Unravelling molecular mechanisms in transcription-coupled nucleotide excision repair

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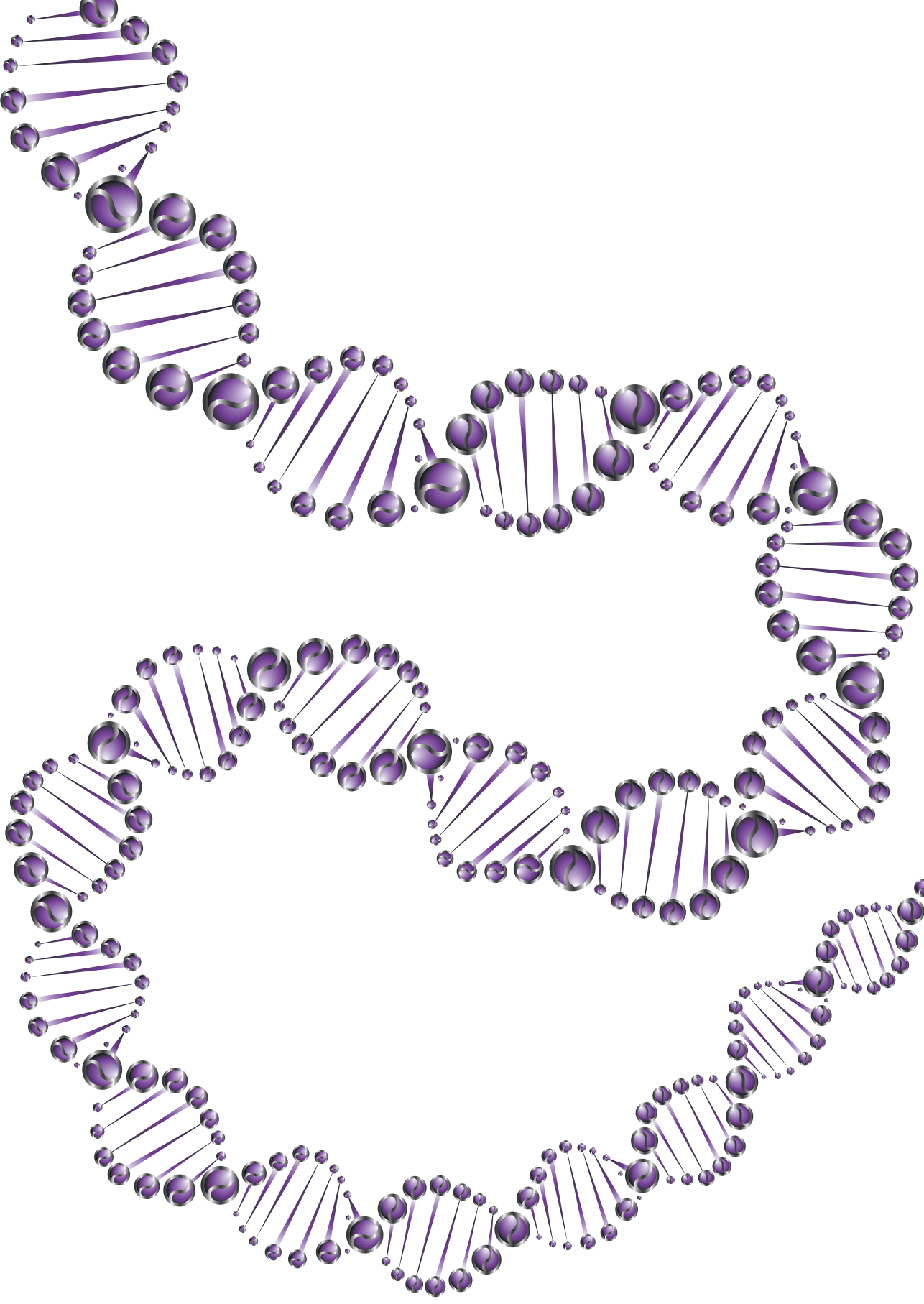

