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Toxic R-loops: cause or consequence of replication stress ?

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Transcription-replication conflicts (TRCs) represent a potential source of endogenous replication stress (RS) and genomic instability in eukaryotic cells but the mechanisms that underlie this instability remain poorly understood. Part of the problem could come from non-B DNA structures called R-loops, which are formed of a RNA:DNA hybrid and a displaced ssDNA loop. In this review, we discuss different scenarios in which R-loops directly or indirectly interfere with DNA replication. We also present other types of TRCs that may not depend on R-loops to impede fork progression. Finally, we discuss alternative models in which toxic RNA:DNA hybrids form at stalled forks as a consequence - but not a cause - of replication stress and interfere with replication resumption.

1. Introduction

R-loops are stable structures that form during transcription when the nascent RNA reanneals with the template DNA, generating an RNA:DNA hybrid and a displaced single-stranded DNA (ssDNA) loop (1). Different techniques have been developed to analyze R-loops, from individual loci to the whole genome level (2). These structures differ by their length, sequence and genomic context. They are very abundant, covering up to 5% of mammalian genomes (3, 4). R-loops play multiple physiological roles such as the regulation of immunoglobulin (Ig) class-switch recombination, CRISPR-Cas9 activity, DSB repair, initiation of mitochondrial DNA replication, chromatin patterning and gene regulation, including the protection of CpG islands against DNA methylation and the regulation of transcription termination (1, 5-8). R-loops represent also a potential source of genomic instability, presumably by increasing transcription-replication conflicts. Indeed, the replication and transcription machineries translocate along the same DNA template and may interfere with each other. TRCs can either occur in a head-on or in a codirectional manner with different outcomes, head-on conflicts being more deleterious than codirectional ones (9, 10). Head-on conflicts have been associated with replication fork slowdown and with increased transcription-associated recombination (11-15). Both events are suppressed by the overexpression of RNase H (16-18), a nuclease degrading the RNA moiety of RNA:DNA hybrids (19). It is therefore generally believed that cotranscriptional R-loops interfere with DNA replication, but the mechanism(s) involved remain poorly understood at the molecular level.

In this review, we discuss several non-mutually exclusive processes by which transcription may interfere with DNA replication in eukaryotic cells, involving or not involving R-loops. We do not elaborate on the many factors that contribute to the formation or to the elimination of R-loops (**Fig. 1**) nor on the human pathologies associated with deregulated R-loop homeostasis as these topics have been extensively covered in recent reviews (1, 5, 6, 20). We rather discuss the multiple strategies used by eukaryotic cells to restrain transcription-replication conflicts in normal growth conditions. We also present alternative models in which RNA:DNA hybrids form at stalled forks as a consequence of replication arrest, and interfere with fork repair mechanisms, as recently reported for double-strand DNA break (DSB) repair.

2. How do R-loops and transcription interfere with DNA replication ?

2.1 Direct effects: R-loops and RNA polymerases

It is now well established that active and backtracked RNA polymerases can directly impede the progression of replication forks in bacteria (21-26). However, the impact of transcription on eukaryotic DNA replication is less well understood. Transcription interferes with the licensing of replication origins by displacing pre-replicative complexes or by altering their chromatin environment (27-34). Transcription can also impede DNA synthesis in specific contexts such as break-induced replication, as D-loop extension is slower and more sensitive to roadblocks than replication forks (35), but can eukaryotic RNA polymerases (RNAPs) actually block a fully functional fork? Among the evidence supporting this view, studies in budding yeast have shown that transcription can induce replication fork pausing (15, 36, 37) (**Fig. 2A**). TRCs are further increased by transcription elongation defects and when inactive RNAPs are retained on chromatin (38). The analysis of the impact of gene orientation and R-loops on the replication of episomes in human cells also revealed that R-loops can induce DNA breaks in an orientation dependent manner (14). Yet, the mechanisms by which RNAPs and R-loops could block replication are still debated (36, 39). In principle, RNA:DNA hybrids could prevent the unwinding activity of the Cdc45-MCM-GINS (CMG) replicative helicase. However, the CMG helicase travels on the leading strand (40) and RNA:DNA hybrids are found on the lagging strand when replication and transcription converge. Although long RNA:DNA hybrids could potentially interfere with lagging-strand synthesis, mechanistic insights on how these structures exactly impede DNA synthesis are still lacking. Interestingly, it has been reported that the replication fork is able to displace RNA:DNA hybrids on the leading strand, when replication and transcription are codirectional (14) and R-loops on either strands do not block fork progression in a reconstituted bacterial DNA replication system (25). Yet, RNA:DNA hybrids are able to block forks when bound to an inactive Cas9 (41), suggesting that protein-bound R-loops are more deleterious to replication than R-loop alone. RNA:DNA hybrids could facilitate the formation of stable secondary structures such as G-quadruplexes on the displaced ssDNA strand (**Fig. 2B**), which would in turn block replication (42, 43). Other possibilities include the cleavage of R-loops by nucleases such as XPF and XPG (44-46) and the formation of nicks on the displaced

ssDNA strand by AID (activation-induced cytidine deaminase) and base-excision repair (47), these ssDNA lesions being converted into DSBs during replication.

