Hypertranscription and replication stress in cancer

Bowry, Akhil; Kelly, Richard; Petermann, Eva

DOI:
10.1016/j.trecan.2021.04.006

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Bowry, A, Kelly, R & Petermann, E 2021, 'Hypertranscription and replication stress in cancer', *Trends in Cancer*, vol. 7, no. 9, pp. 863-877. https://doi.org/10.1016/j.trecan.2021.04.006

Trends in Cancer

CellPress OPEN ACCESS

Review

Hypertranscription and replication stress in cancer

Akhil Bowry,[1] Richard D.W. Kelly,[1,*] and Eva Petermann[1,*]

Replication stress results from obstacles to replication fork progression, including ongoing transcription, which can cause transcription–replication conflicts. Oncogenic signaling can promote global increases in transcription activity, also termed hypertranscription. Despite the widely accepted importance of oncogene-induced hypertranscription, its study remains neglected compared with other causes of replication stress and genomic instability in cancer. A growing number of recent studies are reporting that oncogenes, such as RAS, and targeted cancer treatments, such as bromodomain and extraterminal motif (BET) bromodomain inhibitors, increase global transcription, leading to R-loop accumulation, transcription–replication conflicts, and the activation of replication stress responses. Here we discuss our mechanistic understanding of hypertranscription-induced replication stress and the resulting cellular responses, in the context of oncogenes and targeted cancer therapies.

Highlights

Hypertranscription is defined as a relative increase in global transcription to support proliferation and cell growth.

Oncogenes and oncogenic signaling pathways stimulate the activity of general transcription factors for all three RNA polymerases to induce hypertranscription.

Oncogene-induced hypertranscription correlates with increased R-loop formation and transcription–replication conflicts, resulting in replication stress and genomic instability.

Targeted cancer therapies such as bromodomain and extraterminal motif (BET) and histone deacetylase (HDAC) inhibitors cause widespread hypertranscription and elevated R-loop levels, which are accompanied by replication stress.

Hypertranscription-induced replication stress does not always activate canonical DNA damage response pathways, and recent work suggests that downregulation of homologous recombination could be a frequent event in response to elevated transcription.

Replication stress and hypertranscription in cancer

A major hallmark of cancer is an increased propensity to acquire genomic alterations, known as genomic instability [1]. Replication stress, or replication-associated DNA damage, which occurs when DNA replication fork progression in S phase slows or stalls, is an emerging source of genomic instability in cancer [2]. There is a growing interest in targeting oncogene-induced replication stress as a therapeutic approach in cancer therapy [3]. Expanding research on replication stress in cancer in recent years has begun to increase our mechanistic understanding of oncogene-induced replication stress and genomic instability. Deregulation of replication initiation events, transcription–replication conflicts, reactive oxygen species (ROS), and altered nucleotide metabolism are all potential causes of replication stress in cancer cells [4]. A new mechanism is now emerging, where it is becoming increasingly evident that replication stress can result from the understudied phenomenon of 'hypertranscription'.

The term hypertranscription was coined in the stem cell biology and developmental biology fields to describe a scenario where transcription activity by all three RNA polymerases (RNA Pols) is upregulated globally across the genome to support stem cell proliferation [5]. Rather than specific transcriptional programs being activated, the vast majority of all nascent RNA synthesis, including at 'housekeeping' genes, is upregulated to support cell growth and proliferation. Consequently, hypertranscription describes a relative and not an absolute increase in transcription [5]. Since it cannot be detected by standard gene expression analyses [5] (Box 1), it has been somewhat neglected as a form of transcriptional reprogramming or deregulation, especially in cancer. Over recent years, several studies in neural progenitor cells [6] as well as cancer and non-cancer cells expressing activated oncogenes [7–9] or treated with transcription-targeting drugs [10–13] have suggested a link between excessive transcription and replication stress. In this review we focus on recent advances in our understanding of how the transcription machinery is deregulated by oncogenes and targeted therapies and how this contributes to replication stress and genomic instability.

[1]Institute of Cancer and Genomic Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, UK

*Correspondence:
R.D.W.KELLY@bham.ac.uk (R.D.W. Kelly) and
E.Petermann@bham.ac.uk (E. Petermann).

Box 1. Methods for detecting oncogene-induced hypertranscription and replication stress

Detecting transcription–replication conflicts

There are two methods for the detection of replication stress: the DNA fiber assay and detecting the activation of DNA damage pathways. In the DNA fiber assay, most studies pulse label cells with two thymidine analogs, iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU), at separate intervals, allowing their imaging by fluorescently labeled antibodies and the quantification of DNA fiber length (replication fork speed) or replication structures (e.g., terminated or stalled forks). On the detection of replication stress, cells activate specific kinases involved in the DNA damage response and DNA damage checkpoint activation. Studies generally focus on changes in the number of γH2AX and/or 53BP1 [7–10] nuclear foci; however, some studies also use western blotting detection of phosphorylated RPA, CHK1, and ATR [8,9]. A much more powerful technique examines the enrichment of γH2AX at the genomic level using ChIP-seq. Combining this technique with transcriptional assays such as RNA-seq or DNA–RNA hybrid immunoprecipitation (DRIP-seq) permits the mapping of hypertranscription-induced DNA damage [7,8].

Quantify and mapping R-loops

The detection of DNA–RNA hybrids or R-loops predominantly relies on the DNA–RNA hybrid (S9.6) antibody [117], which can detect RNA/DNA hybrids using a slot blot of genomic DNA [9], immunofluorescence quantification of S9.6 intensity in nuclei [9], and DRIP-seq [7,8]. As mentioned earlier, DRIP-seq is a powerful technique to measure hypertranscription-induced replication conflicts, although the lack of cell-number normalization, the antibody specificity *in vivo*, and subtle protocol differences may reduce the robustness of this procedure [118].

Quantifying hypertranscription

There are a variety of methods used to study hypertranscription [5]. The most widely employed method utilizes the incorporation of the ribonucleotide analog 5-ethynyl uridine (EU) allowing nascent RNA quantification at the single-cell level using fluorescence microscopy or flow cytometry [5–10]. Although a simple yet powerful technique, this RNA quantification method does not strictly reflect the precise definition of hypertranscription, since the precise source of nascent transcription is undefined. Steady-state RNA quantification from equal numbers of cells using the Qubit or Bioanalyzer application has similar caveats. Precise quantification and mapping of hypertranscription requires RNA-seq combined with either spike-in RNAs or cell-number normalization [5]. Nascent RNA-seq technologies such as global run-on sequencing (GRO-seq) [119], precision nuclear run-on sequencing (PRO-seq) [120], and native elongating transcript sequencing, (NET-seq) [121] have in-built normalization and detect the transcriptional output of all RNA Pols.

Oncogenes induce hypertranscription

Activation of oncogenes and oncogenic signaling pathways drives proliferation and cell growth. The additional demands of cell growth are accommodated by oncogene-induced hyperactivation of the basal transcription machinery. A central hallmark of neoplastic transformation is therefore the deregulation of transcription by all three RNA Pols [5,14–16]. In eukaryotic cells, RNA synthesis is performed by RNA Pol I, RNA Pol II and RNA Pol III, which transcribe a unique set of genes and genomic regions. RNA Pols are recruited by the distinct arrangement of general transcription factors (GTFs) that recognize their respective promoters [17,18]. GTFs are essential for the formation of the pre-initiation complex (PIC), which facilitates DNA opening and the ability of RNA Pols to initiate basal RNA transcription. While RNA Pols and their constitutive GTFs are rarely mutated in cancer [15], many GTFs are consistently overexpressed and hyperactivated in tumors, contributing to malignant transformation [15,16,19,20].

Oncogenic pathways such as RAS-ERK and PI3K-mTOR drive cell proliferation and hypertranscription by phosphorylating GTFs involved in RNA Pol I and III transcription [19,20] (Figure 1A,C). rRNA synthesis by RNA Pol I requires both UBF1 and the SL1 complex to mediate RNA Pol I recruitment, promoter binding, and PIC formation [21–24]. Additionally, UBF1 and SL1 help to mediate RNA Pol I transcription elongation in combination with other RNA Pol I-specific elongation factors [25,26]. Overexpression of RRN3 [27] or TBP [28], components of the SL1 complex, is sufficient to increase rRNA synthesis, illustrating how MYC can promote rRNA transcription. ERK1/2 phosphorylates UBF1 and RRN3, facilitating their ability to recruit additional basal transcriptional machinery [29,30]. Similarly, the PI3K pathway can promote RRN3 and UBF1 phosphorylation directly through PI3K [31] or indirectly through AKT, mTORC1, and CK2 activation [31–33] (Figure 1A).

