DNA DAMAGE

Transcription–replication conflicts: how they occur and how they are resolved

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Abstract | The frequent occurrence of transcription and DNA replication in cells results in many encounters, and thus conflicts, between the transcription and replication machineries. These conflicts constitute a major intrinsic source of genome instability, which is a hallmark of cancer cells. How the replication machinery progresses along a DNA molecule occupied by an RNA polymerase is an old question. Here we review recent data on the biological relevance of transcription–replication conflicts, and the factors and mechanisms that are involved in either preventing or resolving them, mainly in eukaryotes. On the basis of these data, we provide our current view of how transcription can generate obstacles to replication, including torsional stress and non-B DNA structures, and of the different cellular processes that have evolved to solve them.

Transcription-coupled repair
Subpathway of the nucleotide excision repair pathway that removes lesions from the template DNA strands at actively transcribed genes.

Torsional stress
Physical stress at the DNA molecule generated by over-rotation of the double helix; manifested as the accumulation of positive or negative supercoils.

Non-B DNA
Any DNA structure that is different from right-handed double helix with 10 nucleotides per turn.

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Genomes are templates for many biological processes, including transcription, epigenetic modifications, DNA replication, DNA repair and chromosome segregation. In several cases, crosstalk between different processes occurring at the DNA may have a positive effect, as in the case of transcription-coupled repair[1]. However, in other cases, the co-temporal activity of two cellular machineries at the same genomic region may cause an encounter that has negative consequences. This is the case for DNA replication and transcription. Research in the past two decades has provided evidence that conflicts between the transcription and replication machineries constitute a considerable natural source of genome instability, which is a hallmark of cancer cells[2]. Given that transcription and replication are two essential processes for cell viability and proliferation and that they occur frequently, a high incidence of encounters between the transcription and replication machineries is to be expected. Transcription-mediated chromatin changes may facilitate firing of DNA replication origins[3], which can have a positive effect on replication initiation but may also lead to collisions that are a potential threat to genome integrity and cell viability.

How the replication machinery progresses along a double-stranded DNA molecule occupied by an RNA polymerase is an old question. Alberts and colleagues[4] elegantly addressed this question using the T4 bacteriophage system *in vitro*; however, current knowledge of the structure of RNA polymerases and the mechanisms of transcription elongation suggests that the factors and mechanisms used by cells to solve such conflicts are more complex than previously foreseen. The relevance of transcription as a source of genome instability, as measured by the rate of point mutations or of recombination and chromosome rearrangements, and the putative mechanisms by which such instability is mediated have been reviewed recently[5–8]. Here, we review recent data on the factors and mechanisms that are involved in either preventing or resolving transcription–replication collisions, and on their potential consequences. In particular, we discuss how the transcription machinery may directly hinder the progression of the replication forks or how transcriptional activity generates obstacles to replication, including torsional stress and non-B DNA structures, and the different solutions the cells have evolved to avoid, minimize or resolve these collisions or their consequences.

How do collisions occur?

A basic difference between the transcription and replication machineries is that the elongating RNA polymerase holoenzyme, which comprises one polymerase subunit, embraces the double-stranded DNA. The nascent RNA chain is synthesized in the active pocket of the RNA polymerase, where it forms a dynamic RNA–DNA hybrid that is 9–11 nucleotides in length (FIG. 1a). By contrast, the elongating DNA polymerase holoenzyme consists of two polymerase subunits (DNA Pol III in bacteria and DNA Pol ε and Pol δ in eukaryotes), each working on a single-stranded DNA (ssDNA) template (FIG. 1b). Furthermore, whereas several active RNA polymerases can simultaneously transcribe the same gene, replisomes move alone and are not followed by a second replication

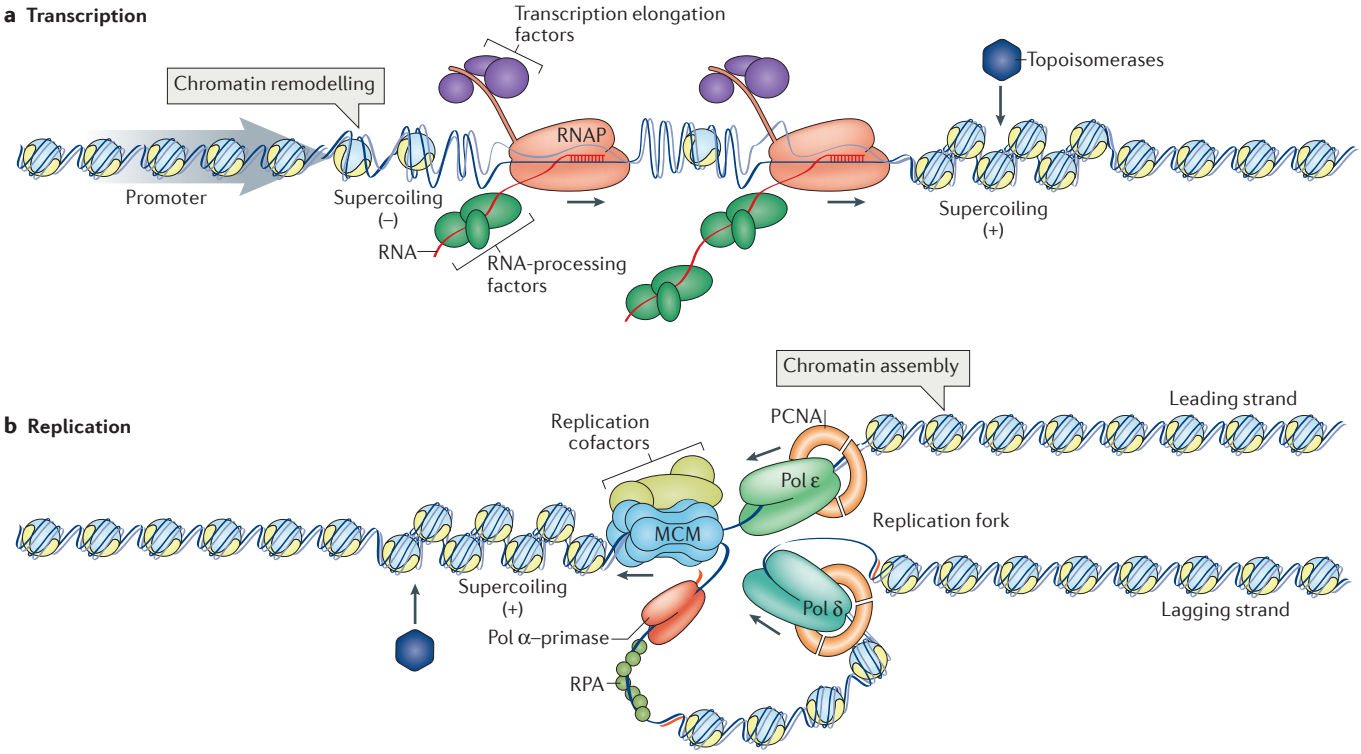

Figure 1 | **Transcription and replication. a** | A small portion of the DNA double helix is unwound by the RNA polymerase (RNAP) to enable transcription (known as 'transcription bubble'). DNA unwinding by the RNAP generates positive and negative supercoiling, which is alleviated by topoisomerases. In eukaryotes, transcription also involves chromatin modification and remodelling. The progression of RNAP requires the activity of transcription elongation factors, and the nascent RNA is co-transcriptionally processed by different factors. **b** | At the replication fork, the DNA helicase minichromosome maintenance complex (MCM) opens the double helix, and DNA polymerase ε (Pol ε) and Pol δ extend the leading and lagging strand, respectively. Synthesis of each new DNA molecule is initiated by the Pol α–primase complex. Lagging-strand synthesis leads to the formation of single-stranded DNA (ssDNA), which is coated with replication protein A (RPA). Fork progression requires the activity of several replication cofactors, including the clamp proliferating cell nuclear antigen (PCNA). DNA unwinding by the replication fork generates positive supercoiling, which is alleviated by topoisomerases. Replication also entails reassembly of recycled and *de novo*-synthesized nucleosomes at the newly synthesized DNA. Arrows indicate the direction of synthesis by RNAP and DNA polymerases.