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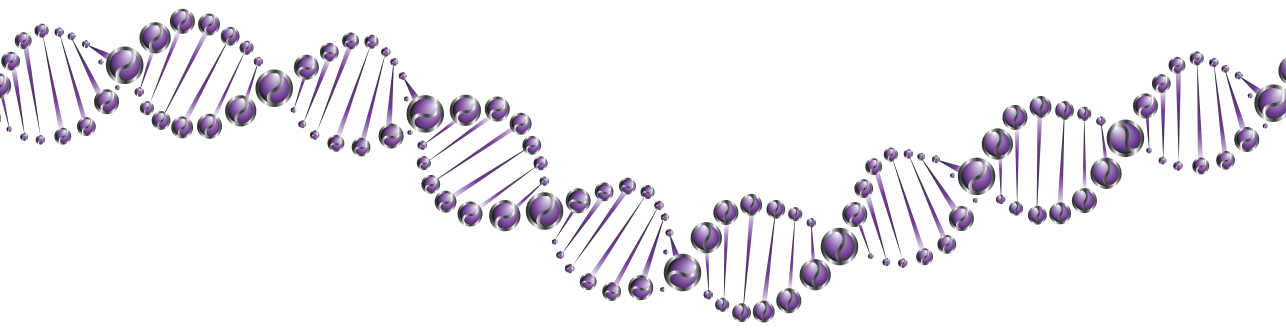
Mechanisms to alleviate transcription-replication collisions: an RNA polymerase II perspective



Abstract

Helix-distorting DNA lesions in actively transcribed DNA strands results in the stalling of RNA polymerase II (RNAPII) and initiation of transcription-coupled DNA repair (TCR) to efficiently remove the damage and restore transcription. Defects in proteins required for the repair of transcription-blocking DNA lesions can cause prolonged stalling of RNAPII. Transcription and replication are essential cellular processes that both use DNA as a template. Therefore, cells have evolved mechanisms to coordinate transcription and replication to minimize collisions between these machineries. However, despite these strategies, collisions between the transcription and replication machineries are inevitable. Mounting evidence suggests that transcription-replication conflicts (TRC) can have serious consequences when they occur between the replication machinery and persistently stalled RNAPII. Here I outline and speculate how cells may deal with persistently stalled RNAPII during DNA replication with a particular emphasis on the potential role of the recently identified ELOF1 pathway.

5

Introduction

Cells are continuously exposed to endogenous (metabolic processes) and exogenous (environmental factors) sources that cause a variety of genomic DNA lesions. These DNA lesions interfere with essential cellular processes, such as transcription and replication, resulting in cell cycle arrest, cell death, and genome instability. To prevent the deleterious consequences of DNA damage, cells have evolved a complex network of complementary DNA damage repair and tolerance mechanisms. One of these pathways is transcription-coupled nucleotide excision repair (TCR), which efficiently removes a wide variety of helix-distorting DNA lesions from actively transcribed DNA strands, including UV-induced cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone photoproducts (6-4PPs) [1, 2]. TCR is initiated when elongating RNA polymerase II (RNAPIIo) is unable to translocate past a transcription-blocking lesion, resulting in the stalling of RNAPIIo and the subsequent assembly of the TCR complex, composed of Cockayne syndrome protein A and B (CSA, CSB) and UV-Stimulated Scaffold Protein A (UVSSA) [3, 4].

Inactivating mutations in the *CSB* and *CSA* genes cause Cockayne syndrome (CS), which is characterized by severe neurodegeneration, growth defects, and photosensitivity without increased cancer predisposition. Inactivating mutations in *UVSSA*, on the other hand, cause UV-sensitive syndrome ($UV^{S}S$). Patients with $UV^{S}S$ also exhibit photosensitivity without increased cancer risk, but unlike patients with CS, they do not develop neurological symptoms or growth defects. We have demonstrated that CSB, CSA, and UVSSA are equally important for the repair of transcription-blocking DNA lesions and therefore the inability to remove these DNA lesions does not explain the severe phenotype seen in CS (chapter 2) [4].