2.2 Indirect effects: R-loops and chromatin

Other mechanisms have been proposed to explain how R-loops could indirectly impede fork progression by triggering additional molecular events such as the formation of compact chromatin structures acting as replication roadblocks (**Fig. 2C**). These structures display histone H3S10 phosphorylation, a modification associated with chromosome condensation, and H3K9 dimethylation, a hallmark of heterochromatin (48, 49). Replication through these dense chromatin structures may also require chromatin remodeling complexes such as SWI/SNF (switch/sucrose non-fermentable), FACT and INO80, even though these complexes were mainly shown to prevent the formation of R-loops (50-54). Together with the more direct mechanisms discussed above, the formation of R-loop mediated heterochromatin contribute indirectly to replication-transcription interference, but other TRC mechanisms that may not involve R-loops must also be considered.

2.3 Topological stress

Replication and transcription machineries and their associated DNA unwinding activities generate DNA topological tension when they converge, as shown in bacterial models (19, 55). In eukaryotes, positive DNA supercoiling represents also a major challenge for both gene expression (56) and DNA replication (57-61). Positive DNA supercoiling is released by topoisomerase I (Top1) upstream of the fork and precatenanes formed during controlled fork rotation are resolved by topoisomerase II downstream of the replisome (59, 62, 63). When Top1 is inhibited, the accumulation of positive supercoiling ahead of the replisome impedes fork progression and may contribute to fork reversal (**Fig. 2D**), a fork protection mechanism promoting replication resumption (64-66). In eukaryotes, topological problems in S phase are exacerbated by the presence of a large number of replication forks and termination sites, which require the coordinated action of topoisomerases and specialized DNA helicases (60, 67). This high level of topological stress could explain why all licensed replication origins are not activated simultaneously in differentiated cells (68). In *Drosophila* embryos, the activation of the zygotic genome after the mid-blastula transition is associated with an increased replication stress

(69), supporting the view that replication and transcription must be tightly coordinated to avoid excessive superhelical constraints.

Another challenge facing eukaryotic DNA replication is related to the presence of a nuclear envelope and mRNA export through nuclear pores (70). Indeed, efficient mRNA export requires physical interactions between the transcription machinery and the nuclear pores, generating topologically constrained domains that are particularly difficult to replicate (71). In budding yeast, these topological domains can be unlocked by the checkpoint-mediated release of RNA polymerases from nuclear pores, facilitating the progression of replication forks (71).

Top1 plays a central role in the resolution of TRCs. Although it is dispensable for viability in budding yeast (57, 72), it is essential for growth in mammalian cells (73). Low levels of Top1 are associated with slower fork progression in mouse and human cells (16, 17). Since this replication defect is at least partially suppressed by transcription inhibitors or by the overexpression of RNase H1, it was proposed that cotranscriptional R-loops contribute to TRCs in these cells. The impact of Top1 depletion on R-loop levels is complex. Although increased negative supercoiling behind RNAPs favor the formation of R-loops, positive supercoiling accumulating when replication and transcription converge is thermodynamically unfavorable to R-loop formation (74), at least ahead of replication forks. In bacteria, it has been recently reported that positive supercoiling is converted to negative supercoiling by DNA gyrases to promote R-loop formation and induce replication stress (RS) (55). However, gyrases are absent in eukaryotes and the mechanism by which R-loops could contribute to RS under topological stress conditions is currently unclear. To complicate the picture, modeling and single-molecule profiling studies have recently revealed that R-loops act as powerful and reversible topological stress relievers (75). Further work is therefore needed to decipher the impact of DNA topology and R-loops in transcription-replication conflicts.

2.4 Checkpoint-mediated fork slowdown

R-loops are very abundant in mammalian genomes, covering over 100 Mb of unique DNA sequences and mostly found at specific loci, such as the promoters and termination regions of highly expressed genes (3, 4, 17, 76). This discrete distribution of R-loops contrasts with their widespread effect on DNA replication (77). Indeed, single-molecule analyses show that the velocity of all forks is reduced in cells with high levels of R-loops, whereas one might expect a

bimodal distribution of fork rates if only a fraction of forks encountered roadblocks (16, 17, 24, 52). Although pervasive transcription may explain this pleiotropic effect of R-loops, another possibility is that R-loops indirectly reduce fork speed by activating checkpoint pathways (14, 78) (**Fig. 2D**). Indeed, it is now well established that the intra-S-phase checkpoint can actively slowdown replication. This mechanism was reported in human cells exposed to very low doses of Camptothecin (79) and in budding yeast after induction of a limited number of DSBs in the genome (80). This pathway may act by targeting key components of the replisome and by promoting fork reversal (81, 82). Using an elegant optogenetic approach to induce the formation of TopBP1 condensates, it was recently shown that the activation of ATR in the absence of DNA lesions is sufficient to slow down replication elongation (83). However, it is worth noting that faster forks have also been observed in conditions associated with increased R-loop levels (84, 85). Moreover, it has been reported that forks encountering R-loops in a head-on orientation activate ATR, whereas encounters in a codirectional orientation rather activate ATM (14). Altogether, these studies indicate that although R-loops may directly contribute to TRCs, they could also regulate fork speed in a more global manner by activating checkpoints.

