrids by DRIP after induction of direct cleavage by endonucleases (Ohle *et al*, 2016). BIRDHs interfered with DSB repair as measured by the recovery of PCR signal overlapping the cleavage site. Similarly, BIRDHs were also detected in *rnh1*Δ *rnh201*Δ *S. cerevisiae* mutants at replication-induced DSBs, affecting the appearance of HR intermediates and products and leading to genetic instability (Ortega *et al*, 2021b). Both of these studies (Ohle *et al*, 2016; Ortega *et al*, 2021b) supported that BIRDHs must be removed for repair to proceed.

The observation that overexpression of RNase H1 in *S. pombe* delayed the detection of PCR signal overlapping the cleavage site, together with the increased sensitivity to damage induction, led to the proposal that BIRDHs might also play a positive role in HR repair (Ohle *et al*, 2016). Along the same lines, RNase H1 overexpression in human cells affected the repair of endonuclease-induced breaks (Lu *et al*, 2018). However, this impairment was observed in both NHEJ and HR assays, which is not concordant with a specific role for BIRDHs in one of the repair pathways. Moreover, RNase H1 overexpression is known to be toxic, likely because of its role in the removal of the RNA primer of Okazaki fragments. Notably, overexpression of active RNase H1 caused no effect in the appearance of repair intermediates or products after replication-induced DSBs in budding yeast, which would have been expected for a positive role for BIRDHs in repair (Ortega *et al*, 2021b).

In addition to RNases H, several other RNases have been shown to accumulate at DSBs, prevent BIRDH accumulation, or affect DSB repair. These include the exoRNases XRN1 (Manfrini *et al*, 2015), XRN2 (Morales *et al*, 2016), and the RNA exosome (Richard *et al*, 2013; Manfrini *et al*, 2015; Marin-Vicente *et al*, 2015; Domingo-Prim *et al*, 2019; Gritti *et al*, 2022), as well as endoRNases, as recently shown for yeast Rad27, which cleaves the telomere repeat-containing RNA TERRA when forming a flap at telomere R-loops (Liu *et al*, 2023). Thus, the activity of RNases could aid in the regulation or progression of different steps of DSB repair, likely through the degradation of RNA ends that extrude from BIRDHs.

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However, as no other RNase has been reported to be able to target DNA–RNA hybrids, in principle, only type H RNases would be able to target BIRDHs directly.

Control of BIRDHs by DNA–RNA helicases

Given the cost of RNA synthesis, cells have evolved abilities to unwind rather than degrade the nascent RNAs sequestered in R-loops during transcription. Therefore, it is not surprising that a number of RNA helicases have been shown to unwind DNA–RNA hybrids, with their inactivation or depletion triggering R-loop accumulation in cells (Fig 3). These include SETX/Sen1, UAP56/DDX39B, DDX19, DDX21, AQR, DDX9, DDX1, among others, as reviewed recently (Garcia-Muse & Aguilera, 2019; Brickner *et al*, 2022). These helicases do not seem to be redundant and it should be noted that not all of them have been sufficiently studied to confirm their ability to unwind hybrids (Luna *et al*, 2019). Moreover, only some of these RNA helicases have been specifically studied at BIRDHs. The first one whose function was related to BIRDHs was the human DEAD box helicase DDX1. This helicase forms DDR foci upon ionizing radiation (IR) that colocalize with γH2AX and ATM. In addition, DDX1-depleted cells were sensitive to RNase H1 overexpression, indicating a dependence on hybrids (Li *et al*, 2008). BIRDHs were then confirmed by DRIP upon DDX1 depletion, which also affected the efficiency of repair by HR (Li *et al*, 2016). Similarly, other helicases have been later reported to impact BIRDH formation or resolution, based on either the fact that they were directly detected at DSBs by live-cell microscopy, immunofluorescence or ChIP, or because their loss caused changes in BIRDH levels and affected HR repair. These include SETX (Cohen *et al*, 2018; Rawal *et al*, 2020), DHX9 (Chakraborty & Hiom, 2021), DDX5 (Mersaoui *et al*, 2019; Yu *et al*, 2020; Sessa *et al*, 2021), DDX17 (Bader *et al*, 2022), and UPF1 (Ngo *et al*, 2021). It is likely that other helicases known to protect cells from harmful R-loops could also have similar role on BIRDHs, but studies are still lacking.