Recent studies have demonstrated that the largest subunit of RNAPII is ubiquitylated on a single lysine residue (RPB1-K1268) in response to UV irradiation (chapter 1) [5–7]. It has been suggested that the UV-induced ubiquitylation of RNAPII-K1268 is essential for the repair of transcription-blocking DNA lesions, but when repair fails, it will lead to processing and removal

of RNAPII from the DNA. CSA is part of an DDB1-CUL4A-RBX1 ubiquitin ligase complex ($CRL4^{CSA}$) that is recruited to lesion-stalled RNAPII in a CSB-dependent manner [4] and plays an important role in the UV-induced ubiquitylation of RNAPII-K1268 [5]. Consistently, CSB and CSA-deficient cells are unable to ubiquitylate and subsequently degrade RNAPII [8], while UVSSA-deficient cells display even faster degradation of RNAPII after UV [8, 9]. Therefore, it has been suggested that prolonged stalling of RNAPIIo on DNA lesions might explain the severe features seen in CS patients (chapter 1) [5, 7]. Moreover, we have recently demonstrated that the RNAPII-associated transcription elongation factor ELOF1 is a core TCR protein that positions the $CRL4^{CSA}$ complex for optimal RNAPII ubiquitylation (chapter 3) [10, 11].

The persistent stalling of RNAPIIo could lead to potentially harmful collisions with the replication machinery. Collisions between the transcription and replication machinery can have serious consequences including DNA breakage, R-loop formation and genome instability [12]. This perspective will focus on different types of transcription-replication conflicts (TRC), the mechanisms cells employ to prevent detrimental collisions, and the consequences of the different types of collisions. In addition, I will speculate on the potential differences between CSB and ELOF1-deficient cells in the replication stress response.

Transcription-replication conflicts

Conflicts between the transcription and replication machinery are a major source of genome instability. Transcription-replication conflicts (TRC) can only occur in the S-phase of the cell cycle, since duplication of DNA is restricted to the S-phase. To reduce the frequency of TRC during the S-phase, cells have evolved mechanisms to coordinate transcription and replication temporally (transcription and replication occur on different moments) and spatially (transcription and replication occur in different spaces in the nucleus [13]. For example, it has been shown that in the early S-phase 95% of ongoing replication sites do not colocalize with transcription sites, suggesting temporal separation of the two processes [14]. Consistent with this idea, replication of transcribed genes leads to a transient reduction of transcriptional activity, except on the transcription start site (TSS) [15]. The TSS showed persistent levels of transcription but required passage into G2/M-phase to complete DNA synthesis [15]. In addition to temporal separation of the two processes, it has been shown that sites of transcription and replication are grouped in distinct separate clusters, indicating spatial separation of the two processes [14]. However, another study demonstrated that replication of long genes is dependent on transcription, since transcription reduces the chromatin density along long genes [16]. This is consistent with other work showing that collisions between the transcription and replication machinery are more likely to occur in long highly transcribed genes [17].

Despite these strategies, collisions between the transcription and replication machineries are inevitable [14]. TRC can occur co-directionally, when the replication and transcription machinery move in the same direction, or head-on, when the machineries move towards each other [13, 18, 19]. Studies in both prokaryotic and eukaryotic cells have demonstrated that head-on encounters

between the replication machinery and elongating RNAPII are more deleterious than co-directional encounters, since head-on collisions leads to impaired replication fork progression, while co-directional encounters do not (Fig. 1a, b) [20, 21]. Consistently, in vitro, the replisome can evict RNAPII from the DNA upon co-directional collisions, allowing replication to continue (Fig. 1a) [22]. In the human genome, many genes contain active replication origins in their promoter and are therefore transcribed and replicated in the same direction [23], which decreases the likelihood of head-on collisions and subsequent replication stress. Interestingly, findings indicate that this bias towards co-directionality does not apply to transcription termination sites (TTS), which are preferentially replicated by an origin located downstream of the gene [24]. These findings suggest that under unperturbed conditions elongating RNAPII only interferes with replication when the transcription and replication machinery move towards each other (head-on).