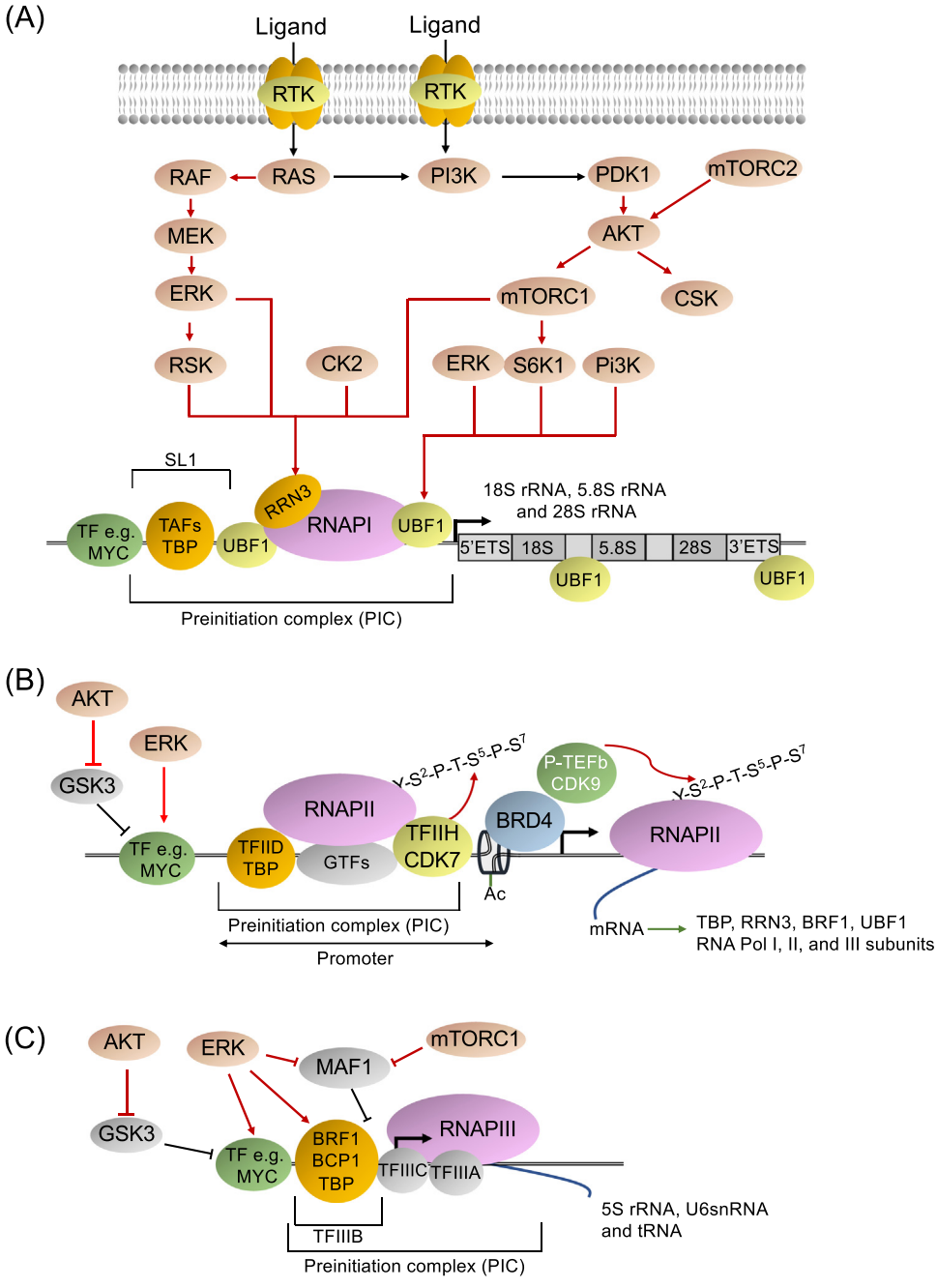


Figure 1. Oncogene-induced hypertranscription. (A) Schematic of growth factor signaling pathways downstream of RAS and PI3K and the regulation of RNA polymerase I (Pol I). UBF1 recruits the SL1 complex and RRN3, mediating RNA Pol I recruitment and promoter binding. UBF1 phosphorylation increases UBF1-Pol I binding. Phosphorylation of RRN3 allows its release from RNA Pol and relieves promoter-proximal pausing. Both UBF1 and SL1 help to mediate elongation in combination with other RNA Pol I-specific elongation factors. (B) Recruitment and activation of RNA Pol II is aided by a preinitiation complex (PIC) formed by the general transcription factors (GTFs) TFIIDA, B, F, E, and H. RNA Pol II phosphorylation on serine 5 of its C-terminal domain (CTD) by TFIIH facilitates the transition from initiation to elongation. Bromodomain-containing protein 4 (BRD4) recruits P-TEFB from its inhibitory 7SK complex, allowing phosphorylation of

(Figure legend continued at the bottom of the next page.)

Oncogene activation also stimulates RNA Pol II activity, altering the expression and activity of many GTFs and transcription factors, such as TBP [34] and C-MYC [35,36] (Figure 1B). C-MYC is also the most frequently amplified gene across all cancer cell types [37]. As a GTF and a constituent component of all RNA Pol PICs, TBP also facilitates RNA Pol I [28], RNA Pol II [9], and Pol III [28] -dependent transcription, and it is known to contribute to cellular transformation [38]. As a transcription factor, C-MYC is a universal mediator of hypertranscription [39,40], regulating the transcription of the vast majority of the human genome, especially genes involved in ribosome biogenesis, protein translation, cell-cycle progression, and metabolism [41,42]. C-MYC directly regulates RNA Pol II target genes that encode RNA Pol I and Pol III subunits or GTFs, such as TBP, RRN3 [43], UBF1 [43,44], and BRF1 [45]. C-MYC also promotes RNA Pol I and Pol III transcription through the interaction with their respective basal transcriptional apparatus [42]. RNA Pol II elongation is further controlled by direct phosphorylation of the C-terminal domain (CTD) of its RPB1 subunit [46]. The CDK7 subunit of TFIIH, the CDK9 subunit of TFIIH, and the CDK9 subunit of P-TEFb phosphorylate serines 5 and 2, present in repeated heptads, to regulate the transition from the initiation to the elongation phase of transcription [18]. Increased P-TEFb activity could play a role in oncogene-induced replication stress.

RNA Pol III transcribes 5S rRNA, tRNA, and other small RNAs involved in RNA processing and translation. The initiation of basal RNA Pol III transcription converges on TFIIIB recruitment to the PIC, which in turn recruits RNA Pol III and facilitates promoter opening [47]. TFIIIB is a multisubunit GTF comprising BRF1 or BRF2, BDP1, and TBP, each of which is critical for RNA Pol III transcription. ERK enhances the function of TFIIIB by phosphorylating and activating BRF1 to stimulate RNA Pol III-dependent transcription of 5S rRNA and tRNA genes [48]. A major regulator of RNA Pol III transcription (and potentially RNA Pol I and II) is the transcriptional corepressor MAF1, which directly interacts with RNA Pol III and inhibits the assembly of TFIIIB by TFIIIC [49]. PI3K-mTORC1 and MAPK-ERK phosphorylation of MAF1 alleviates its ability to inhibit RNA Pol III, permitting transcription [50–52] (Figure 1C).

Oncogene-induced hypertranscription causes replication stress

Transcription can pose obstacles to replication fork progression and thus has the potential to cause replication stress. Replication stress results from an inability to complete genome replication; for example, due to replication fork slowing, stalling, or collapse or a lack of backup sites for replication initiation. Oncogene activation can induce replication stress through a number of mechanisms related to increased proliferation; these include aberrant replication initiation, increased ROS, altered nucleotide metabolism, and hypertranscription [4]. For example, overexpression of the proto-oncogene CYCLIN E, required for the G1 to S phase transition, has been shown to cause replication stress through changes to replication initiation [53,54]. Similarly, H-RAS activation [55] and C-MYC overexpression [56] both increase replication initiation and replication stress.

Alternatively, increases in transcription activity can underlie oncogene-induced replication stress. Recent work has highlighted that hypertranscription may underlie transcription–replication conflicts in the context of oncogene activation. Introduction of the oncogene H-RAS^{V12} into

RNA Pol II CTD serine 2 and transcriptional elongation. Oncogenic signaling stabilizes MYC through direct phosphorylation or preventing GSK3-directed degradation, allowing MYC to recruit coactivators to target genes. Oncogenic activation of RNA Pol II increases the expression of GTF and RNA Pol subunits involved in RNA Pol I-, II-, and III-dependent transcription. (C) RNA Pol III transcription initiation requires TFIIIC binding at tRNA genes or TFIIIA and TFIIIC binding at 5s rRNA genes. TFIIIC recruits TFIIIB, comprising BRF1 or BRF2, BDP1, and TBP, which in turn recruits RNA Pol III. Oncogenic signaling activates BRF1 and inhibits the RNA Pol III transcriptional repressor MAF1. Abbreviations: RNAP, RNA polymerase; TF, transcription factor.

human BJ fibroblasts increases global transcription activity. Together with increased R-loop levels, this results in replication fork slowing, DNA damage, and markers of genomic instability [9]. Hypertranscription and replication stress are both linked to H-RAS^{V12}-induced increase in TBP mRNA and protein levels. Depletion of TBP, small-molecule inhibition of nascent transcription, or overexpression of RNase H1, an endonuclease that specifically degrades the RNA in RNA–DNA hybrids, all counteract H-RAS^{V12}-induced replication stress [9]. As TBP is involved in the activation of all three RNA Pols, it will be interesting to investigate the roles of other, more specific GTFs, as well as RNA Pol I and Pol III, in oncogene-induced replication stress (Figure 2A).

A parallel study showed a similar mechanism using estrogen (E2, 17b-estradiol) stimulation of hormone-starved MCF7 breast cancer cells (Figure 2B). Estrogen had previously been shown to induce features of hypertranscription in MCF7 cells, including upregulation of RNA Pol I and Pol III [57]. Estrogen increases global transcription activity as measured by 5-ethynyl uridine (EU) incorporation and stimulated the formation of R-loops (Box 2) at estrogen-responsive genes [7]. R-loops colocalize with DNA damage markers, and ectopic expression of RNase H1 to suppress R-loop formation reduced estrogen-induced DNA damage and double-strand breaks (DSBs) [7]. At least some estrogen-induced damage is DNA replication dependent, suggesting transcription–replication conflicts [7].