fork. Regardless of whether collisions are co-directional or in head-on orientation (FIG. 2), the replication fork cannot progress past an elongating RNA polymerase, and so their encounters cause conflicts[9,10]. Although replication fork progression may be affected by collisions in both orientations, data suggest that the consequences of collisions are more dramatic in the head-on orientation[11,12]. When encounters were promoted in yeast artificial systems in a head-on orientation, replication pause sites were detected by 2D-gel electrophoresis and hyper-recombination was observed[12]. By contrast, co-directional orientation did not lead to replication pauses or high levels of hyper-recombination. A possible explanation for this difference is that co-directional encounters may in part be resolved once the RNA polymerase terminates transcription.

Cells have developed different strategies to reduce or prevent collisions. In bacteria, there is a genome-wide bias towards co-orientation of replication and transcription[13], and inverting transcriptional units to provoke head-on collisions causes replication impairment, proliferation defects and genome instability[11]. In eukaryotes, a bias towards co-directional replication and transcription is not obvious, but cells seem to have evolved other strategies to reduce head-on collisions. For example, in the *Saccharomyces cerevisiae* highly transcribed ribosomal DNA (rDNA) genes, replication fork barriers (RFBs) block fork progression and thereby prevent harmful encounters with RNA polymerases[14]. In mammalian cells, replication and transcription of rDNA seem to be spatially separated in the nucleoli as a way to avoid collisions[15]. In other regions of the genome, transcription and replication seem to be separated temporally[16]. Analysis of nascent mRNAs of genes encoding replication factors revealed that genes transcribed during early replication are replicated late in S phase, and vice versa[17].

Importantly, however, it is not clear whether the RNA and DNA polymerases ever actually make contact. It is plausible that before the physical connection occurs, transcription- and replication-mediated changes in chromatin and DNA structures attenuate the progression of the polymerases. Therefore, to fully understand conflicts, we need to identify the elements and conditions that affect their occurrence.

Replisomes
Protein complexes with helicase, primase and DNA polymerase activities that conduct DNA replication.

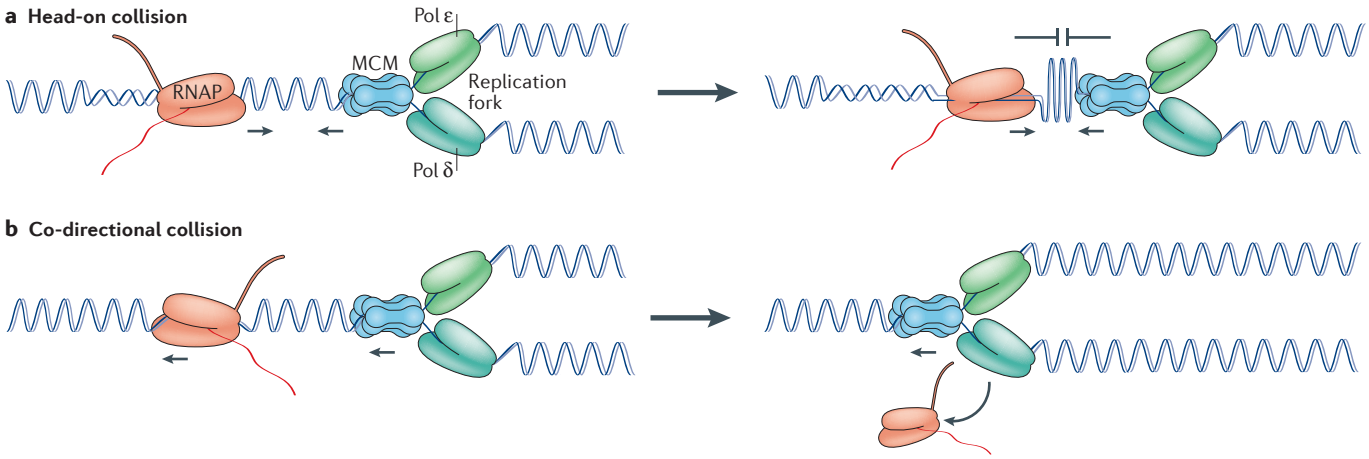

Figure 2 | **Head-on and co-directional transcription–replication collisions. a** | Progression in opposite directions of an RNA polymerase (RNAP) and a replication fork leads to head-on collisions, which induce pausing and blockage of the replication fork, and may lead to its collapse and the formation of DNA breaks. **b** | Progression of an RNAP and a replication fork in the same direction leads to co-directional collisions if the fork moves more quickly than the RNAP. Co-directional collisions can be resolved by displacement of the RNAP from the DNA. MCM, minichromosome maintenance complex; Pol δ, DNA polymerase δ; Pol ε, DNA polymerase ε.

Cis-elements affecting collisions

The transcription machinery may constitute a natural obstacle to replication fork progression, but this interference can be direct or indirect, as the transcription process may also generate structural features that could hinder replication fork progression. These features include changes in DNA supercoiling or secondary DNA structures, such as hairpins, triplex DNA (including hinged (H)-DNA), G-quadruplexes (also known as G-quartets) or RNA–DNA hybrids.

DNA supercoiling. Transcription and replication require the unwinding of the DNA molecule. In transcription, this unwinding leads to positive and negative supercoiling ahead and behind the RNA polymerase, respectively (FIG. 3a). The resulting torsional stress is relieved by DNA topoisomerases, which are classified as type I or type II depending on whether they catalyse the breakage of one or both DNA strands, respectively. In budding yeast, topoisomerase mutants accumulate supercoiling, and the resulting torsional stress prevents both transcription and replication of the highly transcribed rDNA[18], suggesting that supercoiling can block these processes. Later studies in yeast and human cells have shown that both topoisomerase 1 (TOP1; Top1 in yeast) and TOP2 (Top2 in yeast) are crucial for preventing collisions between the transcription and replication machineries[19,20], indicating that unresolved torsional stress can attenuate the progression of both DNA and RNA polymerases and promote transcription–replication conflicts. Genome-wide analysis of the distribution of Top1 and Top2 in replicating budding yeast cells revealed an association between these enzymes and moving replication forks[19,21]. Moreover, cells deficient in both top1 and top2 also accumulate DNA damage[19]. Analyses of replication by DNA combing have revealed that replication forks are slower in yeast and human TOP1-deficient cells than in control cells[20]. Interestingly, in TOP1-depleted human cells, there was an increase in fork stalling that correlated with the accumulation of γH2AX foci in S phase and that was suppressed by inhibition of transcription elongation with cordycepin. Therefore, TOP1 activity can prevent transcription–replication conflicts and their harmful consequences[20].