The key question again would be why there are so many different helicases to do a similar job on apparently the same BIRDH structures. In this regard, it seems that helicases may have specific functions at DSBs, since the effect of their loss is not always the same. For instance, in contrast to DDX1, DHX9 did not redistribute to γH2AX foci upon ionizing radiation but did so after treatment with CPT in a transcription and hybrid-dependent manner (Chakraborty & Hiom, 2021). Thus, each helicase might be specialized in the repair of DNA damage induced by certain agents or in a certain type of lesions. A second possibility is that each helicase could function in a subset of genomic damaged sites. In agreement, not all IR-induced γH2AX foci overlapped with DDX1 (Li *et al*, 2008). Strikingly, another DEAD box helicase, DDX5, is initially excluded from DSB sites induced by laser microirradiation (Yu *et al*, 2020; Sessa *et al*, 2021). However, DDX5 was detected by ChIP upon endonucleolytic induction of breaks (Yu *et al*, 2020; Sessa *et al*, 2021) arguing that the initial exclusion detected is a consequence of the general transcription shutdown triggered by DSBs. The helicase senataxin (SETX) has also been detected at endonuclease-induced breaks in sites undergoing transcription as analyzed genome-wide by ChIP-seq (Cohen *et al*, 2018), and budding yeast SETX ortholog Sen1 accumulates at HO endonuclease-induced DSB, limiting the local accumulation of BIRDHs (Rawal *et al*, 2020). Interestingly, human SETX and DDX5 depletion led to increased BIRDHs at endonuclease-induced DSBs, suggesting that these two helicases are important to counteract BIRDHs (Cohen *et al*, 2018; Sessa *et al*, 2021).

In contrast, the loss of the DEAD-box helicase DDX17 was recently reported to reduce the levels of BIRDHs (Bader *et al*, 2022). Since this reduction was observed exclusively in DSBs occurring at regions that are not R-loop prone in undamaged conditions, and DDX17 depletion affected the DSB signaling cascade at the step of RNF168 ubiquitylation of γH2AX and the subsequent steps of 53BP1 and BRCA1 recruitment, it has been proposed that BIRDHs are required to enable proper DSB signaling (Bader *et al*, 2022). However, given the potential impact of most RNA helicases on transcription and RNA metabolism, it would be useful to show that these helicases do not affect the processing and termination of nascent RNAs trapped around DNA breaks, thus potentially having an indirect impact on repair by altering 3′-end resection or the loading of HR factors. Systematic studies with multiple helicases and assays upon DSB induction as well as genome-wide approaches would be needed to decipher the potential roles of each helicase in BIRDH processing, if any.