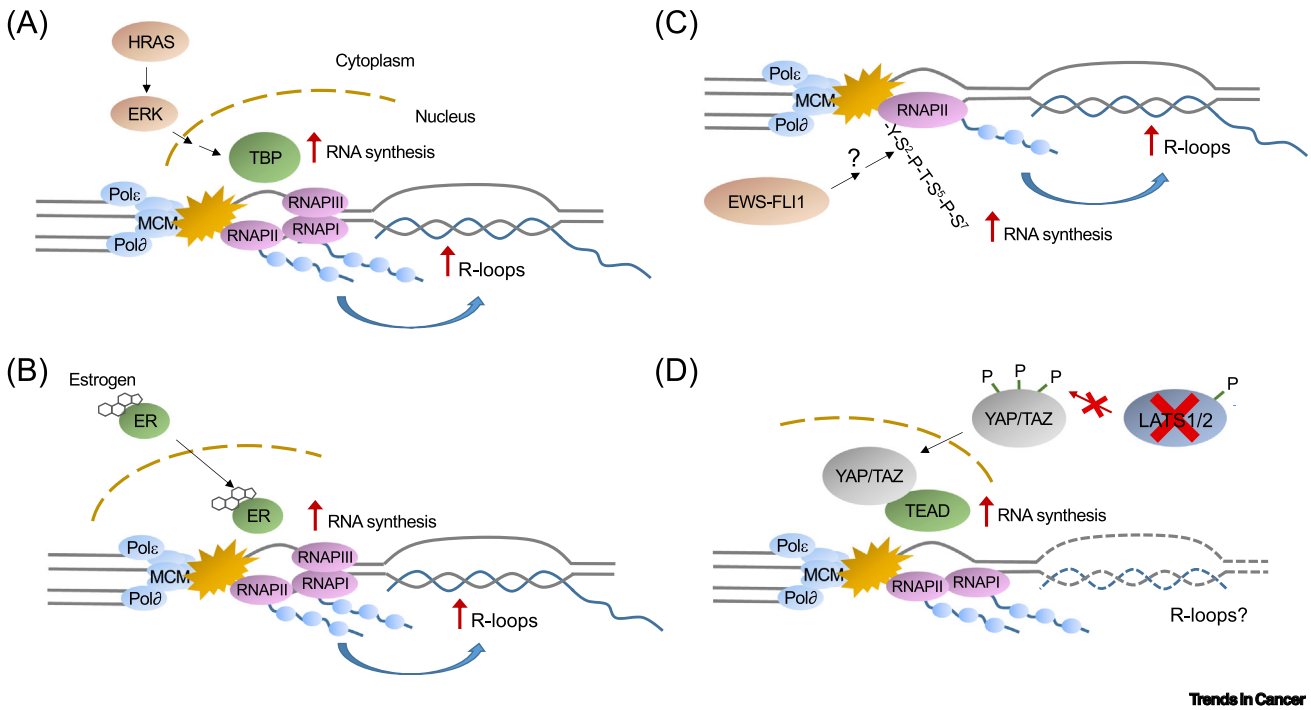


Figure 2. Oncogenic signaling pathways induce transcription and replication stress. (A) H-RAS activation increases global RNA synthesis, at least in part through increased expression of the general transcription factor (GTF) TBP. Elevated RNA synthesis is accompanied by an increase in R-loops and by replication stress. TBP activates all three RNA polymerases (RNA Pols); thus, RAS-induced replication stress could result from increased RNA synthesis by RNA Pol I and Pol III as well as Pol II. (B) Estrogen activates the estrogen receptor (ER) transcription factor, causing a rapid burst in transcription at a large number of responsive genes, including those transcribed by RNA Pol I and Pol III. This is accompanied by increased R-loop formation, replication stress, and DNA breakage. (C) Expression of the EWS–FLI1 fusion oncoprotein, which drives Ewing sarcoma, increases RNA Pol II C-terminal domain (CTD) phosphorylation via an unknown mechanism. Ewing sarcoma cell lines, or cell lines expressing EWS–FLI1, display increased RNA synthesis, increased R-loops, and replication stress. (D) Knockout of the Hippo pathway LATS1/2 kinases leads to hypophosphorylation and nuclear translocation of the YAP/TAZ transcriptional coactivators. Nuclear YAP/TAZ interact with TEAD transcription factors to strongly increase nascent RNA synthesis by RNA Pol I and Pol II, which promotes replication stress. Abbreviation: RNAP, RNA polymerase.

Box 2. Hypertranscription, R-loops, and replication stress

R-loops are three-stranded DNA:RNA hybrids that can form during transcription, when the nascent RNA rehybridizes with the template DNA, leaving a displaced homologous ssDNA [122]. Generally, R-loop formation is favored in RNA Pol II-dependent genes at structural or sequence features such as negative supercoiling, high GC content, and DNA nicks [101,123]. R-loops are important regulators of gene expression, DNA replication, and DNA repair [101,123]; however, unscheduled and persistent R-loops are a source of DNA replication stress, DNA damage, and genome instability [101]. Mechanistically, R-loops are very stable hybrids that can lead to conflicts with replication through the physical restriction of replication fork movement [124]. Inhibition of replication [125–127] or transcription [9] or overexpression of RNase H1, an endonuclease that specially degrades RNA in R-loops [7–9], reduces R-loop levels and alleviates replication stress. R-loops have been linked to DNA damage, and ectopic expression of RNase H1 can lower γH2AX levels [128,129]. R-loop-dependent damage can be both replication dependent and replication independent [130].

R-loops can occur through increased synthesis of both coding and noncoding RNA. Depletion of SPT6, a transcription elongation factor, interferes with the termination of noncoding transcription and enhances the synthesis of extended-length long noncoding RNA (lncRNA) [131]. This extended lncRNA is more prone to the accumulation of R-loops, which promotes replication stress and DNA damage. This underlines the importance of transcription regulation in regulating R-loop levels to ensure minimal damage and genomic instability [131].

The role of R-loop accumulation in replication stress is an emerging field and it has been shown that R-loop-induced replication stress can be observed outside the immediate context of oncogene activation. SMARCAL1 is a protein involved in transcriptional regulation and the processing of DNA structures, mutations of which cause Schimke immuno-osseous dysplasia. The loss of SMARCAL1 leads to an increase in R-loop formation as well as increased γH2AX [132]. Homozygous loss of TOP3B, a topoisomerase involved in relaxing negatively supercoiled DNA, also causes excessive R-loop and RAD51 focus formation combined with an increase in genomic instability. TOP3B loss is potentially associated with renal cancer [133]. Loss of Polycomb group transcriptional repressor proteins such as BMI1 or RNF2, which keep chromatin in a silenced state, slows replication fork progression and leads to increased 53BP1 focus formation and genomic instability [134]. The p53 inhibitor and proto-oncogene MDM2 also interacts with the Polycomb repressive complex. Depletion of MDM2 leads to an increase in R-loops, which was associated with replication fork slowing and increased γH2AX formation [135].

Likewise, the EWS–FLI1 fusion oncoprotein, which causes Ewing sarcoma, has been suggested to increase RNA Pol II occupancy and transcription-associated R-loops genome wide [8]. Again, overexpression of RNase H1 reduces R-loop accumulation and ataxia telangiectasia and Rad3 related (ATR) activation while increasing proliferation, suggesting R-loop-induced replication stress [8]. The interpretation of these data is complicated by the fact that some experiments were performed using a model for inducible EWS–FLI1 expression while others were performed using Ewing sarcoma cell lines, which makes it difficult to ascribe all observations directly to EWS–FLI1. EWS–FLI1 increases transcription and R-loop formation through an unknown mechanism; however, Ewing sarcoma cell lines contain high levels of phosphorylated RNA Pol II [8]. Therefore, it could be speculated that EWS–FLI1 increases R-loops through elevated RNA Pol II transcriptional elongation (Figure 2C).

Several other oncogenes are associated with increased replication stress, although the involvement of hypertranscription is only now becoming apparent. When the genes encoding the Hippo pathway kinases LATS1 and LATS2 are conditionally deleted during mouse brain development, this leads to activation of the main Hippo pathway transcription coactivators YAP/TAZ and increased nascent transcription in neural progenitor cells. This hypertranscription triggers replication stress and DNA damage, inhibiting differentiation and causing widespread apoptosis [6]. This study stands out by performing a thorough nascent transcriptome analysis that nicely illustrates the features of YAP/TAZ-induced hypertranscription, although it did not measure R-loop formation (Figure 2D). In contrast to other studies, transcription inhibition was unable to rescue slow replication fork progression and only rescued fork stalling [6]. Given these findings, it seems reasonable to suspect similar hypertranscription and replication stress phenotypes in cancers where YAP and TAZ are mutually amplified or activated [58].

Hypertranscription in targeted cancer treatment

While oncogenes can increase transcription, targeting transcription is an exciting avenue for cancer therapy. Targeted therapeutics have been developed to inhibit transcription factors [16,59], CDK7 [60], CDK9 [61], CDK12 [62], BET proteins [63], and histone deacetylases (HDACs) [64]. BET proteins and HDACs are interesting therapeutic targets: they both focus on a post-translational modification (PTM), lysine acetylation, and their inhibitors (BETi and HDACi) can both cause hypertranscription and replication stress. Histone acetylation levels are antagonistically governed by histone acetyltransferases (HATs) and HDACs and regulate chromatin structure, DNA accessibility, and transcriptional permissiveness [65,66] (Figure 3A). Although a direct link between histone acetylation and hypertranscription is lacking, in general terms transcriptional output correlates with histone acetylation levels [67]. Histone lysine acetylation decreases nucleosome interactions with negatively charged DNA to loosen chromatin [68,69] and acts as docking site for bromodomain-containing transcriptional activators, such as bromodomain-containing protein 4 (BRD4) [70] (Figure 3A). Similar to oncogenes, BETi and HDACi can cause hypertranscription and replication stress and the associated DNA damage responses.