On the basis of these results, we suggest that DNA supercoiling is transiently accumulating between the advancing transcription and replication machineries, and may be important in the control of their collisions and subsequent adverse effects (FIG. 3a). In theory, this phenomenon should be exacerbated in the case of the head-on orientation. However, convergent transcription, which should create the same topological constraint as head-on collisions, does not pose a major threat to genome integrity and transcription in budding yeast[22]. Alternatively, inefficient resolution of the negative supercoiling accumulating behind the elongating RNA polymerase may also facilitate local melting of the DNA duplex and, consequently, the formation of non-B DNA structures that can block replication fork progression (see below). Indeed, in yeast, divergent transcription was found to enhance chromosome rearrangements[23].

Non-B DNA structures and RNA–DNA hybrids. Some DNA sequences, especially repetitive sequences, can assume non-B DNA structures, such as hairpins, triplex DNA or G-quadruplexes. Such non-B DNA structures have the capacity to stall replication forks and have been correlated with hot spots of DNA double-strand breaks (DSBs) and chromosomal deletions, translocations and other rearrangements[24]. These secondary DNA structures are believed to form preferentially at the ssDNA that is exposed during DNA replication, but they can also be formed during transcription, owing to the negative supercoiling that transiently accumulates behind

Supercoiling
Over- or under-winding of the DNA helix.

Hairpins
DNA structures in which a strand folds on itself and forms intrastrand base pairing.

Triplex DNA
A single-stranded DNA region bound to the major groove of the DNA duplex forming a three-stranded helix, normally at sequences with mirror symmetry.

G-quadruplexes
Four repeats of at least three guanines that can interact to form four-stranded DNA structures.

DNA combing
A method for the analysis of single DNA molecules; it is used for studying DNA replication.

γH2AX foci
A histone H2A variant that is phosphorylated (γH2AX) and forms nuclear foci, which are generally accepted as markers of DNA double-strand breaks.

CpG islands
Chromosomal regions with a high density of non-methylated CpG sequences, which are often located at gene promoters.

the elongating RNA polymerase (FIG. 3). A good example of the putative relevance of non-B structures is provided by G-quadruplexes, which consist of four repeats of at least three guanines that can form four-strand interactions (FIG. 3b). G-quadruplexes can form during lagging-strand replication, as shown at telomeres[25], as well as during transcription: human cells treated with the G-quadruplex ligand pyridostatin show a tight correlation between pyridostatin binding and the formation of γH2AX foci, which is reduced by treatment with the transcription inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)[26]. The idea that G-quadruplex formation can be potentiated behind an elongating RNA polymerase II (RNA Pol II) has been indirectly inferred in yeast expressing the murine G-quadruplex-prone Sμ immunoglobulin switch region, which stimulates recombination under conditions of high transcription levels[27]. The activity of Top1 in these conditions suppresses G-quadruplex-associated recombination, which is consistent with negative supercoiling enhancing G-quadruplex accumulation[28]. It appears that the genomic instability is higher when the orientation of the G-rich strand of the Sμ sequence, with respect to transcription, leaves the G-rich strand in the non-transcribed strand, suggesting that the ssDNA that allows quadruplex formation originates from transcription. Additional support for the idea that non-B DNA structure may contribute to transcription-mediated replication fork stalling comes from studies in mutants of the budding yeast DNA helicase Pif1 and the fission yeast Pfh1 (Pif1 homologue), which unwind G-quadruplexes *in vitro*. The absence of Pif1 or Pfh1 attenuates or halts replication in regions of high G-quadruplex density and in RNA Pol II- and Pol III-highly transcribed genes[29,30].

Another type of transcription-mediated structure able to cause fork stalling that can strongly contribute to transcription–replication conflicts are co-transcriptional RNA–DNA hybrids (also known as R loops when formed outside the transcription bubble; see FIG. 3c). Although they are natural intermediates in class switching recombination and in the initiation of mitochondrial DNA replication, RNA–DNA hybrids are formed under conditions that prevent the proper formation of the ribonucleoprotein particle, as shown in yeast and human cells[31,32]. Evidence from yeast to mammalian cells suggests that RNA–DNA hybrids can form naturally and may constitute an important transcription intermediate that can cause replication fork stalling at telomeres, rDNA regions, CpG islands and other sites at specific Pol II-transcribed genes, including 3′-end regions. RNA–DNA hybrids have been thoroughly and extensively reviewed recently[33–36] and will not be discussed further here. It is important to note, however, that an enrichment of sequences that have a high probability of forming non-B DNA structures or RNA–DNA hybrids and of undergoing transcription–replication conflicts is observed at some fragile sites (BOX 1).

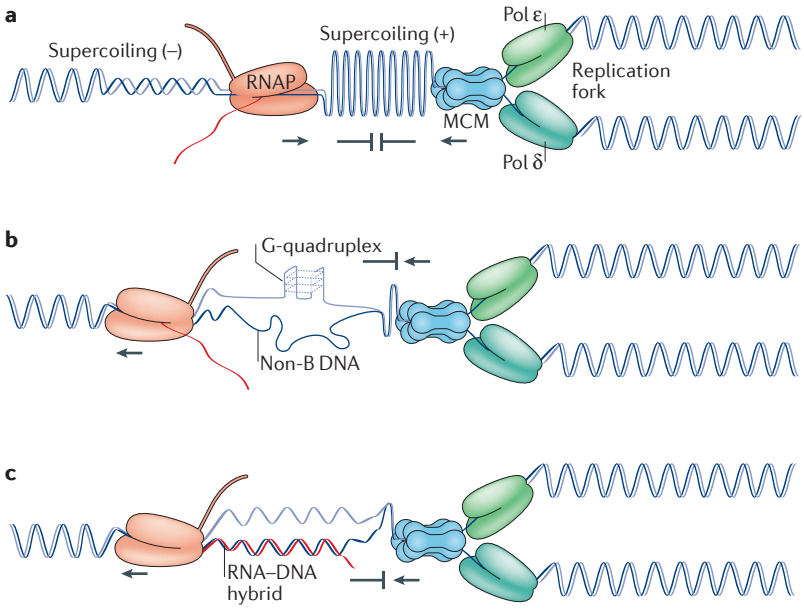

Figure 3 | **Conditions that affect the occurrence of transcription–replication collisions. a** | Convergence of an RNA polymerase (RNAP) and a replication fork when oriented head-on can lead to the accumulation of positive DNA supercoiling between them, which induces pausing of the fork. **b** | The partial unwinding of DNA by the negative supercoiling generated behind the RNAP can enable the formation of non-B DNA structures, such as G-quadruplexes, which may constitute an obstacle for replication fork progression. **c** | Other non-B DNA structures include RNA–DNA hybrids, which also may constitute an obstacle for fork progression. Once a stable non-B DNA structure capable of blocking fork progression is co-transcriptionally formed, the direction of transcription or the presence of the RNAP itself would be in principle irrelevant for the formation of the transcription–replication collision (parts **b** and **c**). MCM, minichromosome maintenance complex; Pol δ, DNA polymerase δ; Pol ε, DNA polymerase ε.