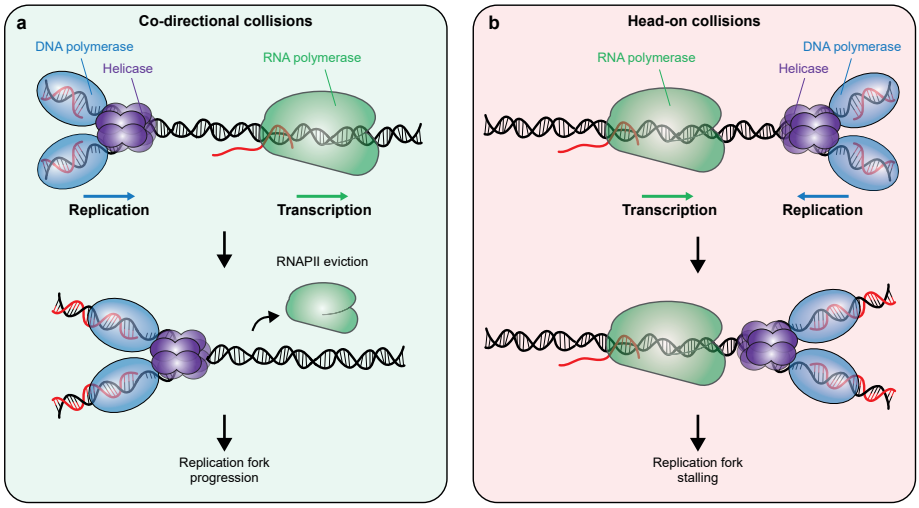


Fig. 1: co-directional vs head-on collisions between the transcription and replication machinery. (**a**) Co-directional collisions with elongating RNAPII do not result in replication stress since the replisome can evict RNAPII from the DNA, allowing replication to continue. (**b**) Upon head-on collisions the replisome is unable to evict RNAPII from the DNA, which results in stalling of the replication fork.

It has been suggested that the differential outcome is caused by excessive R-loop formation upon head-on collisions [19, 25] . R-loops are DNA-RNA hybrid structures that form when nascent RNA hybridizes to the DNA template strand, leaving the non-template as single-stranded DNA (ssDNA). Accumulation of R-loops can cause DNA damage and genome instability. In addition, these structures are also known to block replication fork progression, thereby provoking collisions between the transcription and replication machinery [12, 25] . Interestingly, in a human-cell-based plasmid system, co-transcriptional R-loops are resolved upon co-directional encounters between the replication and transcription machinery [19]. Replicative helicases and replisome-associated

factors, such as Senataxin (SETX) and Fanconi anaemia complementation group M (FANCM), have the ability to resolve R-loops [26–28], which may explain why R-loops do not correlate with co-directional TRC [19]. Importantly, in human cells, inhibiting replication results in a significant increase in R-loops formation [19], and since the human genome is biased towards co-directional collisions [23], this confirms the hypothesis that the replisome can resolve R-loops upon co-directional collisions.

In addition to R-loop formation prior to TRC, there is compelling evidence that R-loops are formed as a consequence of head-on TRC [19, 25]. During transcription, the DNA downstream of RNAPII becomes overtwisted, causing positive supercoiling in front of RNAPII, while the DNA upstream becomes undertwisted, causing negative supercoiling behind RNAPII [29]. Similarly, during replication positively supercoiled DNA accumulates in front of the replication machinery [30]. It is essential for both transcription and replication that the torsional stress is released in a timely manner. In a co-directional TRC the DNA between the replication machinery and the transcription machinery contains both negative and positive supercoils that could diffuse towards each other, thereby releasing the torsional stress [29, 31] . However, in the case of head-on TRC the positive supercoiled DNA in front of the replisome encounters the positive supercoiled DNA in front of RNAPII, resulting in a build-up of positive supercoils. Positive supercoils in one direction causes an equal amount of negative supercoils in the opposite direction [32], the excess of positive supercoils between the transcription and replication machinery will therefore result in an excess of negative supercoils behind the machineries. Negative supercoiled DNA is underwound and therefore sensitive for reannealing of the nascent transcript behind RNAPII, resulting in the formation of R-loops. Consistent with this idea, depletion of Topoisomerase I (TOP1), which is an enzyme that can relieve DNA supercoiling, results in increased formation of R-loops as well as reduced replication fork speed [24, 33, 34]. Importantly, the accumulation of positive supercoils will not only result in R-loop formation, it will also inhibit transcription and replication since positive supercoils prevent DNA unwinding [35]. This is consistent with recent findings, demonstration that R-loop resolution by RNase H1 overexpression could only partially rescue fork progression in TOP1 depleted cells [24].