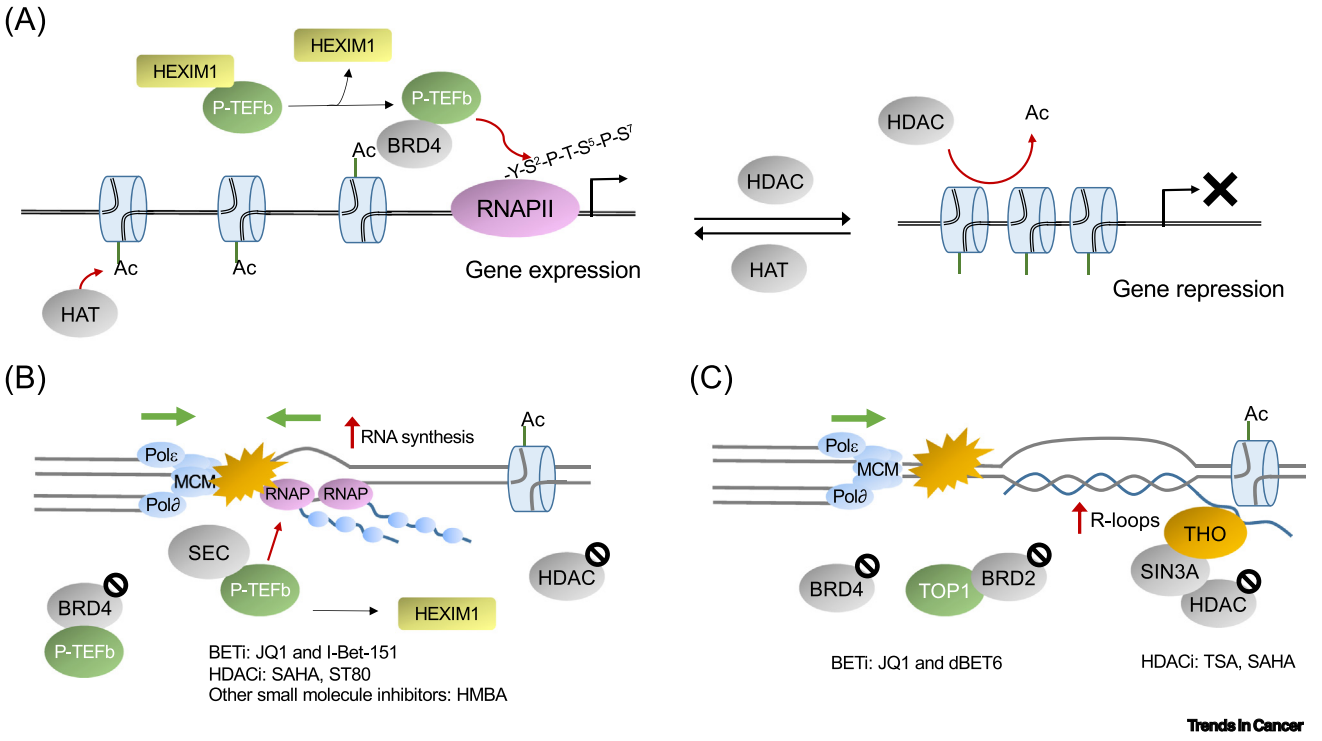


Figure 3. Targeted epigenetic therapies induce transcription–replication conflicts. (A) Histone acetyltransferases (HATs) acetylate chromatin, leading to less compacted chromatin and transcriptional activation. Bromodomain and extraterminal motif (BET) proteins function as transcriptional coactivators and are recruited to acetylated lysines on histone tails. This aids the recruitment of transcription factor complexes such as P-TEFb to activate the transcription of downstream genes. Histone deacetylases (HDACs) remove acetylation, compacting chromatin, which can lead to transcriptional silencing. (B) Small-molecule inhibitors that interfere with transcription, including BET inhibitors (BETis), HDAC inhibitors (HDACis), and HMDA, can release P-TEFb from its inhibitory complex with HEXIM1-7SK and increase the activation of RNA polymerase II (Pol II) via C-terminal domain (CTD) phosphorylation. When BET proteins are inhibited, active P-TEFb can still be recruited by the super-elongation complex (SEC). Increased RNA synthesis can be accompanied by transcription–replication conflicts leading to replication stress. (C) Inhibition or depletion of bromodomain-containing protein (BRD) 4 and BRD2 leads to accumulation of R-loops and associated replication stress. BRD4 is proposed to prevent the buildup of R-loops by promoting the transition of paused to elongating RNA Pol II, and BRD2 suppresses R-loops by stimulating the activity of TOP1. Inhibition of HDAC with trichostatin A (TSA) or suberanilohydroxamic acid (SAHA) also causes accumulation of R-loops and replication stress. A recent model suggests that the HDAC1/2 corepressor complex and the THO RNA processing complex interact to suppress co-transcriptional R-loop accumulation. Abbreviation: RNAP, RNA polymerase.

BET is induce transcription–replication conflicts

The BET bromodomain proteins, comprising BRD2, BRD3, BRD4, and BRDT [71], are transcription regulators that recruit larger protein complexes including transcription factors [72]. BRD4 and BRDT are key regulators of transcriptional elongation by recruiting P-TEFb [73]. Cell-permeable small-molecule BETis such as JQ1 were developed to open a potential new way to target oncogenic transcription. BETis bind competitively to BET bromodomains and prevent their recognition of acetylated lysines, which in turn leads to loss of P-TEFb recruitment, reduced CDK9 phosphorylation of RNA Pol II, DSIF, and NELF resulting in RNA Pol pausing, and reduced transcriptional elongation at genes like *MYC* [74]. Additionally, BETis disrupt the 7SK inhibitory complex of P-TEFb, increasing free and active P-TEFb [75,76] and the transcription of highly expressed histone and noncoding RNA genes [10,77] (Figure 3B). In preclinical studies, BETis have potential anticancer applications including decreased *MYC* expression [63,74,78], increased replication stress [10–12,79], and altered DNA damage checkpoint activation and repair [80–83].

Despite being designed to target transcription, BETis cause replication fork slowing [79]. Recent mechanistic studies suggest that BET inhibition as well as loss of BRD4 or BRD2 can induce replication stress both through hypertranscription and increased R-loop formation [10–13] (Figure 3B,C). BETi treatment increases nascent RNA synthesis early (within 1 h) during treatment across a range of cell lines, although some cell types did not display this response. This was ascribed to increased transcription of highly transcribed histone and other non-polyadenylated noncoding RNA genes. Further investigation demonstrated hypertranscription-induced replication stress and linked this to specific loss of BRD4 activity [10]. Increased R-loop formation and replication stress has also been shown following JQ1 treatment and siRNA depletion of either BRD4 or BRD2 in HeLa (cervical adenocarcinoma), HCT116 (colorectal carcinoma), and U2OS (osteosarcoma) cell lines [11–13] (Figure 3C). It is important to note that increased R-loop formation is not necessarily linked to a burst of hypertranscription, as other mechanisms have been postulated. For example, loss or inhibition of BRD4 prevents the transition of paused to elongating RNA Pol II, leading to the buildup of R-loops [13], and BRD2 suppresses R-loops by interacting with and stimulating the activity of TOP1 [12].

HDAC inhibition causes replication stress

HDACis have been developed with the aim of specifically killing cancer cells. The most widely studied class 1 HDACs (HDAC1, HDAC2, and HDAC3) are the most highly expressed and the main targets of these drugs. The HDACis suberanilohydroxamic acid (SAHA) and depsipeptide have been approved for cutaneous T cell lymphoma [84]; however, HDACis have as yet proved ineffective against solid tumors [64]. HDACs and HATs are implicated in cell cycle regulation, apoptosis, and the DNA damage response [85]. HDACis have pleiotropic effects in cancer cells, with cell death attributed to intrinsic and extrinsic apoptosis activation, impaired DNA replication, replication stress, and a reduced DNA damage response [85,86].

While HDACs are considered transcriptional repressors, loss of HDAC activity leads to both up- and downregulation of gene expression [87]. HDACis can also promote the release of active P-TEFb through the same mechanism as BETis, which may stimulate nascent RNA synthesis [88] (Figure 3B). Treatment with SAHA [89] and specific inhibition or loss of HDAC1/2 [90] and HDAC3 [91,92] leads to rapid replication-fork slowing and replication stress responses. HDAC3 inhibition slows fork speeds as early as 5 min after addition of the drug [91]. In addition, stress is induced without changes in the expression of DNA replication or DNA damage response (DDR) genes, suggesting direct roles for HDACs in maintaining replication fork progression [90]. There are several potential ways by which chromatin changes after loss of HDAC activity might directly perturb DNA replication, such as altered origin firing [93] or accumulation of DNA damage [90]. It

is noteworthy that other histone modifiers, including HATs [94,95], as well as chromatin remodelers and histone chaperones are required for normal replication fork progression and the resolution of transcription–replication conflicts [96,97]. However, hypertranscription has not yet been considered as a potential mechanism and only one study has examined the loss of HDAC activity and transcription–replication conflicts. Loss of SIN3A, an essential scaffold subunit of the SIN3A HDAC1/2 corepressor complex, or HDAC inhibition with SAHA or trichostatin A (TSA) increases R-loop levels in HeLa cells. SIN3A depletion leads to transcription- and R-loop-dependent genome instability, potentially as a consequence of replication fork stalling [98] (Figure 3C). Thus, further studies are required to clarify the mechanistic link between chromatin structure, acetylation status, and transcription–replication conflicts.