Mechanisms for preventing conflicts

Owing to the impact of collisions between the transcription and replication machineries on genomic stability and thereby potentially on disease, cells have evolved mechanisms to prevent such encounters. The factors that minimize collisions include the transcription machinery itself, as well as factors that help or facilitate replication progression along transcribed DNA.

The RNA polymerase transcription apparatus. Some clues to understanding how the RNA polymerase directly contributes to transcription–replication conflicts are starting to emerge from the analysis of several RNA polymerase mutants in bacteria and yeast, but we are still far from having a complete view. A critical step in transcription that seems to be relevant to conflicts is RNA polymerase 'backtracking', which refers to the process by which the RNA polymerase reverses its progression to enzymatically remove the last incorporated ribonucleotide. Backtracking enables transcription elongation to be restarted following a pause caused by hindrances during transcription elongation or as part of a regulatory process to coordinate the different steps of transcription and RNA processing[37]. A backtracked RNA polymerase is able to block replication progression in *Escherichia coli*. Using specific promoter sequences that allow modulation of the polymerase activity, it was shown that a permanently arrested elongating polymerase causes DSBs[38,39]. Such breaks were inferred to be replication dependent, as treatment with the replication

Box 1 | **Fragile sites as hot spots of transcription–replication collisions**

Fragile sites are genomic regions that exhibit constrictions or gaps in metaphase chromosomes following replication stress. They are categorized into two classes: rare fragile sites, which are found in <5% of individuals and arise from trinucleotide repeat expansion, and common fragile sites (CFSs), which are found in all individuals and are not associated with repeat expansion[86]. Fragile sites are frequently enriched in sequences that can stall DNA replication, such as AT-dinucleotide-rich sequences of high DNA flexibility in CFSs, as shown in yeast at FRA16D[87], or in other replication-attenuating sequences, as shown in human cells expressing the rare fragile site FRAXA at the *FMR1* locus[87,88]. Scarcity in origins of DNA replication or inefficient activation of replication in large genomic regions may explain the fragility of some CFSs[89,90]. Interestingly, mapping of CFSs in several human cell lines revealed that they are located mostly within large genes. There is also a high correlation between CFSs and recurrent chromosomal rearrangements observed in cancer cells, and a similar correlation emerged from the analysis of copy number variants, the hot spots of which matched CFSs when located in large regions of active transcription in both human and mouse cells[91,92]. These data suggest that concomitant transcription and replication may lead to fragility. Related to this is the observation that RNA–DNA hybrids accumulate in the long *FHIT*, *WWOX* or *IMMP2L* genes, which harbour the CFSs FRA3B, FRA16D and FRA7K, respectively[93], as well as in the rare fragile sites FXN and FRAXA[61], among others[2]. Recently, a new class of fragile sites, termed early replication fragile sites (ERFSs), were identified and mapped by detecting the localization of replication protein A, which binds to single-stranded DNA, following replication stress using chromatin immunoprecipitation. ERFSs, unlike CFSs, are located near replication origins and within actively transcribed genes, strengthening the possibility that their fragility results from transcription–replication conflicts[94]. Similarly, using the Break-seq technique in cells following exposure to the replication-stress agent hydroxyurea, replication-induced double-strand breaks were mapped preferentially at genes whose expression is induced under replication-stress conditions[95]. Therefore, genomes contain hot spots for transcription–replication collisions, which can manifest as different forms of fragility.

inhibitor hydroxyurea before transcription activation prevented their appearance. The clash between the replisome and the backtracked RNA polymerase was interpreted to be responsible for the formation of DSBs[39]. Consistent with this view, the *E. coli* transcription elongation factors GreA and GreB, which promote the release of backtracked and stalled RNA polymerases, seem to reduce the consequences of conflicts (FIG. 4a). In the absence of GreA and GreB and under substantial transcription activity induced by starvation, replication progression was completely blocked[40]. A similar role was proposed for the yeast transcription elongation factor TFIIS[41], but it remains to be seen whether this activity has any effect on putative collisions.

Direct involvement of the transcription apparatus in modulating transcription–replication conflicts was demonstrated in recent studies using RNA polymerase mutants that compromise the stability of transcription complexes. These RNA polymerase mutants were shown to suppress growth defects of *E. coli* cells lacking factors that help to resolve collisions such as the DNA helicases Rep, UvrD and DinG[42]. These results suggest that less stable transcription complexes may not compromise replication progression because they do not seem to form strong replication obstacles[42]. Also, several yeast RNA Pol II mutants with transcription elongation defects exhibited replication impairment, inferred by 2D gels, bromodeoxyuridine incorporation by DNA polymerases or altered distribution of Rrm3, which is a replicative helicase required for progression of the replication machinery past DNA obstacles[43]. It is likely that following a collision the RNA Pol II is released from the DNA to allow passage of the replisome, as is the case in bacteria[38]. Interestingly, one of these yeast RNA Pol II mutants, the *rpb1-1* mutant (Rpb1 is one of the largest RNA Pol II subunits), has tighter attachment to chromatin than wild-type RNA Pol II, as determined by chromatin immunoprecipitation (ChIP), which supports the idea that RNA Pol II mutants with increased attachment to chromatin could aggravate the consequences of a transcription–replication encounter[43]. These results suggest that the transcription machinery, and RNA Pol II itself, may be involved in managing transcription–replication conflicts depending on the varying feasibilities of their eviction from DNA following a collision. The recent observation that Paf1c (RNA polymerase II-associated factor 1 complex) triggers RNA Pol II degradation at sites of collision[44] supports this view.

Replication fork barriers. Non-nucleosomal protein–DNA complexes that assemble at genes and regulatory elements can obstruct the progression of replication forks. In bacteria, the barrier formed by the transcription complex is able to pause replication forks, and resumption of replication requires specific DNA helicases[45,46]. In yeast, various regions that impede replication fork progression *in vivo* have been identified, the most representative being the RFB found in the 35S rRNA gene in the rDNA[47]. The rDNA region provides the best model to study the impact of replication stress generated by transcription owing to its high transcription rate and high density of replication origins. The replication barrier consists of DNA replication fork-blocking protein Fob1 bound to the specific RFB sequence, which prevents head-on collisions between RNA and DNA polymerases (FIG. 4b). Interestingly, replication fork progression through the RFB–Fob1 complex requires the helicase Rrm3. Deletion of the *rrm3* gene (Δ*rrm3*) increases replication pauses at rDNA, resulting in breakage and accumulation of excised rDNA circles[14,48]. Although Rrm3 could therefore be seen as a complementary factor acting in *trans* to promote replication fork passage through protein barriers, fork pausing in *Δrrm3* mutants is also increased at other pause sites, such as at tRNA genes or telomeres[49,50]. Notably, however, other pause sites are found at highly transcribed genes but are not exacerbated in *Δrrm3* mutants, suggesting that other factors may have roles in the prevention or resolution of collisions[9].