3. How do eukaryotic cells avoid TRCs ?

The data discussed above argues against the view that R-loops represent direct obstacles for DNA replication and call for a reconsideration of the real impact of transcription-replication conflicts on the stability of the genome. Indeed, TRCs are usually studied in pathological conditions, under which replication is challenged by chemical inhibitors or by the absence of key regulators of R-loop formation. For instance, conflicts between replication and transcription have been implicated in chromosome breaks at common fragile sites (34, 86) and at early replicating fragile sites (87), but studies were performed in the presence of replication inhibitors, impairing fork's ability to deal with potential roadblocks, including repetitive DNA sequences. Similarly, genetic screens to identify endogenous sources of DNA damage have been very useful in deciphering the cellular pathways regulating R-loop homeostasis (38, 88-90) (**Fig. 1**), but less in defining how R-loops could threaten genome stability under physiological conditions. As a matter of fact, a recent genome-wide analysis of the distribution of R-loops and RS markers in the human genome revealed that less than 20% of R-loops are associated with RS in unchallenged growth conditions (17). These R-loops did not display distinctive structural features, but were mostly detected

downstream of highly expressed genes (see section 3.3). While the impact of R-loops could be underestimated under these conditions due to the low level of endogenous RS, these data indicate that the vast majority of R-loops do not pose a threat to replication forks. This view is also consistent with the conclusions of another study showing that only a minority of R-loops are genotoxic in budding yeast (91). Direct evidence that RNA:DNA hybrids can directly interfere with eukaryotic DNA replication could eventually come from reconstituted *in vitro* replication systems (92, 93), as previously reported in bacteria (25).

3.1 Replication fork barriers

What is the evidence that transcription interferes with eukaryotic DNA replication in unchallenged growth conditions? The first indication of a transcription-dependent pausing of eukaryotic replication forks was observed by 2D gel electrophoresis at the ribosomal DNA (rDNA) array of *S. cerevisiae* (94, 95) and was later found to be conserved in mammals (96). In yeast, fork arrest occurs downstream of rRNA genes at a well-defined locus called a replication fork barrier (RFB). Fork pausing at the rDNA RFB is an active process, depending both on the binding of a protein called Fob1 to the RFB sequence (97) and on the presence at the replisome of Tof1 and Csm3, two components of the fork protection complex (98, 99). Since RFB arrests forks progressing opposite to the direction of 35S transcription, it is generally believed that Fob1 prevents RNA polymerase I (RNAP1)-mediated genomic instability at the rDNA. However, the loss of Fob1 does not increase – and rather suppresses – homologous recombination at the rDNA array (100, 101), at least in strains containing a normal number of rRNA genes. Together, these data indicate that *S. cerevisiae* cells are fully able to deal with head-on encounters between forks and RNAP1 at a locus accounting for 60% of all RNA synthesis, even in the absence of a functional RFB.

Other replication pause sites in budding yeast are found at tRNA genes (102). Again, fork pausing at these loci does not directly depend on transcription by RNA polymerase III (RNAP3) but on the binding of the transcription factor TFIIB and on the presence of Tof1 and Csm3 at forks (103-105). Upon activation of the replication checkpoint, transcription is transiently shutdown by the eviction of RNAP3 (106) and fork pausing is alleviated by DNA helicases of the Pif1 family (103, 104, 106-108). Fork pausing has also been reported at highly transcribed RNA polymerase II (RNAP2) genes, but generally in cells with altered RNA metabolism or fork

protection mechanisms (12, 15, 36, 37). Under these conditions, arrested forks are rescued by the activation of backup replication origins, promoting the completion of DNA replication and preventing genomic instability (109). When necessary, RNAP2 can also be removed by a conserved pathway involving checkpoint kinases, chromatin remodelers and the degradation of RNA polymerases (**Fig. 2A**) (53, 110-113). Interestingly, a transient activation of the DNA replication checkpoint has recently been reported when *S. cerevisiae* cells are released synchronously into S phase in the absence of exogenous stress (114). This checkpoint activation is due to a spontaneous arrest of replication forks caused by the transient depletion of dNTP pools in early S phase, and not to conflicts with transcription. Altogether, these studies indicate that *S. cerevisiae* cells use different strategies to avoid transcription-replication conflicts. They can also adapt to sudden changes of transcriptional programs when exposed to environmental modifications by delaying replication with stress-activated kinases such as Hog1 (115, 116). But is it also the case for multicellular organisms and their much more complex transcriptional programs?

3.2 Spatial and temporal separation of replication and transcription

Metazoan can also count on a variety of mechanisms to prevent conflicts with transcription during the S phase. These include the spatial and temporal separation of replication and transcription, as illustrated by immunofluorescence studies showing a mosaic distribution of DNA and RNA synthesis in mammalian cells (117). This mutual exclusion of transcription and replication sites was also confirmed at the genome-wide level during *C. elegans* embryonic development and in differentiated human cells (118, 119). Moreover, live-cell analyses of RNA synthesis have shown that transcription is discontinuous at the level of individual genes, with minute-scale fluctuations controlled by Mediator and sub-hour fluctuations controlled by TBP-TATA-box interaction (120, 121). These different levels of temporal separation between RNA and DNA synthesis provide therefore multiple opportunities for replication and transcription to share the same DNA template. However, recent evidence indicate that these protection mechanism can fail in pathological conditions such as those associated with deregulated oncogene expression, under which a massive increase of transcription and R-loops inevitably drive RS, genomic instability and cancer development (122-124).