Impact of BIRDHs on HR repair

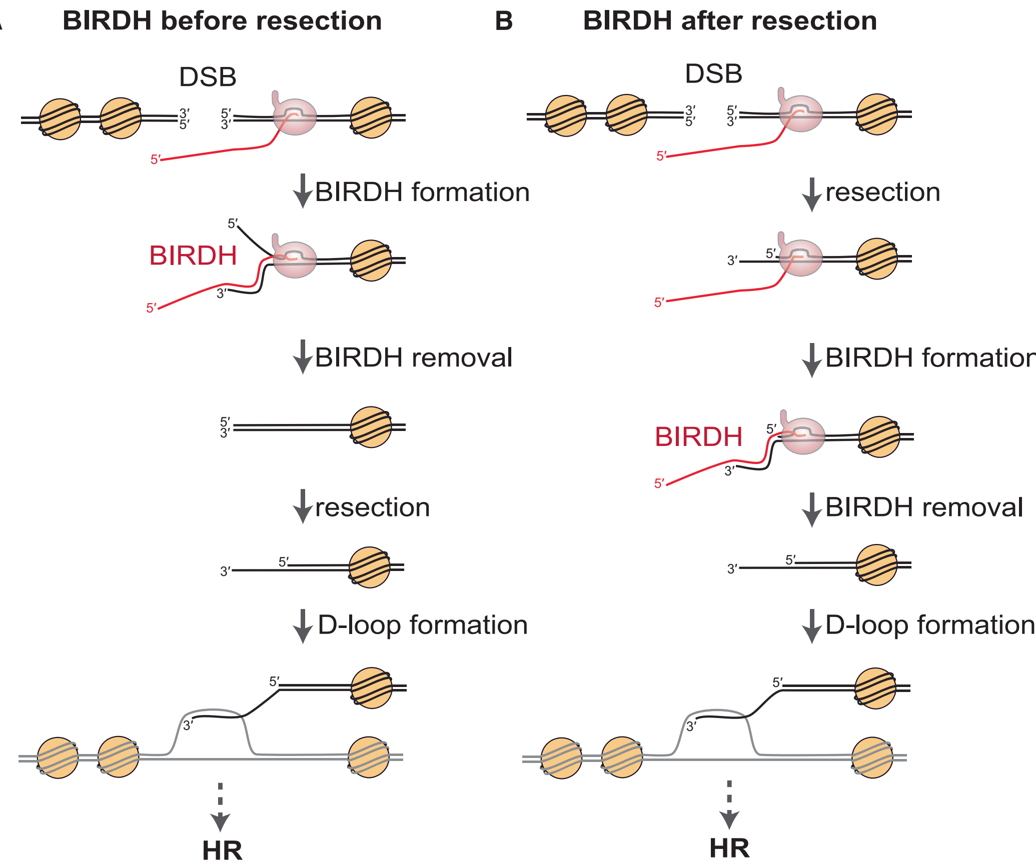
BIRDHs can form at different stages before or even during the repair reaction and this will define the step that will be positively or negatively affected by their formation. Research on the impact of BIRDHs on DSB repair has not always led to the same conclusions, possibly because each study was influenced by the different DSB sources and DSB repair systems used, as discussed above. Disentangling the molecular bases of such differences will help unravel the physiological role of BIRDHs in repair. With this aim we discuss different reports providing insight into (i) the role of BIRDHs in pathway choice, (ii) whether BIRDH removal is required to allow DSB repair, and (iii) whether hybrids are involved at central steps of DSB repair (Fig 5).

BIRDHs and the repair pathway choice

Several studies support that BIRDHs impact the repair pathway choice. Depletion of the helicase senataxin in human cells decreased Rad51 foci formation and increased 53BP1 accumulation after genome-wide endonuclease-induction of DSBs at euchromatin

Figure 5. Potential impact of BIRDHs in DSB repair via HR.

(A) BIRDHs formed before 5′-end resection might impair this step or protect the 3′-end, but would need to be removed for HR to proceed. (B) BIRDHs formed after 5′-end resection might stabilize the 3′-end but would need to be removed for HR to proceed. (C) DNA–RNA hybrids formed at the donor DNA template might either facilitate invasion and D-loop formation or stabilize the D-loop once it is formed. The structure in which R-loops coexist with the invading ssDNA end is termed DR-loop' and the hybrid would also need to be removed for HR to proceed. DNA strands are indicated in different colors (black or gray) to differentiate the homologous sequences.



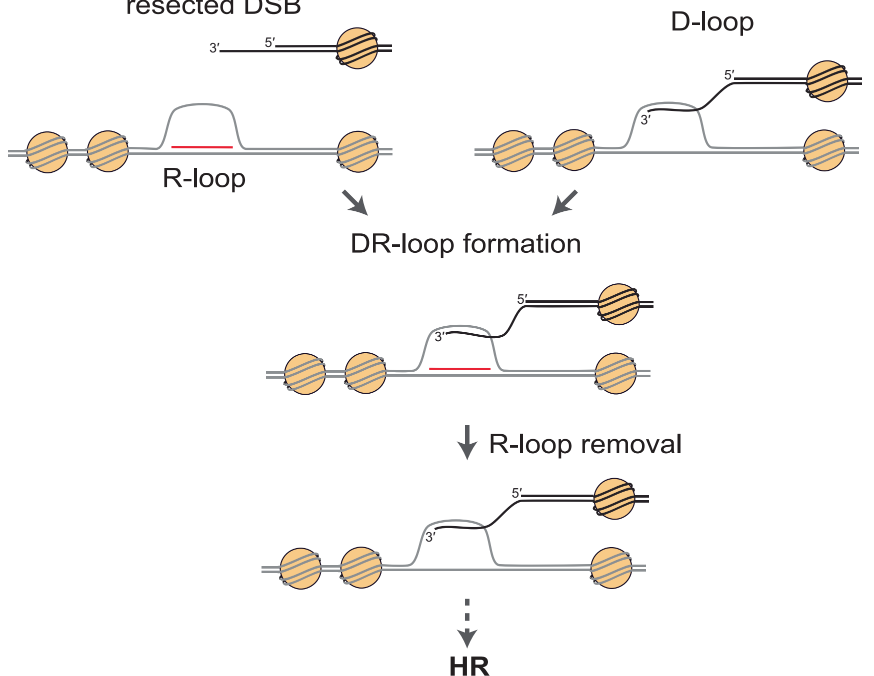


Figure 5.