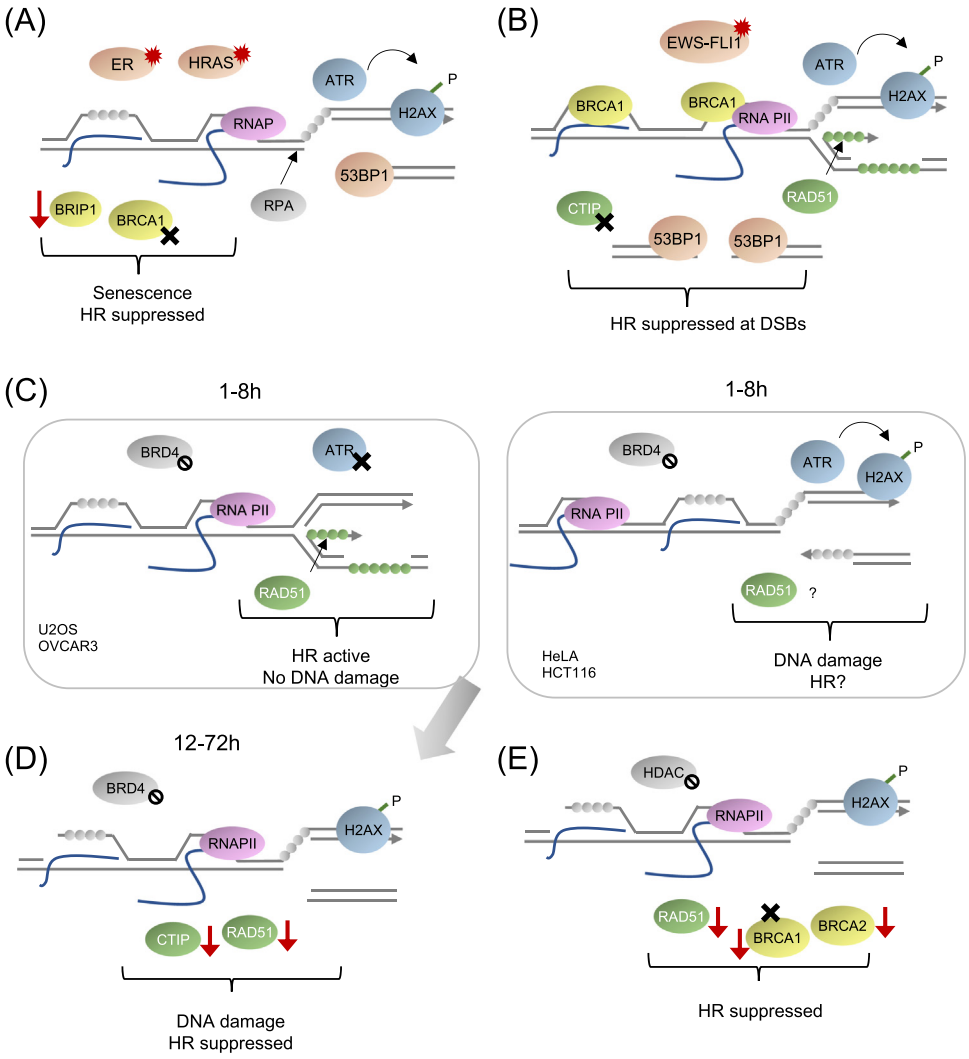
Replication stress responses under conditions of hypertranscription

Replication fork slowing or stalling has the potential to generate extensive stretches of single-stranded DNA (ssDNA), which recruits and activates the replication stress response kinase ATR. ATR phosphorylates a number of downstream targets including checkpoint kinase checkpoint kinase 1 (CHK1) and histone H2AX (γH2AX), which aid fork stabilization and fork restart [99] (Figure 4A). A subsequent collapse of stalled forks generates DSBs, leading to the extensive recruitment of DSB response factors such as 53BP1 and BRCA1 to initiate DNA repair pathways [100]. One such pathway is homologous recombination (HR), which may play important roles in the response to hypertranscription-induced replication stress [8,10] and R-loop-induced DSBs [101]. HR can promote the restart of stalled replication forks and repair DSBs and ssDNA gaps [102,103]. RAD51 recombinase, the central HR protein, also promotes replication fork regression into a four-way Holliday junction or 'chicken-foot' structure (Figure 4B). This slows fork progression and could allow time for replication blocks to be removed [104]. In response to replication stress, RAD51 forms visible nuclear foci, which seem to be specific to the repair of single-stranded gaps and DSBs, while RAD51 functions at stalled forks do not involve focus formation [102,103].

Hypertranscription induced by estrogen, by H-RAS^{V12}, and in Ewing sarcoma cell lines is associated with typical replication stress responses, including ATR, replication protein A (RPA), and CHK1 phosphorylation and increased numbers of γH2AX and 53BP1 foci, indicating DSB formation [7–9] (Figure 4A,B). Estrogen-induced γH2AX foci and DSBs are both enriched at R-loops, supporting transcription–replication conflicts and replication fork collapse [7]. Ewing sarcoma cells also display these DNA damage responses, as well as high levels of spontaneous RAD51 foci compared with controls (Figure 4B). However, they have an impaired ability to further activate HR in response to ionizing radiation. Expression of the EWS–FLI1 fusion oncoprotein in U2OS cells significantly reduces the ability to use HR for DSB repair in a reporter construct. This is restored on BRCA1 overexpression or the depletion of 53BP1. This inability to repair DSBs could potentially be directly linked to the sequestration of BRCA1 to elongating transcription machinery and R-loops, through its interaction with phosphorylated RNA Pol II in an EWS–FLI1-dependent manner. This prevents BRCA1-dependent removal of 53BP1 to facilitate the transition from NHEJ to HR [8]. The EWS–FLI1 oncoprotein thereby indirectly inhibits HR. Interestingly, oncogenic H-RAS^{V12} also inhibits HR by downregulating *BRIP1* expression, thus promoting BRCA1 dissociation from chromatin [105] (Figure 4A).

Replication stress response to BETis and HDACis

BETis, and the depletion of BRD4, are unusual in that they do not always activate a replication stress response despite fork slowing (Figure 4C). Neither the formation of RPA nor γH2AX foci is observed during BETi-induced fork slowing in U2OS cells [10]. BET inhibition and BRD4 depletion have been reported to suppress ATR signaling in U2OS cells [80]. OVCAR3 cell lines also



Trends in Cancer

Figure 4. Replication stress responses during hypertranscription. (A) Activated H-RAS and estrogen receptor (ER) induce canonical replication stress responses. However, homologous recombination (HR) becomes inactivated during RAS-induced senescence due to downregulation of BRIP1 expression, which in turn leads to loss of BRCA1 recruitment. As BRCA1 is required for end resection and RAD51 loading, this results in the suppression of HR. (B) Hypertranscription induced by EWS–FLI1 is accompanied by canonical replication stress responses and increased RAD51 recruitment into nuclear foci. However, the additional activation of HR [e.g., at DNA double-strand breaks (DSBs)] is suppressed, possibly because BRCA1 is sequestered at excessive R-loops. (C) Short-term bromodomain and extraterminal motif (BET) inhibitor treatments (1–8 h) induce cell line-specific replication stress responses. Despite replication fork slowing, the U2OS and OVCAR3 cancer cell lines do not display extensive replication protein A (RPA) recruitment or ataxia telangiectasia and Rad3 related (ATR) activation. This could be due to a lack of single-stranded DNA (ssDNA) formation as well as inhibition of ATR signaling by the BET inhibitor treatment. However, RAD51 is recruited and forms foci, suggesting that extensive stretches of ssDNA are present. By contrast, HeLa and HCT116 cells display normal ATR activation, which could suggest that more DNA damage is induced and/or ATR is not inhibited. It is currently unknown whether RAD51 is activated under these conditions. (D) Long-term BET inhibitor treatments (12–72 h) induce DNA damage and activate the DNA damage response. They also lead to inactivation of HR due to downregulation of the gene expression of *RAD51* and *RBBP8* (CTIP; involved in end resection during HR). (E) Histone deacetylase (HDAC) inhibition (4–24 h) suppresses HR by reducing protein levels of RAD51 as well as other HR factors such as BRCA1 and BRCA2, which are required for RAD51 loading. HDAC inhibition can also prevent the recruitment of BRCA1 to DSBs. Replication stress responses are simplified for clarity and omit, for example, ATM, which phosphorylates histone H2AX in response to DSBs. Abbreviation: RNAP, RNA polymerase.

show JQ1-induced suppression of DNA damage signaling, which was ascribed to downregulation of *TOPBP1* and *WEE1* expression [81]. Furthermore, comet assays to directly measure DNA breaks show little or no DNA damage induction by JQ1 [83,106]. Nevertheless, a recent study reported an opposite effect where γH2AX was increased after JQ1 treatment in U2OS cells [12]. In other cancer cell lines, such as HeLa and HCT116, JQ1 and BRD4 loss can induce DNA damage signaling. In these reports, DNA fiber assays suggest no fork slowing but severe fork stalling, and therefore the nature of transcription–replication conflicts occurring under these conditions is uncertain [11,13]. The impact of BETis in increasing transcription and replication stress is cell-line dependent and is likely to be influenced by other factors that remain to be investigated. So far, only a limited number of cell lines has been investigated, and there is no clear correlation with genetic alterations related to the DNA damage response, such as p53 loss or alternative lengthening of telomeres.