Excessive accumulation of torsional stress and R-loops can lead to replication fork collapse and the formation of DNA double-strand breaks (DSBs). Replication fork collapse is defined as replication forks that lost the ability to perform DNA synthesis [36]. It has been suggested that TOP1 prevents replication fork collapse by suppressing the formation of R-loops [24, 37]. Indeed, TOP1-depleted cells show accumulation of DSBs at TTS, which are preferentially replicated in a head-on orientation [24]. Furthermore, cells have developed a variety of mechanisms to quickly resolve R-loops and prevent R-loop induced genome instability, one of which is via RNase H1 [38, 39]. RNase H1 is upregulated upon R-loop accumulation and is capable of degrading the RNA engaged in R-loops [38]. It has been shown that RNase H1 is most likely recruited to R-loops via direct protein-protein interactions with replication protein A (RPA) [40]. In addition to promoting RNase H1 recruitment to R-loops, RPA also activates the ATM- and Rad3-related (ATR) pathway [19, 40, 41]. ATR signalling results in activation

of proteins involved in the Fanconi anemia (FA) pathway, particularly FANCM, which uses its translocase activity to directly resolve R-loops [28]. Moreover, there are several helicases that have been implicated in resolving R-loops, including Aquarius (AQR), SETX, DHX9, and DDX39B [27, 42–44]. It has been shown that R-loops that accumulate in the absence of these helicases are processed into DSBs by the endonucleases XPF and XPG in a CSB-dependent manner [43].

In conclusion, head-on collisions cause transient fork stalling due to positive supercoiling between the transcription and replication machinery as well as R-loop formation behind RNAPII. Excessive accumulation of torsional stress and R-loops is prevented by the combined actions of TOP1, activation of the ATR pathway, and helicases. Following the release of torsional stress and the resolution of R-loops, RNAPII will be removed from the DNA thereby allowing replication to continue (Fig. 2a). However, in the absence of these factors, torsional stress and R-loops can accumulate, leading to replication fork collapse, DNA breakage, and genome instability (Fig. 2b).

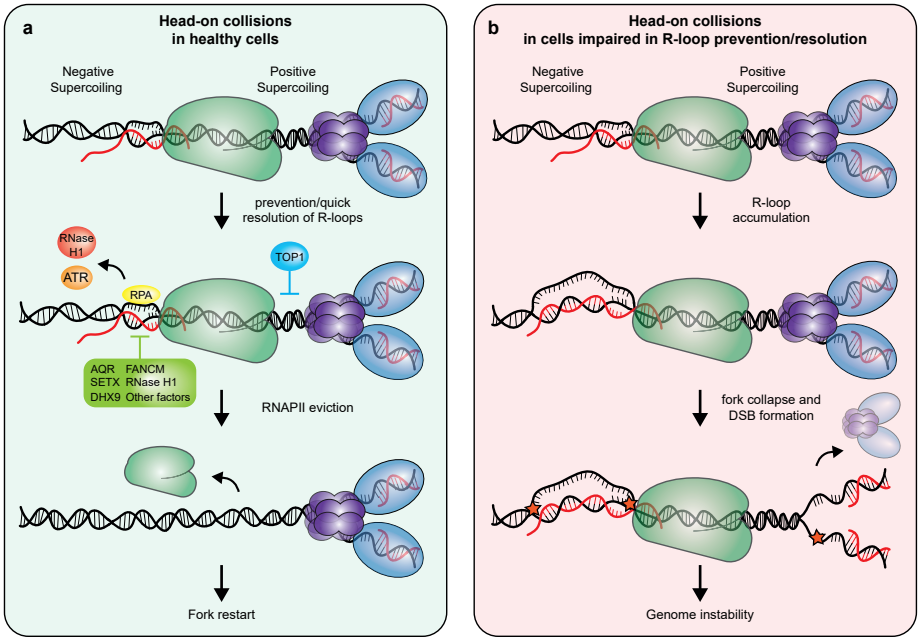


Fig. 2: Head-on collisions can lead to genome instability. Head-on collisions lead to transient fork stalling due to positive supercoiling between the transcription and replication machinery as well as R-loop formation behind RNAPII. (**a**) In healthy cells, excessive accumulation of positive supercoils and R-loops is prevented by the combined actions of TOP1, activation of the ATR pathway, and helicases. Following the release of torsional stress and the resolution of R-loops, RNAPII will be removed from the DNA thereby allowing replication to continue. (**b**) In the absence of these factors, torsional stress and R-loops can accumulate, leading to replication fork collapse, DNA breakage, and genome instability.