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regions (Cohen *et al*, 2018). This effect was suppressed by the transcription elongation inhibitors cordycepin and 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB). In agreement with channeling DSB repair towards NHEJ, loss of senataxin triggered a mild decrease of HR and a concomitant mild increase of NHEJ determined using reporter systems, causing translocations and large XPF-dependent deletions and decreasing survival due to translocations mediated by BLM and POLD3 DNA synthesis (Brustel *et al*, 2018; Cohen *et al*, 2018, 2022). Since the repair pathway choice is governed by the resection process, these results could imply an impairment of resection that would also explain the increase of NHEJ. Interestingly, although resection of these DSBs was unaffected upon senataxin inhibition (Cohen *et al*, 2018), impaired resection and non-canonical resection that neither requires Sgs1 to unwind DNA nor Exo1 to degrade DNA ends was reported in budding yeast mutants of the senataxin ortholog Sen1 (Rawal *et al*, 2020), further suggesting that senataxin-mediated removal of BIRDHs is important for the canonical resection process.

Interestingly, loss of other human helicases that prevent BIRDH accumulation correlates with phenotypes compatible with impaired resection. This is the case for the loss of DDX5 or DHX9, which reduced RPA signal and impaired HR (Yu *et al*, 2020; Chakraborty & Hiom, 2021; Sessa *et al*, 2021). Moreover, DDX5 loss reduced EXO1 recruitment and ssDNA accumulation (Yu *et al*, 2020) and DHX9 loss increased NHEJ efficiency and RIF1 accumulation on chromatin (Chakraborty & Hiom, 2021). Similarly, loss of the RBP hnRNPD induced BIRDH accumulation and impaired resection (Alfano *et al*, 2019). Thus, these results in human cells support that BIRDHs impair resection, implying that they are formed before this step. In agreement, CtIP depletion induces accumulation of catalytically inactive RNase H1 at laser microirradiation sites (Makharashvili *et al*, 2018). Reduced RPA signal was also reported in *rnh1*Δ *rnh201*Δ fission yeast mutants (Ohle *et al*, 2016), but RNase H1 overexpression caused RPA to extend throughout long regions, indicating that BIRDHs might prevent harmful over-resection that could lead to genome instability. On the other hand, although no defect in resection was reported upon depletion of DDX1, the maintenance of ssDNA at resected ends was reduced, impairing Rad51 and RPA foci formation (Li *et al*, 2016). Interestingly, DDX1 interacts with RPA-coated ssDNA (Marechal *et al*, 2014). It would be worth investigating whether DDX1 might help remove BIRDHs and protect ssDNA from degradation by RPA loading. Although it is possible that these phenotypes are not specifically due to a role for these helicases at BIRDHs, taken together, these results generally support that BIRDHs impair DNA resection.

BIRDHs could interfere with the DNA resection reaction directly, given that the presence of an RNA strand at the DNA end could prevent DNA exonuclease activity. In support of this hypothesis, whereas short stretches of ribonucleotides at the 5′ terminus might aid EXO1 action *in vitro*, long DNA–RNA hybrids inhibit EXO1 resection activity (Daley *et al*, 2020). Alternatively, BIRDHs could limit resection by promoting the binding of NHEJ factors that protect DNA ends from degradation. It has been recently shown *in vitro* and *in vivo* that the *S. pombe* Ku protein binds to BIRDHs (Audoynaud *et al*, 2023). In agreement, preferential binding of NHEJ factors to IR or bleomycin-induced damage at transcribed regions has been reported (Chakraborty *et al*, 2016). The role of hybrids in protecting DNA ends from degradation seems similar at DSBs and replication forks, where the RNA primers in Okazaki fragments have been shown to contribute to engage Ku at stalled forks to protect nascent DNA strands from nuclease action (Audoynaud *et al*, 2023).