The RECQL5 DNA helicase. Human RecQ-like ATP-dependent DNA helicase Q5 (RECQL5), a member of the RecQ family of DNA helicases, is to date the protein with the best-characterized active role in preventing transcription–replication collisions. RECQL5 forms a stable complex with RNA Pol II, and several *in vivo* and *in vitro* studies have indicated that it has a negative regulatory role in transcription elongation[51]. ChIP followed by sequencing (ChIP–seq) analysis with an RNA Pol II-targeting antibody revealed that transcription upregulation in cells lacking *RECQL5* increases

Break-seq
A technique to map chromosome breaks based on DNA double-strand break labelling and next-generation sequencing.

Bromodeoxyuridine
A synthetic analogue of the thymidine nucleoside; it is used to follow DNA synthesis.

RecQ family
DNA helicase proteins characterized by their helicase domain, which is essential for ATP binding and hydrolysis, and the RecQ domain, which is required for DNA binding.

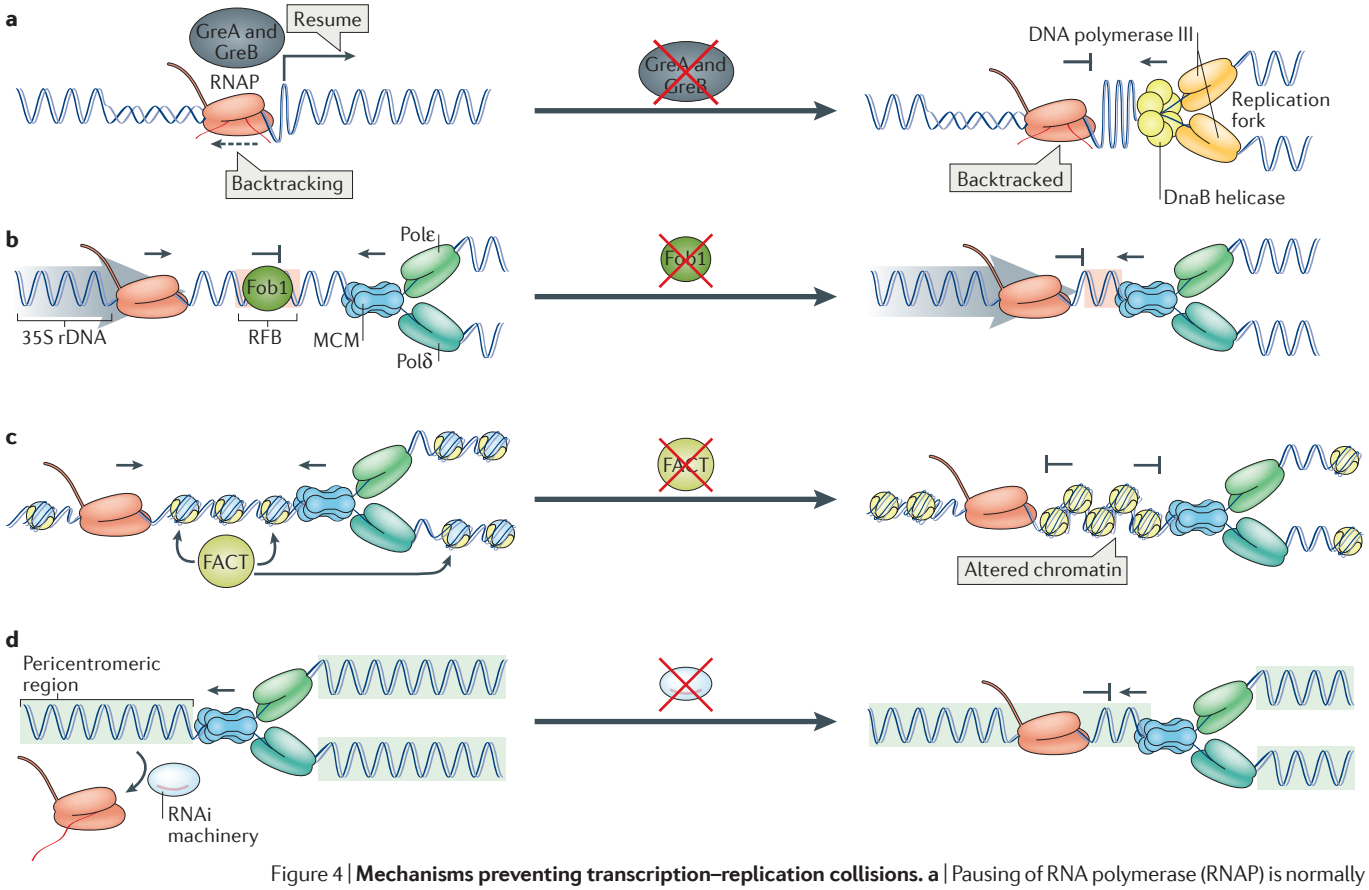

Figure 4 | **Mechanisms preventing transcription–replication collisions. a** | Pausing of RNA polymerase (RNAP) is normally resolved by backtracking, which disengages the 3′ end of the RNA molecule from the active site and leads to back and forth sliding of the RNAP. In *Escherichia coli*, the GreA and GreB RNA-cleavage factors stimulate the removal of the extruded RNA and the reactivation of transcription. Without GreA and GreB, the RNAP might stall and become an obstacle for the replication fork, leading to transcription–replication collisions. **b** | The replication fork barrier (RFB) site is a DNA sequence located near the 3′ end of rRNA genes, which prevents transcription–replication conflicts in the budding yeast. DNA replication fork-blocking protein Fob1 is required for RFB activity, as without it there is no replication fork arrest at the RFB, resulting in transcription–replication collisions. **c** | Chromatin remodelling by the FACT (facilitates chromatin transcription) complex facilitates transcription as well as replication fork progression. Without FACT, altered chromatin reorganization results in transcription–replication collisions. **d** | At pericentromeric regions in fission yeast, co-transcriptional RNAi releases the RNAP, thereby enabling completion of DNA replication. Without the RNAi machinery, failure to release the RNAP during S phase results in transcription–replication collisions. MCM, minichromosome maintenance complex; Pol α, DNA polymerase α; Pol δ, DNA polymerase δ; Pol ε, DNA polymerase ε.

transcription pausing, arrest and backtracking, suggesting that uncontrolled and high transcription rates lead to transcriptional stress[52]. RECQL5 associates with the replicative DNA sliding clamp proliferating cell nuclear antigen (PCNA), which suggests that RECQL5 is also involved in replication. Accordingly, RECQL5-deficient cells fail to incorporate bromodeoxyuridine under conditions of replication stress and rapidly accumulate DNA damage, and these effects can be alleviated by fully arresting replication with the replication inhibitor aphidicolin[53]. Importantly, spontaneous DSBs accumulate in RECQL5-depleted cells during replication, but only in association with RNA Pol II transcription, as the spontaneous breaks are located in transcribed genes and transcription inhibition eliminated their appearance[54]. Furthermore, RECQL5 has been shown to have a role in suppressing genome rearrangements that are preferentially associated with common fragile sites (BOX 1) and transcribed genes[52]. These data have led to the proposal that RECQL5 prevents transcription–replication collisions. Recently, RECQL5 was also ascribed a role in preventing the formation of RNA–DNA hybrids. Apparently, RECQ5 promotes TOP1 sumoylation by facilitating the interaction with the PIAS1–SRSF1 E3 ligase complex. This modification is necessary for the binding of TOP1 to RNA Pol II and for the efficient recruitment of mRNA-processing factors to transcriptionally active sites, thereby reducing the formation of RNA–DNA hybrids, as inferred by the increased levels of such hybrids in cells defective in RECQL5-dependent TOP1 sumoylation[55]. Therefore, RECQL5 may maintain genome integrity by actively limiting the occurrence of transcription–replication conflicts and/or by reducing the accumulation of non-B DNA structures generated during transcription that could enhance replication blockage (FIG. 3).