3.3 Co-orientation of replication and transcription

Another important level of coordination concerns the co-orientation of replication and transcription to avoid frontal collisions. The genome of bacteria species such as *B. subtilis* has evolved to position a vast majority of the genes codirectionally with forks (125). In contrast, pathogenesis and stress survival genes are positioned in a head-on orientation relative to forks in pathogenic bacteria, which increases R-loop mediated DNA damage and favors targeted evolution (126, 127). Evolution could not shape metazoan genomes the same way because of the complexity and the diversity of their gene expression programs. Yet, they used another trick to limit transcription-replication conflicts by evolving flexible replication programs that adapt to transcription by activating origins upstream of highly expressed genes (128-130). In mammals, origin efficiency is determined in part by ORC1 binding near transcription start sites (TSS), early replication correlating directly with high transcription levels (131). As a consequence, active genes are preferentially replicated codirectionally with transcription (128) and replication stress marks such as phospho-RPA32 (S33) are only detected when forks reach the transcription termination site (TTS) (17).

Altogether, these data support a model in which TSS and gene bodies are replicated codirectionally, whereas TTS are replicated in a head-on orientation and could be viewed as traffic lights, arresting forks to avoid collisions with RNA polymerases inside gene bodies (**Fig. 3A**). In this model, positive supercoiling that accumulates when transcription and replication converge is used as a signal to slow down replication forks. Given that transcription is discontinuous at the level of individual cells, forks could simply restart after transcription bursts, once topological constraints are relieved (**Fig. 3B**). Central to this model is the activation of replication origins near the TSS of active genes and the absence of replication initiation in gene bodies. This model may not apply to very long genes that may contain intergenic origins. However, these genes generally show lower expression levels. Interestingly, it has been reported that a novel class of replication origins, called oncogene-induced origins, are found in gene bodies of cells overexpressing oncogenes such as *CCNE1* and *MYC*, which induce a premature entry into S phase (123). These intragenic origins are normally inactivated by transcription in G₁, as reported earlier for *Xenopus* embryos (132). However, they would persist after oncogene induction due to the shortening of their G₁ phase, resulting in increased TRCs and genomic instability.

4. R-loops: cause or consequence of fork arrest?

The results discussed above seem to argue against a model in which R-loops directly block the progression of DNA replication forks, at least in normal growth conditions. Yet, most of these studies also show that the overexpression of RNase H largely rescues the slow replication phenotype associated with increased R-loops. This apparent discrepancy could be explained by an alternative model in which toxic R-loops are not a cause, but rather a consequence of replication fork arrest (**Fig. 4**). Indeed, RNA:DNA hybrids could form or get trapped downstream of stalled forks due to the presence of ssDNA gaps and could interfere with replication restart. This view is supported by a recent study showing that the lack of DNA damage checkpoint and post-replicative repair (PRR) factors could favor the formation of RNA:DNA hybrids downstream of arrested forks (133). In this context, hybrids formation would be facilitated by the persistence of ssDNA gaps in PRR mutants. They could also result from RNAPs progressing along the lagging strand after the MUS81-dependent cleavage of the leading strand of stalled forks (134). R-loops could also result from pervasive transcription, which could take advantage of the fact that nascent chromatin is extensively remodeled to promote the resection of newly replicated DNA (135). However, direct evidence for the recruitment of RNAP at stalled forks is still lacking.

An important question that remains to be addressed is the mechanism by which RNA:DNA hybrids could interfere with fork recovery. Stalled forks are restarted by homologous recombination (HR)-mediated mechanisms that could be affected by the presence of RNA:DNA hybrids (136). Indeed, a growing body of evidence indicates that RNA:DNA hybrids also form at DSBs in different organisms, from yeasts to human cells (137-140). Both RNAP2 and RNAP3 have been implicated in the formation of these RNA:DNA hybrids (137, 141, 142). They are required to initiate homologous recombination (HR) but need to be removed to complete HR-mediated DSB repair, even though the impact on RNA:DNA hybrids on DNA end resection is still debated (143). In the absence of the Senataxin helicase, involved in the processing of RNA:DNA hybrids (144), HR is impaired and DSBs are repaired by non-homologous end joining, at the expense of increased chromosomal rearrangements (139, 140). Besides Senataxin, many factors involved in the homeostasis of R-loops and implicated in TRCs are also required to process RNA:DNA hybrids at DSBs, including MRN (142), XRN2 (145), RAD52 and XPG (146), the exosome (147) and the DDX1 and DDX5 helicases (148, 149). BRCA2 is also

implicated in this process through the recruitment of DDX5 (150) and RNase H2 (151) to DSBs containing RNA:DNA hybrids. As it has been proposed for DSB repair, an attractive possibility could be that RNA:DNA hybrids play a positive role at stalled forks but eventually need to be removed to complete the fork restart process.

5. Conclusion and perspectives

In conclusion, the mechanisms by which RNA:DNA hybrids interfere with DNA replication are more varied than initially thought and are probably not mutually exclusive. Although they could impede fork progression by acting directly or indirectly as roadblocks, they could also interfere with the restart of arrested forks, as they impede DSB repair. In cells that are unable to process R-loops, these hybrids could also delay the repair of replication-borne DSBs, inducing a persistent DNA damage checkpoint response that would in turn slow down replication elongation. These data indicate that RNA:DNA hybrids play a central role in the complex interplay between transcription, replication and DSB repair, but further work is required to understand how these important functions are regulated at the molecular level.

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Figure legends

Figure 1. Nuclear factors implicated in R-loop metabolism and in the resolution of TRCs.