Thus, although beneficial at stalled forks, the potential ability of BIRDHs to prevent resection could be detrimental for most of the DSBs. However, it could also help regulate the repair pathway choice at certain genomic regions. This seems to be the case in the Ig S regions, where R-loops impair resection to promote NHEJ, responsible for class switching (Refaat *et al*, 2023). Along the same lines, DDX1 has been shown to resolve G4 structures at Ig S regions to promote R-loops and efficient class switching (Ribeiro de Almeida *et al*, 2018). In this case, DNA–RNA hybrids formed at Ig S regions seem to ensure that the subsequently formed DSB is not resected, thus favoring NHEJ-mediated class switching.

An important characteristic to be considered is whether hybrids accumulate at both sides of a DSB or only at one. DNA–RNA hybrids have been detected at both sides of a DSB in budding and fission yeast (Ohle *et al*, 2016; Ortega *et al*, 2021b). The fact that an RNA molecule generated prior to the DSB hybridizes only with its template DNA implies that the structure of such hybrids is asymmetrical in terms of the two DNA ends. Hybrids could form with the 5′ end at one side of the break and with the 3′ end on the other side. However, strand-specific detection of hybrids in *S. pombe* has revealed their presence on the forward DNA strand upstream of the cleavage site and on the reverse DNA strand downstream of the cleavage site suggesting that BIRDHs may be formed exclusively at the 3′ ended strands (Ohle *et al*, 2016; Fig 4A). Similarly, BIRDHs were detected at 3′ but not at 5′ ended strands in human cells (Liu *et al*, 2021). This would be compatible with hybrids being formed at already resected ends (Fig 4B). However, BIRDHs are not affected by ssDNA availability (Cohen *et al*, 2022). It seems to be possible that BIRDHs can form at 3′ ended strands both before and after resection (Fig 5A and B). In addition to the evidence that BIRDHs impair resection, there are also reports that support a positive role of BIRDHs in repair, based on the observation of active BIRDH accumulation at DSBs. For instance, DROSHA depletion reduces BIRDHs at transcribed DSB sites (Lu *et al*, 2018; Bader *et al*, 2022); the helicase UPF1 promotes BIRDHs at sub-telomeric regions and promotes resection (Ngo *et al*, 2021); and the loss of the helicase DDX17 decreased RPA foci, impaired resection and reduced BIRDH levels (Bader *et al*, 2022). These results support that BIRDH formation, rather than accidental, might be induced to fulfill a task at a particular repair step. In this sense, it is conceivable that BIRDHs aid resection if DNases target R-loops, as previously discussed (Aguilera & Gomez-Gonzalez, 2017). Indeed, it has been proposed that human RAD52 promotes XPG activity to process DNA–RNA hybrids around a DSB to leave ssDNA overhangs that would resemble resected DSBs, thus favoring a process of transcription-associated HR repair (Yasuhara *et al*, 2018). Therefore, one possible model to cover all observations could be that although BIRDHs limit the action of nucleases and the loading of RPA impairing resection, they can exert positive roles to protect 3′ ends or if targeted by R-loop processing DNases. Further work would be required to determine whether this would be a frequent and programmed step in DSB repair or a sporadic event.

DDR and BIRDH removal during repair

Whether formed before or after resection, and whether having a positive or negative impact on this step, BIRDHs must be removed

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to proceed with the repair reaction. It is thus possible to imagine that BIRDHs are a kind of "dirty ends" that require processing before repair. In this sense, SWI/SNF chromatin remodelers, which are essential DDR components, have been recently shown to promote not only RAD52 but also RNase H recruitment to DSBs to facilitate repair (Davo-Martinez *et al*, 2023). Moreover, evidence suggests that DNA damage favors functional interactions between HR factors and DNA–RNA hybrid resolvases. This is the case for BRCA1 with senataxin (Hatchi *et al*, 2015), BRCA1 with DHX9 and USP52 (Matsui *et al*, 2020; Chakraborty & Hiom, 2021) or BRCA2 with RNH2 (D'Alessandro *et al*, 2018), XRN2 with DDX5 (Mersaoui *et al*, 2019) and BRCA2 with DDX5 (Sessa *et al*, 2021). The biological relevance of these interactions can be twofold, either to facilitate recruitment of hybrid resolvases to DSB sites, or of DSB repair factors to DNA–RNA hybrids, given their potential to be associated with DSBs. In any case, a defect in DSB repair caused by the loss of a hybrid resolvase could also be the indirect consequence of the recruitment of repair factors to R-loop sites, as shown for the EWS1-FLII factor that blocks BRCA1-mediated repair by sequestering BRCA1 at R-loop sites in Ewing sarcoma (Gorthi *et al*, 2018).