Chromatin remodelling. In eukaryotes, transcription and replication occur in the context of highly structured chromatin. Following replication, the chromatin state is maintained by coupling the deposition of recycled parental histones with newly synthesized histones on the duplicated DNA, which is carried out by histone chaperones and chromatin-remodelling complexes. Even though not much work has been done on the role of chromatin remodelling in diminishing transcription–replication conflicts, evidence exists about the importance of such factors in collisions. This has been clearly shown for the histone chaperone FACT (facilitates chromatin transcription) complex, which was initially found to be required for chromatin remodelling during transcription but is also involved in DNA replication[56–58]. Yeast and human cells lacking FACT complex activity have high levels of transcription–replication collisions, exhibiting fork progression impairment that correlates with increased genomic instability. However, when transcription was inhibited with cordycepin in FACT-depleted human cells, the rate of fork progression was restored to normal[59]. Therefore, chromatin-reorganizing factors, such as FACT, can prevent collisions by promoting replication fork progression through transcribed regions (FIG. 4c). It would certainly be interesting to see whether this view would also apply to the INO80 chomatin-remodelling complex, as it was shown in budding yeast that Ino80 cooperates with the transcription factor Paf1c to trigger RNA Pol II degradation at sites of collision[44].

It is worth noting that RNA–DNA hybrids also accumulate in FACT-depleted cells, suggesting that either replication forks often stall at regions containing RNA–DNA hybrids or that RNA–DNA hybrids are formed as a consequence of transcription–replication collisions. The negative DNA supercoiling that locally accumulates behind a stalled RNA polymerase, putative suboptimal messenger ribonucleoprotein assembly or inefficient chromatin remodelling associated with a transcription–replication collision could create the conditions to favour co-transcriptional R-loop formation. The observation that different states of heterochromatin or chromatin condensation, as identified by the phosphorylation of histone H3 Ser10 or the dimethylation of histone H3 Lys9 (H3K9me2) in *S. cerevisiae*, *Caenorhabditis elegans* and human cells, correlate with the formation of co-transcriptional RNA–DNA hybrids[60] suggests that RNA–DNA hybrids can trigger chromatin compaction, which might also contribute to replication fork stalling at transcribed regions. Indeed, the expansion of triplet repeats in the *FXN* and *FMR1* genes (BOX 1) induced their silencing through the deposition of H3K9me3 and heterochromatinization, and led to the accumulation of RNA–DNA hybrids[61]. Therefore, chromatin compaction following transcription seems to be a contributor to transcription–replication conflicts that needs further exploring (FIG. 4c).

Finally, gene silencing at pericentromeric regions in *Schizosaccharomyces pombe* through the establishment of heterochromatin provides additional support for the role of chromatin organization in the occurrence of transcription–replication conflicts. Such silencing requires the RNAi machinery to facilitate recruitment of chromatin modifiers by siRNA, and it has been proposed that the RNAi machinery is involved in the release of RNA polymerase at pericentromeric regions to prevent collisions[62] (FIG. 4d). ChIP–seq analysis of the RNAi machinery mutant *Δdcr1* showed that RNA polymerase accumulates during S phase at specific pericentromeric repeats, which correlate with sites of siRNA accumulation in normal cells, therefore defining sites of RNA polymerase release to allow replication completion. Importantly, replication fork stalling at such pericentromeric repeats was reduced in mutants of either transcription or replication initiation. Finally, failure to release RNA polymerase due to the absence of RNAi machinery results in DNA damage[62]. These results open up the possibility that the capacity of the RNAi machinery to regulate gene expression and modulate chromatin structure may have an important role in transcription–replication conflicts, but this role has yet to be deciphered. Consistent with this view, genome-wide analysis has shown that RNAi activity in releasing the RNA polymerase is not restricted to heterochromatin regions but also occurs at sites of replication stress, such as at highly transcribed genes and rRNA and tRNA genes[63].

Conflicts and the DNA damage response

The major consequence of transcription–replication conflicts is genome instability. In most cases, this is triggered by chromosome breakage that occurs as a result of replication fork blocking and collapse, which can generate DSBs; therefore, transcription–replication conflicts are expected to be sensed and resolved by the DNA damage response (DDR) and various repair pathways.

Prevention of transcription–replication conflicts by the DDR. There is no evidence that the DDR senses transcription–replication collisions directly, but fork blockage could be sufficient to trigger DNA damage checkpoints, as it can result in the uncoupling of leading- and lagging-strand synthesis, generating a long stretch of ssDNA[64,65] (FIG. 5). It is likely that unless a DSB is produced, which activates the ataxia telangiectasia mutated (ATM; Tel1 in budding yeast)-dependent checkpoint, a transcription–replication collision will activate the ataxia telangiectasia and Rad3 related (ATR; Mec1 in budding yeast)-dependent replication checkpoint, which senses stretches of ssDNA and protects the integrity of replication forks. Evidence that the Mec1 checkpoint is involved in transcription–replication collisions has been provided for tRNA transcription in budding yeast. The tRNA transcription cycle involves assembly of a pre-initiation complex (PIC) comprising RNA Pol III and two DNA binding factors, TFIIIB and TFIIIC. At tRNA genes, transcription can act as a RFB during normal cell proliferation because the PIC interferes with fork progression[49,66], but the Mec1 replication checkpoint can prevent replication fork stalling by dismantling the PIC in budding yeast[67]. This may be a conserved mechanism to reduce transcription–replication collisions at tRNA genes; in *Drosophila melanogaster*, removal of Rpp30 (RNaseP protein p30), which is required for correct pre-tRNA processing, leads to increased replication stress and checkpoint activation[68].

DNA damage response (DDR). A network of DNA damage repair and checkpoint factors that function together to repair DNA lesions.