Collisions with paused or stalled RNAPII complexes

Even though it is generally believed that head-on TRC are more deleterious, co-directional collisions can be problematic upon collision with a paused or stalled RNAPII complex. Regulated pausing of RNAPII in early elongation and controlled release of paused RNAPII complexes is critical for maintenance of transcriptional integrity. This scheduled pausing of RNAPII is caused by reversal of RNAPII, also referred to as backtracking, in which the 3' end of RNA is displaced from the active site of RNAPII, thereby trapping RNAPII in a temporarily transcriptionally inactive state [45]. Transcription factor TFIIS stimulates cleavage of the transcript by RNAPII, leading to resumption of transcription elongation. Importantly, human cells expressing a TFIIS mutant that is unable to stimulate transcript cleavage show increased genomic instability [46].

In yeast, it has been shown that co-directional collisions with backtracked RNAPII leads to the formation of DSBs, while head-on TRC do not [47]. Consistent with this, upon collisions with stalled RNAPII complexes distinct DNA damage response (DDR) pathways are activated, co-directional collisions triggers activation of the Ataxia–telangiectasia-mutated (ATM) pathway, while head-on collisions triggers activation of the ATR pathway [19]. The ATM pathway plays a critical role in the repair of DSBs, which supports the hypothesis that co-directional collisions could result in the formation of potentially toxic DSBs. It is important to note, that head-on collisions can cause DSB formation upon depletion of TOP1. Moreover, even though both WT and TOP1-depleted cells show activation of the ATR pathway [24], it is likely that in TOP1-depleted cells the ATM pathway will also be activated due to the formation of DSBs.

Helix-distorting DNA lesions in actively transcribed DNA strands also result in stalling of RNAPII, which triggers the initiation of TCR to efficiently remove the lesion and restore transcription [2]. The UV-induced ubiquitylation of RPB1-K1268 is essential for the repair of transcription-blocking DNA lesions, but when repair fails it will lead to processing and removal of RNAPII from the lesion, preventing persistent stalling of RNAPII which could cause potentially harmful collisions with the replication machinery [5]. Ubiquitylation and proteasomal degradation of RNAPII is not specific to UV-induced transcription stress, but occurs upon transcription-stress in general. For example, RNAPII is ubiquitylated in response to cisplatin induced DNA damage as well as treatment with the transcription elongation inhibitor α-amanitin [48, 49]. It is important to note that it is currently unknown if RPB1-K1268 ubiquitylation is specific to UV-induced transcription stress, given that in response to DSBs the RPB1 subunit is ubiquitylated on K48 by the HECT-type E3 ubiquitin ligase WWP2 [50]. In the next section I will discuss the replication-stress response in TCR-deficient cells.

The replication stress response in TCR-deficient cells

CSB and CSA-deficient cells are unable to ubiquitylate and subsequently degrade RNAPII [8], causing prolonged stalling of RNAPIIo at DNA lesions, which could result in potentially harmful conflicts with the replication machinery. In contrast, UVSSA-deficient cells display even faster degradation of RNAPII after UV [8, 9], making it likely that UV-induced collisions between the transcription and replication machinery are less prevalent in UVSSA-deficient cells. Considering

that ELOF1 is required for UV-induced RPB1-K1268 ubiquitylation [10, 11], it is to be expected that loss of ELOF1 also causes prolonged stalling of RNAPIIo and potentially harmful conflicts with the replication machinery.

Indeed, we (chapter 3) and others recently demonstrated that replication fork progression is reduced in CSB and ELOF1-deficient cells upon the induction of transcription-blocking DNA lesions with UV irradiation or Illudin S treatment [10, 11]. Importantly, fork progression was partially restored in ELOF1-deficient cells upon mild transcription inhibition [11], supporting the idea that impaired fork progression upon loss of ELOF1 is due to conflicts between the transcription and replication machinery. Interestingly, while our findings indicate that CSB and ELOF1-deficient cells are equally impaired in replication fork progression [10], another study demonstrated that the reduction in fork progression was stronger in ELOF1-deficient cells [11], suggesting that ELOF1 might also have a CSB-independent role in dealing with DNA damage-induced replication stress (discussed later). These seemingly different results could be caused by a clonal effect or differences in study design, such as Illudin S treatment vs UV irradiation. It is therefore crucial that fork progression is analysed in multiple CSB and ELOF1-KO clones after induction of transcription-blocking DNA lesions with UV irradiation and Illudin S. Nevertheless, our data does show that replication fork progression is more strongly reduced upon loss of CSB and ELOF1 together, which is consistent with the idea of an CSB-independent role for ELOF1 [10]. Although it is likely that the reduced fork progression in CSB and ELOF1-deficient cells is caused by TRC, it would be important to directly detect TRC, for example, by monitoring interactions between the transcription and replication machinery using a proximity-ligation assay between PCNA and RNAPII [19].