R-loops form during transcription when nascent transcripts anneal to the DNA template, forming an RNA:DNA hybrid and displacing the non-transcribed DNA strand. When DNA replication and transcription converge, the replicative helicase (CMG) progresses on the DNA strand that is opposite to the RNAP and to the RNA:DNA hybrid. Factors preventing the formation of R-loops include fork-associated factors such as components of the Fanconi pathway, DNA topoisomerase I (Top1) and RNA biogenesis factors, such as the THO/TREX complex and the splicing factor SRSF1. These factors prevent R-loop formation by sequestering the nascent RNA and hindering its annealing to DNA. Other factors in the resolution or the degradation of R-loops. These include various RNA/DNA helicases and translocases that unwind RNA:DNA hybrids. Nucleases such as RNase H hydrolyze the RNA moiety in the RNA:DNA hybrids and nucleases such as XPF and XPG directly cleave R-loops. See the following references for details (1, 5, 6). RNAP: RNA polymerase, Pol ϵ : DNA polymerase ϵ , Pol δ : DNA polymerase δ , CMG: CDC45, MCM2-7, GINS complex.

Figure 2. Transcription-replication conflicts in eukaryotic cells. Transcription and R-loops can interfere with DNA replication by different non-exclusive mechanisms. (A) Frontal collisions between the replisome and active or stalled RNAPs could induce fork arrest, activate the checkpoint kinase Mec1^{ATR} and promote the eviction and the degradation of the RNAP (see text for details). (B) In a head-on orientation, the RNA:DNA hybrid cannot directly interfere with the unwinding activity of the CMG helicase as it is located on the opposite strand. Yet, secondary structures such as G-quadruplexes (G4 DNA) could form on the non-template strand and block replication. This ssDNA strand can also be targeted by the activation-induced cytidine deaminase (AID) to generate uridines (U) in the DNA, which are subsequently processed by BER. R-loops can also be cleaved by nucleases such as XPF and XPG. These ssDNA breaks are converted into DSBs upon fork passage. (C) R-loops have been implicated in the formation of dense chromatin structures, characterized with a high density of histone H3 phosphorylated on serine 10 (p-H3S10) and histone H3 dimethylated on lysine 9 (H3K9me2). These structures are intrinsically difficult to replicate and interfere with the progression of replication forks. (D) Positive DNA

supercoiling accumulates when transcription and replication converge, hampering the separation of DNA strands and slowing down both processes. Paused forks often undergo a complex process called fork reversal, which promotes the resection of nascent DNA and facilitates fork restart by recombination-mediated mechanisms. Positive supercoiling can be relieved locally by the DNA topoisomerase I (Top1; see text for details). **(E)** Activation of ATR or ATM by a limited number of transcription-dependent DNA breaks or stalled replication forks is sufficient to slow down forks throughout the genome in a checkpoint-dependent manner (see text for details).

Figure 3. Model of TRC avoidance in the human genome. **(A)** In gene-rich regions, replication origins are often positioned upstream of the promoter of highly expressed genes. On average, these genes are therefore replicated codirectionally with transcription, which limits head-on conflicts. **(B)** When replication and transcription converge, the accumulation of positive DNA supercoiling could be used as a signal for replication slow down. Since transcription is a discontinuous process at the level of individual cells, replication could resume in between two bursts of transcription.

Figures 4. RNA:DNA hybrids: cause or consequence of RS ? **(A)** In classical models of TRCs, RNAP and/or R-loops interfere with DNA replication by blocking fork progression. In this model, RNase H is required to remove R-loops ahead of forks and prevent fork arrest. **(B)** In alternative models, RNA:DNA hybrids could also form or accumulate behind stalled forks as a consequence of fork arrest and interfere with fork restart processes, such as fork reversal and resection. RNA:DNA hybrids could result from pervasive transcription on nascent chromatin and would be favored by the presence of ssDNA gaps. Alternatively, RNA:DNA hybrids could preexist and could persist after fork passage. In these models, RNase H activities would be required to promote fork restart.