RAD51 can promote fork regression and slow fork speeds at low levels of replication stress where there is no ATR activation [104]. Even when not activating ATR, BETis still cause RAD51 focus formation and RAD51-dependent replication fork slowing, which is required to prevent a BETi-induced DNA damage response. Other factors involved in fork regression, SMARCAL1, ZRANB3, and PARP activity, are also required for BETi-induced fork slowing [10]. This suggests that BETis may induce fork regression, which may help to prevent DNA damage. Interestingly, longer treatments with BETis induce loss of HR proficiency across a number of cell lines due to repression of the *RAD51* and *RBBP8* (CTIP) genes [82,83] (Figure 4D). These data suggest that early during BETi treatment, transcription–replication conflicts activate a HR response that is then followed by the suppression of HR protein expression and loss of HR proficiency. It has been suggested that RNA synthesis and R-loop formation at the damage site is directly required for HR [107,108]. It might therefore be speculated that hypertranscription could facilitate this mechanism and thereby stimulate the RAD51 focus formation observed early during BETi treatment or after the activation of EWS–FLI1 [8,10]. It is also possible that downregulation of recombination factors is a way for cells to adapt to long-term dysregulated RNA synthesis under these conditions.

Like BET inhibition, HDACis can suppress the replication stress response, including cell-cycle checkpoint signaling [86]. As with BETis, there are conflicting results, with some reports showing HDACis having no effect on fork speeds [86] or promoting RAD51 focus formation [90]. Histone acetylation is pro-recombinogenic and HDACis could be suspected to promote HR. However, prolonged HDAC inhibition (4–24 h) appears to consistently suppress HR by reducing protein levels of RAD51, as well as other HR factors such as BRCA1 and BRCA2 (Figure 4E) [86,109]. Inhibition of HDAC1/2 has also been reported to inhibit the recruitment of HR factors to DSBs [110].

Concluding remarks

In conclusion, recent work has revealed that increased transcription can be a mechanism of oncogene-induced DNA damage, providing a molecular link between upregulation of the transcription machinery and genomic instability in cancer. This model is currently positioned alongside alternative or additional explanations such as altered nucleotide metabolism [111]. It will be interesting to investigate the impact on additional oncogenic signaling pathways such as PI3K/mTOR on hypertranscription and replication stress (see Outstanding questions).

Furthermore, recent developments suggests that cancer therapeutics can cause hypertranscription-induced replication stress as well. It is an unanswered question how inhibition of BET proteins and HDACs is toxic for cancer cells more than for non-cancer cells, as these

Outstanding questions

How common is the induction of hypertranscription-associated replication stress in different oncogenic signalling pathways or the loss of tumor suppressors?

What are the individual contributions of RNA Pol I, II, and III to hypertranscription-induced replication stress?

What is the relationship between hypertranscription and increased R-loop formation?

What are the mechanisms behind the apparent cell line-specific differences in BET inhibitor-induced hypertranscription and replication stress?

To what extent does hypertranscription contribute to replication-independent DNA damage?

Is the inhibition of homologous recombination a universal cellular response to hypertranscription-induced replication stress and could this have implications for cancer treatment?

targets regulate global transcription. Understanding the role of BET proteins and HDACs in preventing transcription–replication conflicts could be vitally important for therapeutic targeting in the future. Currently, conflicting results obtained with both types of inhibitors highlight the importance of paying attention to treatment conditions and cell type.

Many of the key studies cited here showed that hypertranscription-associated DNA damage occurs predominantly in S phase, but replication-independent DNA damage was also observed [7,9,11–13]. Hypertranscription may therefore also increase transcription-associated DSBs. These can result from abortive action of DNA topoisomerases, mainly in regulatory elements [112–114]. They can require RAD51 for repair and, if unrepaired, contribute to genomic instability [114–116]. However, some studies reported no link between transcription levels and DSB formation [115,116]. This would require further investigation; for example, by testing whether hypertranscription alters the frequency or genomic location of DSBs induced by the DNA topoisomerase 2 inhibitor etoposide [112].

Finally, the inhibition of HR that has been frequently observed in the cited studies is an intriguing finding that raises the question of whether downregulation of HR could be a general response to hypertranscription. Another unanswered question is whether increased R-loop formation constitutes a good indicator of hypertranscription, as R-loops could also be increased through loss of R-loop-suppressing activities. We suggest that it may be useful to measure nascent transcription in addition to R-loop levels when investigating transcription–replication conflicts.

Acknowledgments

This work was supported by a Cancer Research UK Programme Foundation Award to E.P. (C25526/A28275).

Declaration of interests

No interests are declared.

References

1. Hanahan, D. and Weinberg, R.A. (2011) Hallmarks of cancer: the next generation. *Cell* 144, 646–674
2. Gaillard, H. *et al.* (2015) Replication stress and cancer. *Natl. Rev.* 15, 276–289
3. Dobbelstein, M. and Sorensen, C.S. (2015) Exploiting replicative stress to treat cancer. *Nat. Rev. Drug Discov.* 14, 405–423
4. Kotsantis, P. *et al.* (2018) Mechanisms of oncogene-induced replication stress: jigsaw falling into place. *Cancer Discov.* 8, 537–555
5. Percharde, M. *et al.* (2017) Hypertranscription in development, stem cells, and regeneration. *Dev. Cell* 40, 9–21
6. Lavado, A. *et al.* (2018) The Hippo pathway prevents YAP/TAZ-driven hypertranscription and controls neural progenitor number. *Dev. Cell* 47, 576–591.e578
7. Stork, C.T. *et al.* (2016) Co-transcriptional R-loops are the main cause of estrogen-induced DNA damage. *eLife* 5, e17548
8. Gorthi, A. *et al.* (2018) EWS–FLI1 increases transcription to cause R-loops and blocks BRCA1 repair in Ewing sarcoma. *Nature* 555, 387
9. Kotsantis, P. *et al.* (2016) Increased global transcription activity as a mechanism of replication stress in cancer. *Nat. Commun.* 7, 13087
10. Bowry, A. *et al.* (2018) BET inhibition induces HEXIM1- and RAD51-dependent conflicts between transcription and replication. *Cell Rep.* 25, 2061–2069.e2064
11. Lam, F.C. *et al.* (2020) BRD4 prevents the accumulation of R-loops and protects against transcription–replication collision events and DNA damage. *Nat. Commun.* 11, 4083
12. Kim, J.J. *et al.* (2019) Systematic bromodomain protein screens identify homologous recombination and R-loop suppression pathways involved in genome integrity. *Genes Dev.* 33, 1751–1774
13. Edwards, D.S. *et al.* (2020) BRD4 prevents R-loop formation and transcription-replication conflicts by ensuring efficient transcription elongation. *Cell Rep.* 32, 108166
14. White, R.J. (2005) RNA polymerases I and III, growth control and cancer. *Nat. Rev. Mol. Cell Biol.* 6, 69–78
15. Bywater, M.J. *et al.* (2013) Dysregulation of the basal RNA polymerase transcription apparatus in cancer. *Natl. Rev.* 13, 299–314
16. Bradner, J.E. *et al.* (2017) Transcriptional addiction in cancer. *Cell* 168, 629–643
17. Vannini, A. and Cramer, P. (2012) Conservation between the RNA polymerase I, II, and III transcription initiation machineries. *Mol. Cell* 45, 439–446
18. Cramer, P. (2019) Organization and regulation of gene transcription. *Nature* 573, 45–54
19. Sharifi, S. and Bierhoff, H. (2018) Regulation of RNA polymerase I transcription in development, disease, and aging. *Annu. Rev. Biochem.* 87, 51–73
20. Willis, I.M. and Moir, R.D. (2018) Signaling to and from the RNA polymerase III transcription and processing machinery. *Annu. Rev. Biochem.* 87, 75–100
21. Friedrich, J.K. *et al.* (2005) TBP–TAF complex SL1 directs RNA polymerase I pre-initiation complex formation and stabilizes upstream binding factor at the rDNA promoter. *J. Biol. Chem.* 280, 29551–29558
22. Cavanaugh, A.H. *et al.* (2002) Rrn3 phosphorylation is a regulatory checkpoint for ribosome biogenesis. *J. Biol. Chem.* 277, 27423–27432