Alternatively, in addition to promoting the recruitment of hybrid resolvases, the DDR machinery might also take advantage of factors with a role in R-loop removal present at the site before the DSB occurred. This seems to be the case for the DDX5 helicase, which is present in transcribed chromatin and retained at DSBs through an interaction with BRCA2 that stimulates its intrinsic DNA–RNA unwinding activity to remove BIRDHs (Sessa *et al*, 2021). Accordingly, DDX5 ChIP showed BRCA2-dependent enrichment at DSBs suggesting that a portion of the pre-existing DDX5 is actively retained at DSBs by BRCA2 to counteract BIRDHs during HR stages downstream of the resection step (Sessa *et al*, 2021). In this sense, it would be interesting to explore whether the accumulation of hybrids observed in DSB repair-deficient cells, such as BRCA2-deficient cells (Bhatia *et al*, 2014), is in part due to the accumulation of unrepaired DSBs, which could be extended to any condition compromising DSB repair. Along this line of thought, other HR repair mutants may also show high levels of hybrids, due to their enrichment at unrepaired DSBs.

The DDR seems to exert a further layer of regulation of repair by the post-transcriptional modification of the RNA, which could also occur within BIRDHs. As such, ATM triggers the localization of METTL3, which catalyzes the most common RNA modification, m6A, at BIRDHs through its interaction with RNAPII, thus ensuring that this modification is present in transcribed chromatin when it breaks (Zhang *et al*, 2020). This modification impacts the stability of hybrids (Abakir *et al*, 2020; Zhang *et al*, 2020). Moreover, the DDR induces RNA editing, such as A-to-I deamination by ADAR proteins, which was shown to promote the recruitment SETX and BRCA1 for efficient resection and HR (Jimeno *et al*, 2021). Other RNA modifications might also impact DNA damage repair, as shown for m5C (Chen *et al*, 2020), although its role at BIRDHs has not been addressed. Although unintuitive, a role for RNA modifications in BIRDH stability and DSB repair, whether direct or indirect, should be further explored.

R-loops at the donor DNA template

Once DNA DSBs have been resected to allow loading of RAD51, a question emerging is whether DNA–RNA hybrids could still regulate DSB repair. Interestingly, a recent study (Ouyang *et al*, 2021) suggested that hybrids can further facilitate HR at downstream steps. Transcription-dependent hybrids were detected by DRIP not only at the DSB region but also at the donor DNA region in an intramolecular direct-repeat system in human cells. This suggests that D-loop formation could either be preceded by a *trans* RNA invasion so that the RNA would serve as a homology searcher itself, opening the path to the ssDNA end (R to D-loop switch), or stabilize the invaded ssDNA end once D-loops have been formed (Fig 5C). Interestingly, the formation of the structure that will result from the coexistence of R-loops at the donor template with the invading ssDNA end, termed "DR-loop," is stimulated *in vitro* by the RAD51-associated protein 1 (RAD51AP1) and, in agreement with its role *in vivo*, RAD51AP1 depletion reduced hybrid detection at the donor region. Moreover, RAD51AP1 promoted the stimulation of HR by either transcription or RNA tethering, suggesting that RAD51AP1-mediated hybrid formation promotes HR (Ouyang *et al*, 2021).

This mechanism was proposed to support the ALT pathway at telomeres in the absence of RAD52 (Kaminski *et al*, 2022). In this case, TERRA R-loops seem to favor D-loop formation indirectly by facilitating G4 structures in the displaced ssDNA strand (Yadav *et al*, 2022). Globally, these results suggest that DNA–RNA hybrids formed during HR would favor the recombination event. Along the same lines, it has been recently shown that the displaced strand of R-loops can be invaded by break DNA ends to promote transcription-coupled spontaneous insertions (Min *et al*, 2023) further indicating that R-loops in a donor homologous template favor invasion. These results are of relevance in the context of transcription-associated genetic instability in non-dividing cells, as R-loop invasion by DNA break ends leads to mutagenic insertions that are independent of replication.