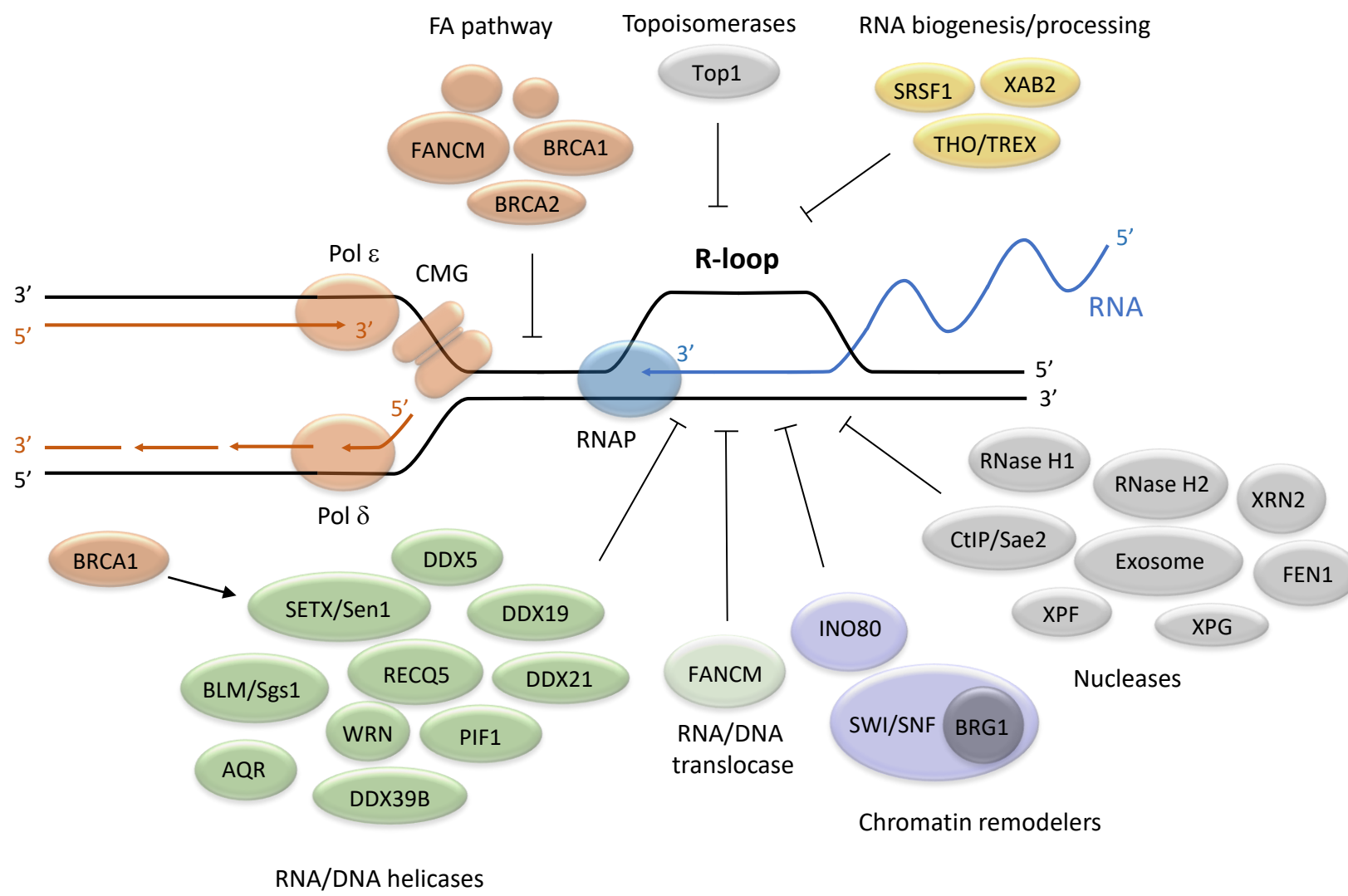


Fig. 1

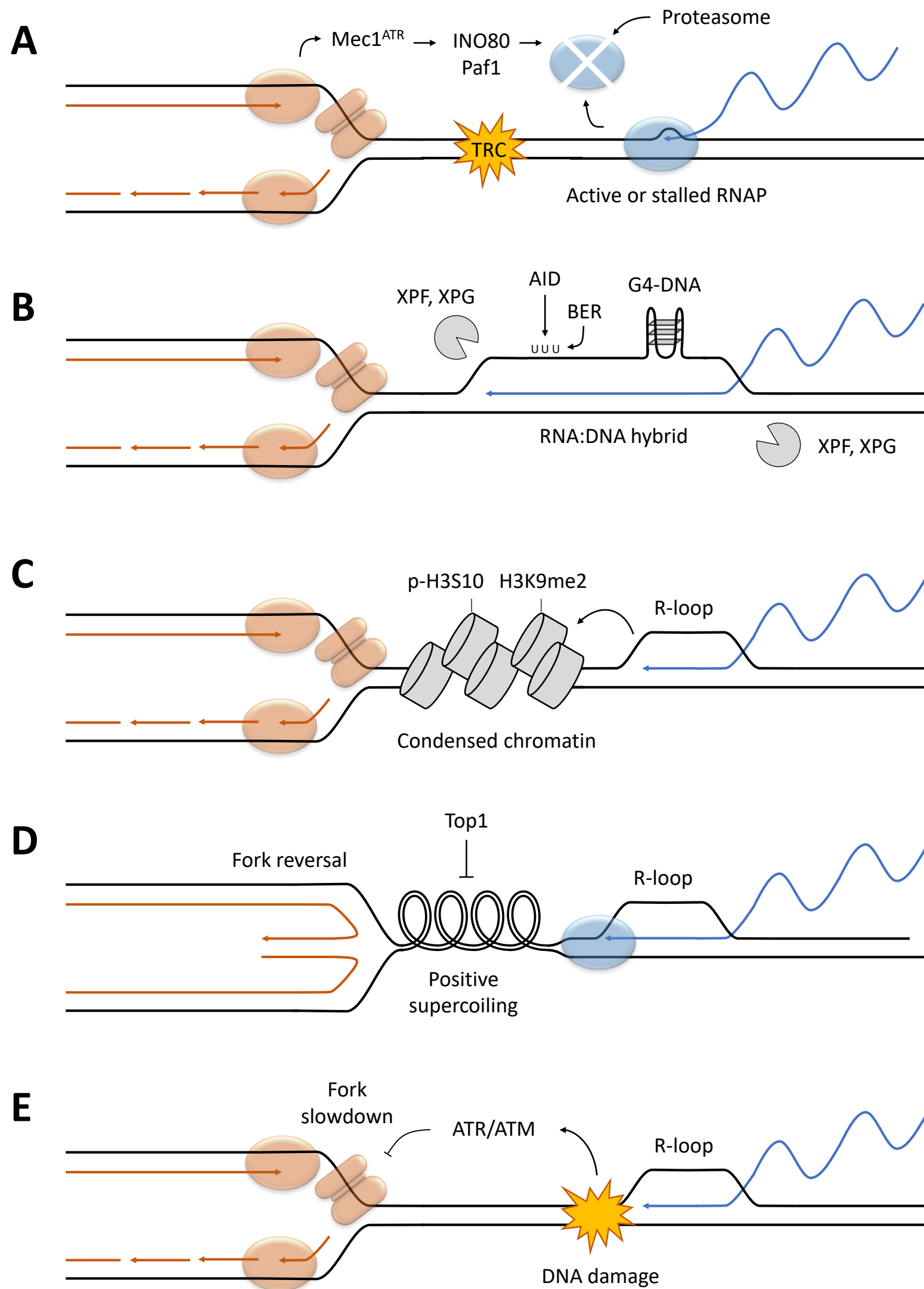