The response to replication stress involves phosphorylation of H2AX (γH2AX) as well as accumulation of 53BP1 foci. Indeed, both CSB-KO and ELOF1-KO cells show accumulation of 53BP1 upon UV irradiation and an increase in γH2AX foci in replicating cells 15 hr after Illudin S treatment [10, 11]. Importantly, even though both CSB-KO and ELOF1-KO cells show a marked increase in Illudin S-induced γH2AX foci in S-phase cells, γH2AX foci increases even further in CSB/ELOF1-dKO cells [10]. Furthermore, the UV-induced increase in 53BP1 foci is more pronounced in ELOF1-deficient cells compared to CSB-deficient cells, which is in line with their earlier observation that the reduction in fork progression was stronger in ELOF1-deficient cells [11]. These combined findings are consistent with the hypothesis that ELOF1 might also have a CSB-independent role in dealing with DNA damage-induced replication stress.

γH2AX and 53BP1 foci also form in response to DNA breakage, including DSBs [51]. Co-directional collisions with persistently stalled backtracked RNAPII can lead to replication fork collapse and the formation of DSBs [47]. However, it is unclear if encounters between the replication fork and persistently stalled RNAPIIo at DNA lesions also leads to replication fork collapse and DSB formation or if cells employ alternative pathways to remove RNAPIIo from the DNA allowing replication to continue (Fig. 3a, b). A way to distinguish between these possibilities is to monitor activation of the ATR and the ATM pathway. The ATR pathway is primarily activated upon replication stress that involves replication fork stalling without the formation of DSBs (Fig. 3a), whereas co-directional collisions accompanied by the formation of DSBs results in activation of the

ATM pathway (Fig. 3b) [19]. Moreover, it will be important to directly monitor co-directional and head-on collisions between the replisome and lesion-stalled RNAPII in TCR-deficient cells using plasmid-based systems [19, 47], to determine if TRC in these cells result in accumulation of DSBs. Furthermore, if DSBs accumulate, it would be of interest to determine if these dependent on the orientation of replication relative to the direction of transcription.

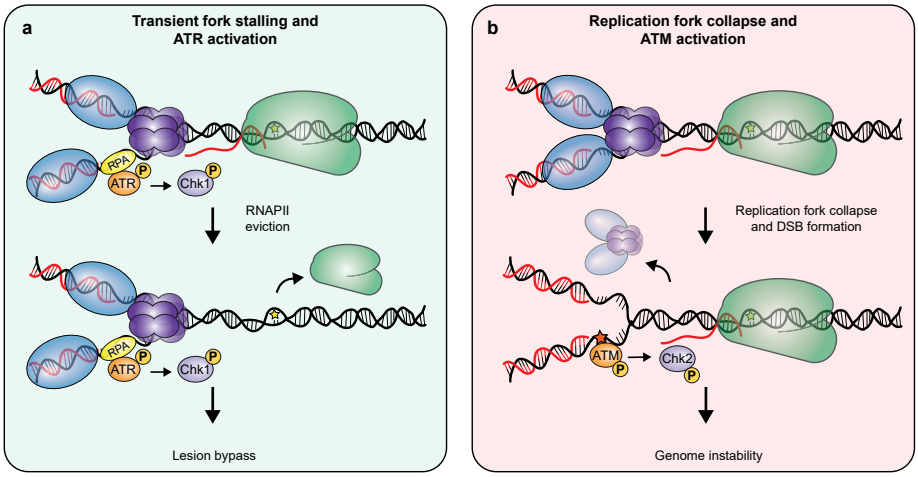


Fig. 3: Hypothetical models depicting the potential outcomes of co-directional collisions with persistently stalled RNAPII at DNA lesions. (**a**) The replication fork can transiently stall upon collision with RNAPII, which results in ATR/Chk1 signaling. Activation of the ATR pathway may signal to other factors for the removal of RNAPII after which the replication machinery can bypass the lesion and resume replication. (**b**) The replication fork persistently stalls upon collision with RNAPII, which results in replication fork collapse, DSB formation, and activation of the ATM pathway.