23. Lin, C.-Y. *et al.* (2006) CK2-mediated stimulation of Pol I transcription by stabilization of UBF–SL1 interaction. *Nucleic Acids Res.* 34, 4752–4766
24. Goodfellow, S.J. and Zomerdijk, J.C.B.M. (2012) Basic mechanisms in RNA polymerase I transcription of the ribosomal RNA genes. *Subcell. Biochem.* 61, 211–236
25. Stefanovsky, V. *et al.* (2006) Growth factor signaling regulates elongation of RNA polymerase I transcription in mammals via UBF phosphorylation and r-chromatin remodeling. *Mol. Cell* 21, 629–639
26. Denissov, S. *et al.* (2011) A model for the topology of active ribosomal RNA genes. *EMBO Rep.* 12, 231
27. Rossetti, S. *et al.* (2016) Mammary epithelial morphogenesis and early breast cancer. Evidence of involvement of basal components of the RNA polymerase I transcription machinery. *Cell Cycle* 15, 2515–2526
28. Zhong, S. *et al.* (2004) Epidermal growth factor enhances cellular TATA binding protein levels and induces RNA polymerase I- and III-dependent gene activity. *Mol. Cell. Biol.* 24, 5119–5129
29. Stefanovsky, V.Y. *et al.* (2001) An immediate response of ribosomal transcription to growth factor stimulation in mammals is mediated by ERK phosphorylation of UBF. *Mol. Cell* 8, 1063–1073
30. Zhao, J. *et al.* (2003) ERK-dependent phosphorylation of the transcription initiation factor TIF-IA is required for RNA polymerase I transcription and cell growth. *Mol. Cell* 11, 405–413
31. Drakas, R. *et al.* (2004) Control of cell size through phosphorylation of upstream binding factor 1 by nuclear phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. U. S. A.* 101, 9272–9276
32. Bierhoff, H. *et al.* (2008) Phosphorylation by casein kinase 2 facilitates rRNA gene transcription by promoting dissociation of TIF-IA from elongating RNA polymerase I. *Mol. Cell. Biol.* 28, 4988
33. Panova, T.B. *et al.* (2006) Casein kinase 2 associates with initiation-competent RNA polymerase I and has multiple roles in ribosomal DNA transcription. *Mol. Cell. Biol.* 26, 5957–5968
34. Johnson, S.A. *et al.* (2000) Transcriptional regulation of the TATA-binding protein by Ras cellular signaling. *Mol. Cell. Biol.* 20, 5000–5009
35. Lavoie, H. *et al.* (2020) ERK signalling: a master regulator of cell behaviour, life and fate. *Nat. Rev. Mol. Cell Biol.* 21, 607–632
36. Hoxhaj, G. and Manning, B.D. (2020) The PI3K–AKT network at the interface of oncogenic signalling and cancer metabolism. *Natl. Rev.* 20, 74–88
37. Beroukhim, R. *et al.* (2010) The landscape of somatic copy-number alteration across human cancers. *Nature* 463, 899–905
38. Johnson, S.A. *et al.* (2003) Increased expression of TATA-binding protein, the central transcription factor, can contribute to oncogenesis. *Mol. Cell. Biol.* 23, 3043–3051
39. Nie, Z. *et al.* (2012) c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. *Cell* 151, 68–79
40. Lin, C.Y. *et al.* (2012) Transcriptional amplification in tumor cells with elevated c-Myc. *Cell* 151, 56–67
41. Poortinga, G. *et al.* (2015) Targeting RNA polymerase I to treat MYC-driven cancer. *Oncogene* 34, 403–412
42. Campbell, K.J. and White, R.J. (2014) MYC regulation of cell growth through control of transcription by RNA polymerases I and III. *Cold Spring Harb. Perspect. Med.* 4, a018408
43. Poortinga, G. *et al.* (2011) c-MYC coordinately regulates ribosomal gene chromatin remodeling and Pol I availability during granulocyte differentiation. *Nucleic Acids Res.* 39, 3267–3281
44. Poortinga, G. *et al.* (2004) MAD1 and c-MYC regulate UBF and rDNA transcription during granulocyte differentiation. *EMBO J.* 23, 3325–3335
45. Sansom, O.J. *et al.* (2007) Myc deletion rescues Apc deficiency in the small intestine. *Nature* 446, 676–679
46. Corden, J.L. (1990) Tails of RNA polymerase II. *Trends Biochem. Sci.* 15, 383–387
47. Turowski, T.W. and Tollervey, D. (2016) Transcription by RNA polymerase III: insights into mechanism and regulation. *Biochem. Soc. Trans.* 44, 1367–1375
48. Felton-Edkins, Z.A. *et al.* (2003) The mitogen-activated protein (MAP) kinase ERK induces tRNA synthesis by phosphorylating TFIIIB. *EMBO J.* 22, 2422–2432
49. Johnson, S.S. *et al.* (2007) Mammalian Maf1 is a negative regulator of transcription by all three nuclear RNA polymerases. *Mol. Cell* 26, 367–379
50. Kantidakis, T. *et al.* (2010) mTOR associates with TFIIIC, is found at tRNA and 5S rRNA genes, and targets their repressor Maf1. *Proc. Natl. Acad. Sci. U. S. A.* 107, 11823–11828
51. Shor, B. *et al.* (2010) Requirement of the mTOR kinase for the regulation of Maf1 phosphorylation and control of RNA polymerase III-dependent transcription in cancer cells. *J. Biol. Chem.* 285, 15380–15392
52. Orioli, A. *et al.* (2016) Human MAF1 targets and represses active RNA polymerase III genes by preventing recruitment rather than inducing long-term transcriptional arrest. *Genome Res.* 26, 624–635
53. Ekholm-Reed, S. *et al.* (2004) Deregulation of cyclin E in human cells interferes with prereplication complex assembly. *J. Cell Biol.* 165, 789–800
54. Jones, R.M. *et al.* (2013) Increased replication initiation and conflicts with transcription underlie cyclin E-induced replication stress. *Oncogene* 32, 3744–3753
55. Di Micco, R. *et al.* (2006) Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* 444, 638–642
56. Dominguez-Sola, D. *et al.* (2007) Non-transcriptional control of DNA replication by c-Myc. *Nature* 448, 445–451
57. Hah, N. *et al.* (2011) A rapid, extensive, and transient transcriptional response to estrogen signaling in breast cancer cells. *Cell* 145, 622–634
58. Calses, P.C. *et al.* (2019) Hippo pathway in cancer: aberrant regulation and therapeutic opportunities. *Trends Cancer* 5, 297–307
59. Bushweller, J.H. (2019) Targeting transcription factors in cancer – from undruggable to reality. *Natl. Rev.* 19, 611–624
60. Fisher, R.P. (2019) Cdk7: a kinase at the core of transcription and in the crosshairs of cancer drug discovery. *Transcription* 10, 47–56
61. Bacon, C.W. and D'Orso, I. (2019) CDK9: a signaling hub for transcriptional control. *Transcription* 10, 57–75
62. Iniguez, A.B. *et al.* (2018) EWS/FLI confers tumor cell synthetic lethality to CDK12 inhibition in Ewing sarcoma. *Cancer Cell* 33, 202–216.e206
63. Filippakopoulos, P. and Knapp, S. (2014) Targeting bromodomains: epigenetic readers of lysine acetylation. *Nat. Rev. Drug Discov.* 13, 337–356
64. Falkenberg, K.J. and Johnstone, R.W. (2014) Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. *Nat. Rev. Drug Discov.* 13, 673–691
65. Verdin, E. and Ott, M. (2015) 50 Years of protein acetylation: from gene regulation to epigenetics, metabolism and beyond. *Nat. Rev. Mol. Cell Biol.* 16, 258–264
66. Sheikh, B.N. and Akhtar, A. (2019) The many lives of KATs – detectors, integrators and modulators of the cellular environment. *Nat. Rev. Genet.* 20, 7–23
67. Hebbes, T.R. *et al.* (1988) A direct link between core histone acetylation and transcriptionally active chromatin. *EMBO J.* 7, 1395–1402
68. Luger, K. and Richmond, T.J. (1998) The histone tails of the nucleosome. *Curr. Opin. Genet. Dev.* 8, 140–146
69. Robinsn, P.J. *et al.* (2008) 30 nm Chromatin fibre decompaction requires both H4-K16 acetylation and linker histone eviction. *J. Mol. Biol.* 381, 816–825
70. Kanno, T. *et al.* (2004) Selective recognition of acetylated histones by bromodomain proteins visualized in living cells. *Mol. Cell* 13, 33–43
71. Wu, S.-Y. and Chiang, C.-M. (2007) The double bromodomain-containing chromatin adaptor Brd4 and transcriptional regulation. *J. Biol. Chem.* 282, 13141–13145
72. Rahman, S. *et al.* (2011) The Brd4 extraterminal domain confers transcription activation independent of pTEFb by recruiting multiple proteins, including NSD3. *Mol. Cell. Biol.* 31, 2641–2652