Fig. 2

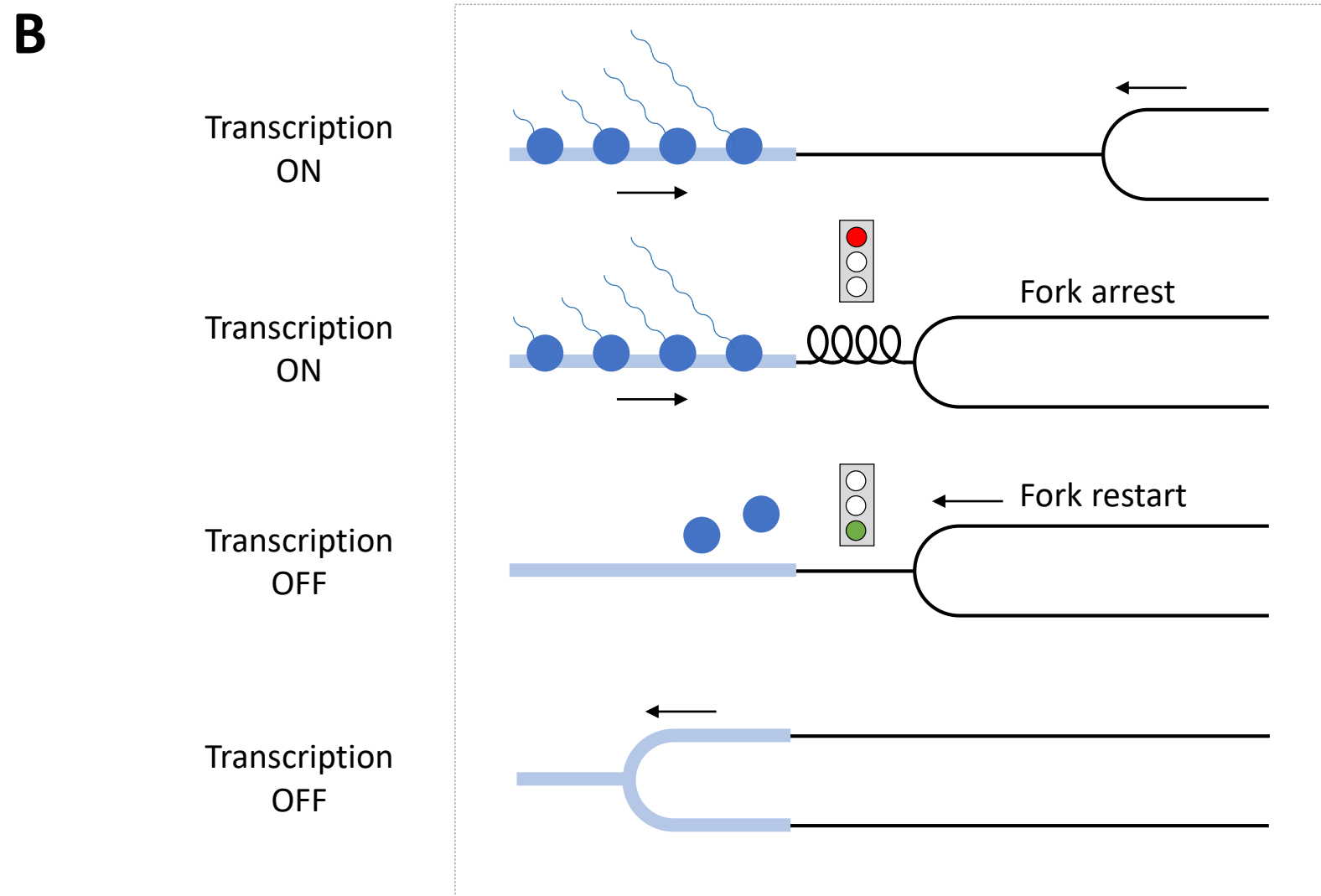
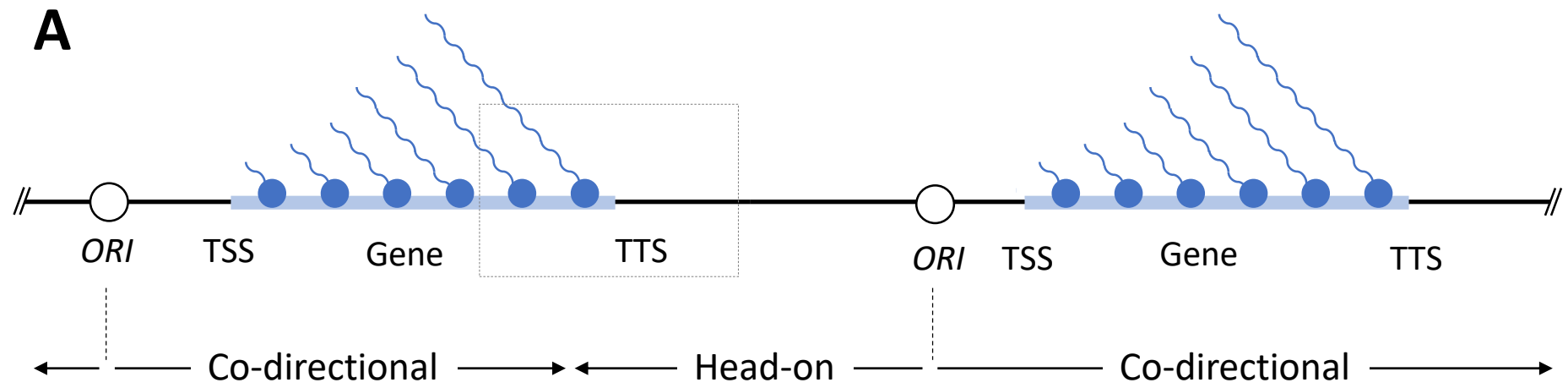