Nevertheless, HR events may have different outputs and regulation when occurring in the same DNA molecule (intramolecular HR), or in different molecules, such as the sister-chromatid or the homologous chromosome (intermolecular HR). Intriguingly, the *hpr1* mutation of the THO complex, which confers high levels of R-loops, increases intramolecular gene conversion but has no effect on intermolecular gene conversion (Santos-Rosa & Aguilera, 1994). Indeed, there was no positive effect detected for BIRDHs in intermolecular yeast recombination systems (Ortega *et al*, 2021b). It is thus also possible that DSB-induced changes in supercoiling or chromatin that expand from the break site facilitate the formation of hybrids at the donor region located in the same DNA molecule that was cleaved, with such hybrids being DR-loops. It would be interesting to assess whether these structures would be compatible with HR occurring between alleles located in different molecules, or whether they only occur at HR between intramolecular repeats.

As in early steps of HR, hybrids potentially formed within DR-loops would also need to be transient and removed by helicases or RNases in order for downstream recombination steps to proceed. Indeed, several data support that this is the case at potential telomeric DR-loops. Senataxin depletion enhanced the presence of RAD51AP1 at telomeres (Kaminski *et al*, 2022) and knockdown of RNase H1 impaired ALT implying that telomeric R- to D-loop switch requires R-loops to be transient (Yadav *et al*, 2022). Thus, regardless of their potential impact on D-loop formation, DR-loops will need to

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Box 1. In need of answers

- What is the frequency at which a BIRDH can be formed at a single DSB?
- Does the frequency of BIRDHs depend on specific topological or chromatin features of the regions where they can form?
- Is BIRDH formation affected by the conditions in which the DSB happens, such as cell-cycle stage, developmental stage, tissue, or aging?
- Can RNAP be recruited *de novo* to broken DNA to synthesize RNA to a significant level as to have a key physiological role in repair?
- Which DNA–RNA hybrid resolvases can act at BIRDHs? What is the specificity of these different enzymes at R-loops versus BIRDHs? Are they specific for different kind of DSBs or at different genomic locations?
- Is removal of BIRDHs regulated by the DDR?
- Can BIRDHs form before and after DSB resection? Do BIRDHs interfere with resection directly or via the binding of proteins?
- Does R-loop targeting by nucleases favor DSB resection?
- Is there any role for the different RNA modifications at BIRDHs and HR?
- Is a homologous sequence prone to R-loop formation easier to be found by Rad51 nucleofilaments than a non-R-loop prone homologous sequence? Can DSB induce hybrid formation in a different homologous DNA molecule? If so, does this impact repair?

be removed to proceed with the DSB repair reaction, thus revealing both positive and negative impacts on HR.

Concluding remarks

Despite occurring at very low frequency, DNA–RNA hybrids formed sporadically as a byproduct of transcription along the transcribed genome hold great biological relevance because R-loops are a source of transcription-replication conflicts and DNA breaks. Thus, their impact in cell physiology and development may be very high, as indeed is the case for spontaneous DNA lesions such as DSBs, also occurring sporadically. DSBs occurring at regions with ongoing transcription induce the appearance of a different type of DNA–RNA hybrids, BIRDHs. Regardless of the controversy of whether these can also be formed as a consequence of *de novo* transcription or not, there are reports that support either a positive or a negative role of BIRDHs in DSB repair. The results suggest that BIRDHs might have different effects depending on the system used or the type of event analyzed in each study. An unsolved question is whether BIRDHs are structures that have evolved to become a key intermediate in the repair of DSBs, particularly in HR. If that were the case, rather than being a sporadic event, we would expect BIRDHs to form in a controlled manner, for which we have no evidence so far. Rather, they seem to be structures that can eventually form at transcribed regions undergoing a DSB, which, depending on their topological structure or perhaps chromatin features, can favor the repair reaction or constitute an obstacle to it; thus responding more to a local and specific phenomenon than to a general rule. To be able to answer this and other intriguing questions (Box 1), we require deeper knowledge on BIRDHs, particularly with respect to the upstream molecular process, whether they are genetically controlled, the frequency at which they occur and the DNA structural and functional features that may affect their formation and resolution.

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Belén Gómez-González: Writing – original draft; writing – review and editing.
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The authors declare that they have no conflict of interest.

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