73. Jang, M.K. *et al.* (2005) The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription. *Mol. Cell* 19, 523–534
74. Xu, Y. and Vakoc, C.R. (2017) Targeting cancer cells with BET bromodomain inhibitors. *Cold Spring Harb. Perspect. Med.* 7, a026674
75. Bartholomeeusen, K. *et al.* (2012) Bromodomain and extra-terminal (BET) bromodomain inhibition activate transcription via transient release of positive transcription elongation factor b (P-TEFb) from 7SK small nuclear ribonucleoprotein. *J. Biol. Chem.* 287, 36609–36616
76. Chaidos, A. *et al.* (2014) Potent antimyeloma activity of the novel bromodomain inhibitors I-BET151 and I-BET762. *Blood* 123, 697–705
77. Bhadury, J. *et al.* (2014) BET and HDAC inhibitors induce similar genes and biological effects and synergize to kill in Myc-induced murine lymphoma. *Proc. Natl. Acad. Sci. U. S. A.* 111, E2721–E2730
78. Mertz, J.A. *et al.* (2011) Targeting MYC dependence in cancer by inhibiting BET bromodomains. *Proc. Natl. Acad. Sci. U. S. A.* 108, 16669–16674
79. Da Costa, D. *et al.* (2013) BET inhibition as a single or combined therapeutic approach in primary paediatric B-precursor acute lymphoblastic leukaemia. *Blood Cancer J.* 3, e126
80. Zhang, J. *et al.* (2018) BRD4 facilitates replication stress-induced DNA damage response. *Oncogene* 37, 3763–3777
81. Karakashev, S. *et al.* (2017) BET bromodomain inhibition synergizes with PARP inhibitor in epithelial ovarian cancer. *Cell Rep.* 21, 3398–3405
82. Yang, L. *et al.* (2017) Repression of BET activity sensitizes homologous recombination-proficient cancers to PARP inhibition. *Sci. Transl. Med.* 9, eaal1645
83. Sun, C. *et al.* (2018) BRD4 inhibition is synthetic lethal with PARP inhibitors through the induction of homologous recombination deficiency. *Cancer Cell* 33, 401–416.e8
84. Li, Y. and Seto, E. (2016) HDACs and HDAC inhibitors in cancer development and therapy. *Cold Spring Harb. Perspect. Med.* 6, a026831
85. Newbold, A. *et al.* (2016) How do tumor cells respond to HDAC inhibition? *FEBS J.* 283, 4032–4046
86. Goder, A. *et al.* (2018) HDAC1 and HDAC2 integrate checkpoint kinase phosphorylation and cell fate through the phosphatase-2A subunit PR130. *Nat. Commun.* 9, 764
87. Dovey, O.M. *et al.* (2013) Histone deacetylase 1 and 2 are essential for normal T-cell development and genomic stability in mice. *Blood* 121, 1335–1344
88. Fujinaga, K. *et al.* (2015) Visualization of positive transcription elongation factor b (P-TEFb) activation in living cells. *J. Biol. Chem.* 290, 1829–1836
89. Conti, C. *et al.* (2010) Inhibition of histone deacetylase in cancer cells slows down replication forks, activates dormant origins, and induces DNA damage. *Cancer Res.* 70, 4470–4480
90. Bhaskara, S. *et al.* (2013) Histone deacetylases 1 and 2 maintain S-phase chromatin and DNA replication fork progression. *Epigenetics Chromatin* 6, 27
91. Wells, C.E. *et al.* (2013) Inhibition of histone deacetylase 3 causes replication stress in cutaneous T cell lymphoma. *PLoS One* 8, e68915
92. Summers, A.R. *et al.* (2013) HDAC3 is essential for DNA replication in hematopoietic progenitor cells. *J. Clin. Invest.* 123, 3112–3123
93. Aggarwal, B.D. and Calvi, B.R. (2004) Chromatin regulates origin activity in *Drosophila* follicle cells. *Nature* 430, 372–376
94. Kim, J.J. *et al.* (2020) PCAF-mediated histone acetylation promotes replication fork degradation by MRE11 and EXO1 in BRCA-deficient cells. *Mol. Cell* 80, 327–344.e328
95. Agudelo Garcia, P.A. *et al.* (2020) Histone acetyltransferase 1 is required for DNA replication fork function and stability. *J. Biol. Chem.* 295, 8363–8373
96. Kurat, C.F. *et al.* (2017) Chromatin controls DNA replication origin selection, lagging-strand synthesis, and replication fork rates. *Mol. Cell* 65, 117–130
97. Herrera-Moyano, E. *et al.* (2014) The yeast and human FACT chromatin-reorganizing complexes solve R-loop-mediated transcription–replication conflicts. *Genes Dev.* 28, 735–748
98. Salas-Armenteros, I. *et al.* (2017) Human THO–Sin3A interaction reveals new mechanisms to prevent R-loops that cause genome instability. *EMBO J.* 36, 3532–3547
99. Saldivar, J.C. *et al.* (2017) The essential kinase ATR: ensuring faithful duplication of a challenging genome. *Nat. Rev. Mol. Cell Biol.* 18, 622–636
100. Panier, S. and Boulton, S.J. (2014) Double-strand break repair: 53BP1 comes into focus. *Nat. Rev. Mol. Cell Biol.* 15, 7–18
101. Wells, J.P. *et al.* (2019) R loops and their composite cancer connections. *Trends Cancer* 5, 619–631
102. Petermann, E. *et al.* (2010) Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair. *Mol. Cell* 37, 492–502
103. Piberger, A.L. *et al.* (2020) PrimPol-dependent single-stranded gap formation mediates homologous recombination at bulky DNA adducts. *Nat. Commun.* 11, 5863
104. Zellweger, R. *et al.* (2015) Rad51-mediated replication fork reversal is a global response to genotoxic treatments in human cells. *J. Cell Biol.* 208, 563–579
105. Tu, Z. *et al.* (2011) Oncogenic RAS regulates BRIP1 expression to induce dissociation of BRCA1 from chromatin, inhibit DNA repair, and promote senescence. *Dev. Cell* 21, 1077–1091
106. Pawar, A. *et al.* (2018) Resistance to BET inhibitor leads to alternative therapeutic vulnerabilities in castration-resistant prostate cancer. *Cell Rep.* 22, 2236–2245
107. Ohle, C. *et al.* (2016) Transient RNA–DNA hybrids are required for efficient double-strand break repair. *Cell* 167, 1001–1013.e1007
108. D'Alessandro, G. *et al.* (2018) BRCA2 controls DNA:RNA hybrid level at DSBs by mediating RNase H2 recruitment. *Nat. Commun.* 9, 5376
109. Adimoolam, S. *et al.* (2007) HDAC inhibitor PCI-24781 decreases RAD51 expression and inhibits homologous recombination. *Proc. Natl. Acad. Sci. U. S. A.* 104, 19482–19487
110. Fukuda, T. *et al.* (2015) Class I histone deacetylase inhibitors inhibit the retention of BRCA1 and 53BP1 at the site of DNA damage. *Cancer Sci.* 106, 1050–1056
111. Aird, K.M. *et al.* (2013) Suppression of nucleotide metabolism underlies the establishment and maintenance of oncogene-induced senescence. *Cell Rep.* 3, 1252–1265
112. Gothe, H.J. *et al.* (2019) Spatial chromosome folding and active transcription drive DNA fragility and formation of oncogenic MLL translocations. *Mol. Cell* 75, 267–283.e212
113. Bouwman, B.A.M. and Crosetto, N. (2018) Endogenous DNA double-strand breaks during DNA transactions: emerging insights and methods for genome-wide profiling. *Genes (Basel)* 9, 632
114. Hazan, I. *et al.* (2019) Activation of oncogenic super-enhancers is coupled with DNA repair by RAD51. *Cell Rep.* 29, 560–572.e564
115. Dellino, G.I. *et al.* (2019) Release of paused RNA polymerase II at specific loci favors DNA double-strand-break formation and promotes cancer translocations. *Nat. Genet.* 51, 1011–1023
116. Ballinger, T.J. *et al.* (2019) Modeling double strand break susceptibility to interrogate structural variation in cancer. *Genome Biol.* 20, 28
117. Boguslawski, S.J. *et al.* (1986) Characterization of monoclonal antibody to DNA.RNA and its application to immunodetection of hybrids. *J. Immunol. Methods* 89, 123–130
118. Vanoosthuyse, V. (2018) Strengths and weaknesses of the current strategies to map and characterize R-loops. *Noncoding RNA* 4, 9
119. Core, L.J. *et al.* (2008) Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters. *Science* 322, 1845–1848
120. Kwak, H. *et al.* (2013) Precise maps of RNA polymerase reveal how promoters direct initiation and pausing. *Science* 339, 950–953
121. Churchman, L.S. and Weissman, J.S. (2011) Nascent transcript sequencing visualizes transcription at nucleotide resolution. *Nature* 469, 368–373
122. Huertas, P. and Aguilera, A. (2003) Cotranscriptionally formed DNA:RNA hybrids mediate transcription elongation impairment and transcription-associated recombination. *Mol. Cell* 12, 711–721
123. Costantino, L. and Koshland, D. (2015) The yin and yang of R-loop biology. *Curr. Opin. Cell Biol.* 34, 39–45

124. Hamperl, S. *et al.* (2017) Transcription–replication conflict orientation modulates R-loop levels and activates distinct DNA damage responses. *Cell* 170, 774–786.e719
125. Wellinger, R.E. *et al.* (2006) Replication fork progression is impaired by transcription in hyperrecombinant yeast cells lacking a functional THO complex. *Mol. Cell. Biol.* 26, 3327
126. Gan, W. *et al.* (2011) R-loop-mediated genomic instability is caused by impairment of replication fork progression. *Genes Dev.* 25, 2041–2056
127. Tuduri, S. *et al.* (2009) Topoisomerase I suppresses genomic instability by preventing interference between replication and transcription. *Nat. Cell Biol.* 11, 1315–1324
128. Chernikova, S.B. *et al.* (2012) Deficiency in mammalian histone H2B ubiquitin ligase Bre1 (Rnf20/Rnf40) leads to replication stress and chromosomal instability. *Cancer Res.* 72, 2111
129. Paulsen, R.D. *et al.* (2009) A genome-wide siRNA screen reveals diverse cellular processes and pathways that mediate genome stability. *Mol. Cell* 35, 228–239
130. Sollier, J. *et al.* (2014) Trascription-coupled nucleotide excision repair factors promote R-loop-induced genome instability. *Mol. Cell* 56, 777–785
131. Nojima, T. *et al.* (2018) Deregulated expression of mammalian lncRNA through loss of SPT6 induces R-loop formation, replication stress, and cellular senescence. *Mol. Cell* 72, 970–984.e977
132. Pugliese, G.M. *et al.* (2019) Inducible SMARCAL1 knockdown in iPSC reveals a link between replication stress and altered expression of master differentiation genes. *Dis. Model. Mech.* 12, dmm039487
133. Zhang, T. *et al.* (2019) Loss of TOP3B leads to increased R-loop formation and genome instability. *Open Biol.* 9, 190222
134. Sanchez, A. *et al.* (2020) Transcription–replication conflicts as a source of common fragile site instability caused by BMI1-RNF2 deficiency. *PLoS Genet.* 16, e1008524
135. Klusmann, I. *et al.* (2018) Chromatin modifiers Mdm2 and RNF2 prevent RNA:DNA hybrids that impair DNA replication. *Proc. Natl. Acad. Sci. U. S. A.* 115, E11311