Fig. 3

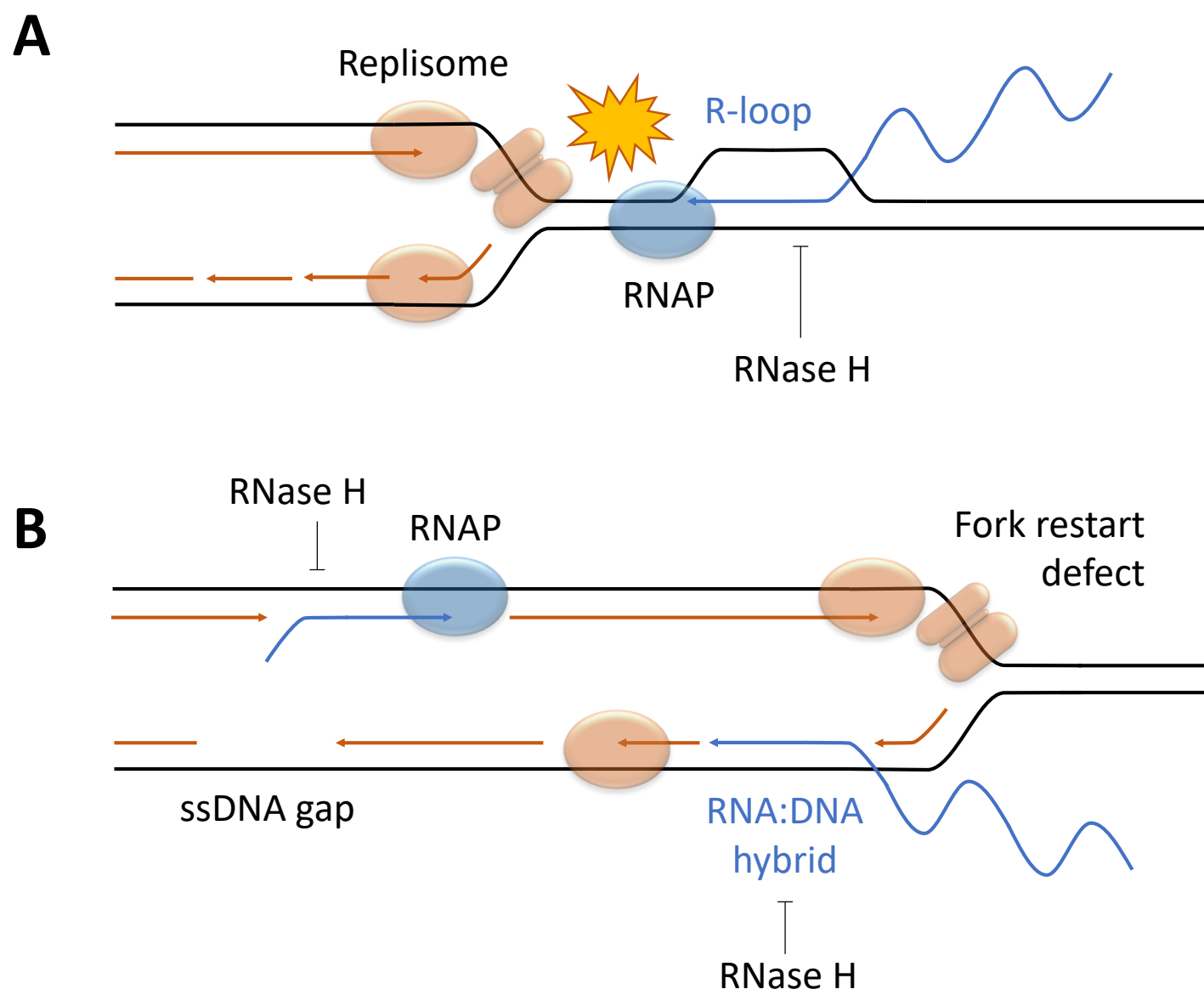


Fig. 4