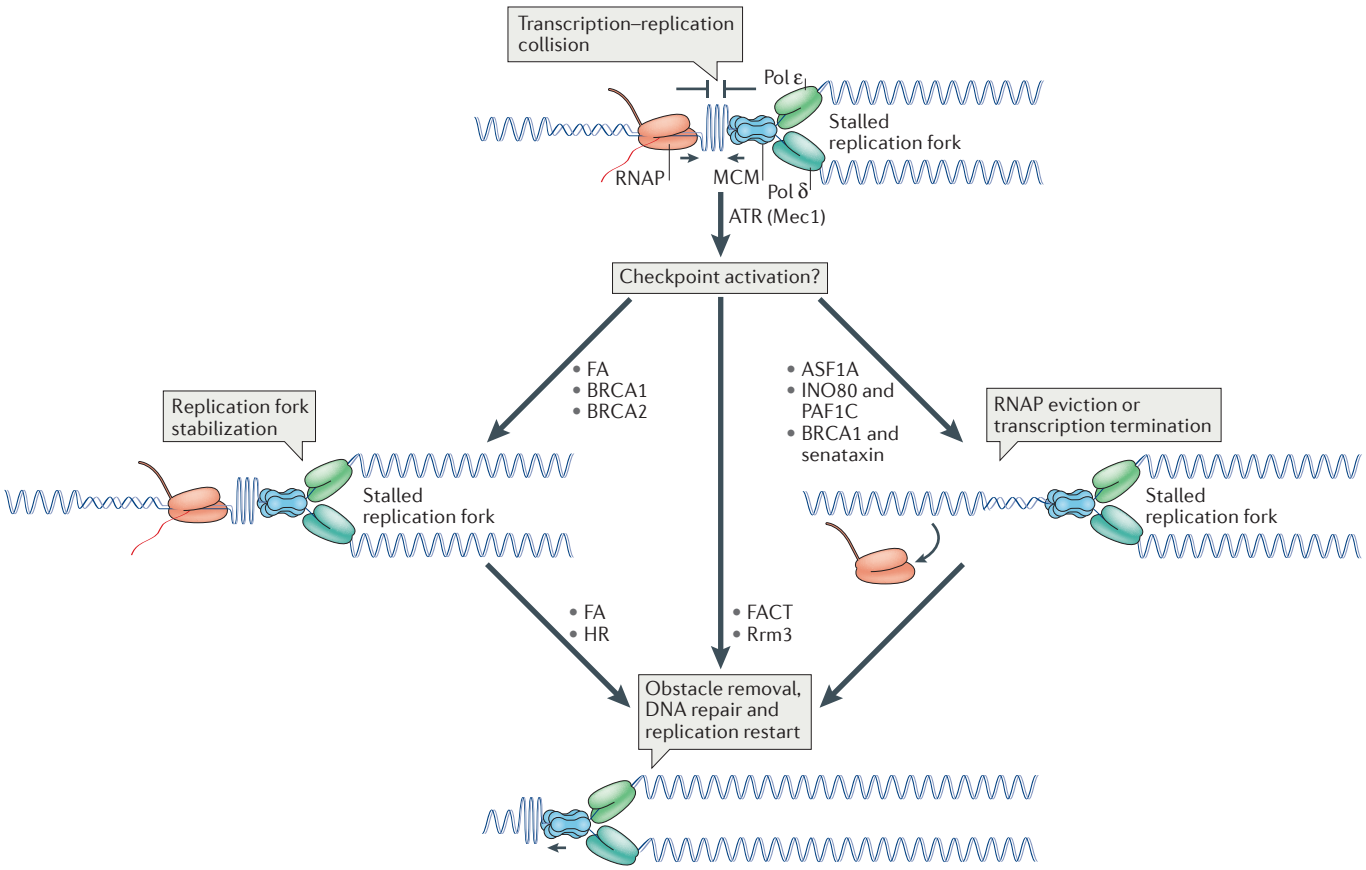

Figure 5 | **Resolving transcription–replication collisions to avoid genome instability.** A stalled replication fork can activate the ataxia telangiectasia and Rad3 related (ATR; Mec1 in budding yeast)-dependent checkpoint to solve collisions and avoid their consequences. Specific factors are recruited to resolve or prevent the obstacle, including DNA helicases (such as Rrm3), mRNA-processing proteins or chromatin-remodelling complexes (such as FACT (facilitates chromatin transcription)). In addition, the RNA polymerase (RNAP) could be released at transcription termination sites, with the help of BRCA1 and the helicase senataxin or the chromatin-remodelling complex INO80 and transcription factor complex PAF1C, as a way to avoid the collisions. Similarly, at tRNA transcription sites, RNA Pol III is directly evicted during S phase. Stabilization and resumption of stalled forks at transcribed DNA regions can occur via DNA repair factors, such as those of the Fanconi anaemia (FA) repair pathway, including the tumour suppressors BRCA1 and BRCA2. Finally, different DNA repair pathways can act at collision sites, if these degenerate into DNA lesions. Although direct involvement of the ATR-dependent checkpoint has been reported in some examples, the degree of its involvement in other cases is yet unknown. ASF1A, anti-silencing function protein 1 homologue A; HR, homologous recombination; MCM, minichromosome maintenance complex; Pol δ, DNA polymerase δ; Pol ε, DNA polymerase ε.

A similar involvement of the DDR in reducing transcription–replication conflicts is observed in human cells when using hydroxyurea and doxorubicin, which are genotoxic agents that interfere with DNA replication by depleting dNTP pools and by inhibiting topoisomerase II, respectively, and that induce fork stalling preferentially in regions of actively expressed genes. Under these conditions, the ATR checkpoint ameliorates transcription–replication collisions by promoting ATR-mediated degradation of the histone chaperone ASF1A (anti-silencing function protein 1 homologue A). This leads to histone eviction, RNA Pol II release and transcription repression[69]. Related to this, it has been shown in budding yeast that RNA Pol II is removed from transcribed genes located near firing origins of replication after exposure to hydroxyurea to avoid further impairment of replication fork progression. This removal depends on Mec1 in cooperation with the chromatin-remodelling complex Ino80 and the transcription factor Paf1c[44]. Therefore, checkpoint-mediated transcription repression, putatively involving RNA Pol II eviction, may protect genome integrity by reducing transcription–replication collisions (FIG. 5).

Upon its activation, ATR phosphorylates many downstream targets, including the tumour suppressor p53, to coordinate the DDR. Recently, p53 was shown to be involved in preventing transcription–replication conflicts by reducing topological stress, as p53 deficiency increases sensitivity to topoisomerase inhibitors and culminates in replication-dependent accumulation of DNA damage, both of which can be reversed by transcription inhibition[70]. However, whether these effects are due to a specific role of p53 as part of the replication and transcription machineries or whether they are due to spatial and

Phosphomimetic
Proteins with amino acid substitutions that simulate their phosphorylated state.

temporal regulation of transcription during replication is still unclear.

Dealing with transcription–replication conflicts in eukaryotes is further complicated by the fact that transcription is coupled with RNA splicing, maturation and nuclear export. A subset of transcribed genes is localized proximally to nuclear pores, presumably to facilitate the nuclear export of their transcripts. This phenomenon is known as 'gene gating' and is mediated by RNA-binding proteins such as the transcription export 2 complex (TREX-2) and nucleoporins[71,72]. Interestingly, mutations in some of these gene-gating factors partially suppress fork instability in checkpoint mutants, suggesting that in the absence of a functional checkpoint, if replication forks are stalled in loci located at the nuclear pore, chromatin cannot be released from the pores to allow replication restart[73]. It is possible that the persistence of transcribed chromatin at the nuclear periphery and the accumulation of torsional stress due to the fixation of DNA to the pore that restricts supercoil release are impediments to replication fork progression. If this is indeed the case, the Mec1 and Rad53 checkpoint kinases could trigger the release of the fork blocked from the nuclear pore, allowing completion of replication[73]. It is interesting to note that Mec1 activation results in phosphorylation of the Rrm3 and Pif1 DNA helicases, which, as mentioned above, assist replication at obstacles in budding yeast. Both helicases have been associated with stalled replication forks, and combined removal of both suppresses the increase in fork reversal and cell lethality in the absence of a functional Rad53 under conditions of replication stress, suggesting that both replication fork reversal and cell lethality depend on the unregulated activity of Rrm3 and Pif1 (REF. 74). Indeed, under conditions of replication stress, the expression of phosphomimetic *rrm3* mutants lessen the *rad53* mutation defects. Although the role of Rrm3 is not specific to replication forks that are stalled at sites of collisions, and includes other types of protein obstacles that require restart of replication forks, it seems plausible that the Mec1 checkpoint surveillance mechanism helps to reduce transcription–replication collisions and their consequences through its action on Rrm3.

Conflict resolution by the BRCA and Fanconi anaemia proteins. An emerging question is whether specific DNA repair pathways are active at transcription–replication collision sites following activation of the DDR activation by the DNA damage checkpoints. Although thorough analysis of this issue is required, insight into the role of specific DNA repair pathways in preventing transcription-associated genomic instability, including instability caused by RNA–DNA hybrids, is emerging. For example, it has recently been shown that the tumour suppressors BRCA1 and BRCA2, which are involved in DSB repair, help to prevent the formation of or remove RNA–DNA hybrids[75,76]. Although this function could be related to the DSB repair function of BRCA proteins, the fact that they are components of the Fanconi anaemia pathway, which repairs interstrand crosslinks and was recently shown to prevent the collapse of stalled replication forks[77], suggests that these proteins may have a key role in resolving replication fork stalling resulting from transcription–replication conflicts (FIG. 5). Accordingly, it has recently been demonstrated that the Fanconi anaemia repair pathway contributes to preventing transcription–replication conflicts from resulting in DNA lesions, in particular those linked to the accumulation of RNA–DNA hybrids. Reducing the number of RNA–DNA hybrids by inhibiting transcription with cordycepin or by directly removing them with RNaseH alleviated the DNA damage observed in cells lacking Fanconi anaemia complex subunits[78,79]. The Fanconi anaemia pathway may function at sites of collisions, in particular those involved with RNA–DNA hybrid accumulation, by repairing the DNA breaks resulting from replication fork arrest caused by RNA–DNA hybrids to facilitate resumption of replication[79]. Specifically, FANCM (Fanconi anaemia complementation group M) was shown to have RNA–DNA branch migration activity *in vitro*, which has led to the suggestion that the Fanconi anaemia factors could help to remove RNA–DNA hybrids *in vivo*[78], but this has not been demonstrated.

A genome-wide analysis revealed that BRCA1 is enriched in actively transcribed genes at regions of transcription termination, and this enrichment is mediated by the direct interaction with senataxin[80], which is an RNA–DNA helicase involved in RNA Pol II transcription termination that was shown to supress RNA Pol II collisions at transcription termination sites[81,82]. Indeed, senataxin and BRCA1 were shown to interact with each other to suppress the formation of RNA–DNA hybrids at transcription termination sites[80]. This finding, together with the parallel observation that BRCA2 interacts with TREX-2 (REF. 75), favours the hypothesis that DNA repair proteins and the transcription apparatus cooperate to respond to transcription–replication conflicts (FIG. 5). It would be interesting to explore whether this is related to the purification of the RNA Pol II complex together with

Box 2 | Oncogenes and transcription–replication collisions

An oncogene refers to a gene that, when mutated, contributes to the development or progression of cancer, whereas the term proto-oncogene is reserved for its wild-type allele. Oncogenes generally regulate cell division, cell differentiation and/or cell death. This is the case for the oncogene *MYC*, which regulates the transcription of several genes that control cell growth and cell cycle progression[2]. As replication stress and genomic instability are hallmarks of cancer cells[96], it seems plausible that oncogenes may increase the rate of transcription–replication conflicts, which can serve as a source of genomic instability. Altered expression of cyclin E or oncogenic RAS induces chromosomal fragility at sites that colocalize with large genes and only partially overlap with the canonical, replication stress-induced fragile sites[97]. Oncogene expression can negatively affect replication by promoting activation of replication origins, as shown for MYC and cyclin E[98,99]. Using DNA combing and cell-free extracts derived from *Xenopus laevis* eggs, it has been shown that Myc increases activation of early-replicating origins, resulting in elevated fork collapse and subsequent DNA damage accumulation[100]. Although such replication fork collapses may occur independently of transcription, the excess of active replication forks may increase the probability of collisions. Consistent with this view, DNA damage resulting from replication impairment by cyclin E overexpression was partially suppressed by the transcription inhibitor cordycepin, suggesting that collisions can indeed contribute to oncogene-induced replication stress[101]. In the future, it will be important to determine the general relevance of this phenomenon in cancer cells and to explore the possibility of using transcription–replication collisions as a selective target in cancer therapy.

DSB repair factors in yeast[83]. Of note, the yeast mitogen-activated protein kinase Hog1, which is also a transcription factor that responds to osmotic stress, was shown to delay replication timing by affecting early-origin firing and replication fork progression through direct action on Mrc1, which is a replisome component[84] and a substrate of the replication checkpoint protein Mec1 (REF. 85). These data suggest that under conditions of transcriptional burst — for example, in response to environmental stress — the coordinated action on both the transcription and replication machineries could be crucial in the management of transcription–replication conflicts.

Conclusions and perspectives

We do not yet have a molecular understanding of how replication forks traverse DNA regions undergoing transcription. Evidence accumulated in the past two decades indicates that an important natural source of genome instability stems from transcription–replication conflicts. The biomedical relevance of transcription–replication conflicts is emphasized by several cancer-prone conditions or human diseases, such as Fanconi anaemia, ataxia-ocular apraxia type 2 or amyotrophic lateral sclerosis type 4, which are caused by mutations in genes that are involved in preventing or solving such conflicts. We have recently started to identify factors and putative mechanisms that may contribute to either diminishing the frequency of collisions or resolving them in a way that limits their negative consequences, such as replication stress and DNA breaks. However, to decipher the mechanisms by which replication forks replicate DNA undergoing transcription without compromising genome integrity, we still need to identify DNA sequences or secondary structures, or specific chromatin features at collision hot spots. We also need to determine the role of torsional stress and chromatin remodelling in either promoting or preventing transcription–replication collisions, or the mechanisms by which the DDR senses such collisions or resolves them. This will necessitate a better understanding of the dynamics of replication and transcription machineries in response to different types of obstacles, from DNA lesions to protein barriers, and will not only help us to understand how cells execute proper replication of their entire genome, bypassing the putative barriers generated by transcription, but also to clarify the importance of transcription–replication collisions as a source of DNA damage, in particular of oncogene-induced replication stress and DNA damage (BOX 2), and the possibility of using transcription–replication collisions as selective targets in cancer therapy.

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Competing interests statement
The authors declare no competing interests.