The global response to replication stress

Cells deficient in the repair of transcription-blocking DNA lesions show higher levels of UV-induced apoptosis compared to TCR-proficient cells. Importantly, the increase in DNA damage-induced apoptosis in CSB-deficient cells is dependent on replication, suggesting that collisions between the replisome and persistently stalled RNAPII induces cell death [52, 53]. In line with this, knockout of either CSB or ELOF1 results in the UV-induced upregulation of CDKN1A (p21) and CDKN2A (p16) and downregulation of Lamin B1 [10], which are hallmarks of senescent cells [54, 55]. Interestingly, CSB has also been shown to regulate p21 levels directly by binding to the promoter of p21, thereby preventing p21 transcription and replication-induced senescence [56]. Since the loss of ELOF1 results in the upregulation of p21, even though these cells still have functional CSB, an intriguing possibility would be that CSB dissociates from the p21 promoter to induce senescence in cells that experience persistent replication stress. In addition, loss of CSB or ELOF1 results in upregulation of Cyclin E (CCNE1) [10]. *Cyclin E* is an oncogene that regulates the transition from the G1 to the S-phase of the cell cycle. It is therefore not surprising that aberrant upregulation of Cyclin E leads to deregulation of the cell cycle and replication stress. Interestingly, it has been shown

that overexpression of Cyclin E also leads to increased origin firing and increased TRC [57, 58].

Unrepaired DNA damage is extremely toxic to cells and is a major contributor to the development of cancer, it is therefore likely that DNA damage-induced apoptosis prevents the development of cancer in CS patients [59]. Interestingly, the mitochondrial stress markers, NOXA [60], ATF4 [61], and DDIT3 [61] are upregulated in both CSB and ELOF1-KO cells in response to UV irradiation [10]. Mitochondrial dysfunction has been implicated in the development of neurological symptoms in CS patients [62]. This would indicate that hypomorphic mutations in ELOF1, if viable, may also cause mitochondrial dysfunction and possibly neurodegeneration.

CSB-independent role for ELOF1 in preventing replication fork collapse

Even though it is evident that persistent stalling of RNAPIIo in TCR-deficient cells causes replication stress, there is compelling evidence that indicates that ELOF1 also has a TCR-independent role in the replication stress response. We (chapter 3) and others recently demonstrated that ELOF1-deficient cells, but not CSB-deficient cells, are sensitive to compounds that cause replisome stalling, such as the DNA polymerase αinhibitor CD437 and the DNA crosslinker mitomycin C (MMC) [10, 11]. This is in agreement with genome-wide CRISPR screens showing that loss of ELOF1, but not CSB, CSA, or UVSSA, sensitizes cells to genotoxic agents that interfere with DNA replication [63].

The precise function of ELOF1 in the replication stress response is currently unknown, however, it has been suggested that cells become more dependent on this second ELOF1 pathway when canonical TCR fails [10]. Indeed, CSB/ELOF1-dKO cells are more sensitive to compounds that induce transcription-blocking DNA lesions, such as Illudin S and Irovulven, than either single KO, while this is not the case for CSB/CSA-dKO cells [10]. Interestingly, ELOF1-deficient cells are unable to restart transcription following treatment with MMC [11], which induces DNA crosslinks that block replication, indicating that the role of ELOF1 in replication is linked to transcription. This is in agreement with other data showing that loss of ELOF1 in CSA-deficient cells causes an additive sensitivity to UV irradiation in cycling but not in non-cycling cells [11]. Altogether these findings demonstrate that ELOF1 functions in a second pathway that deals with DNA damage in a transcription and replication-dependent manner. The constitutive interaction between ELOF1 and RNAPII is essential for the repair of transcription-blocking DNA lesions [10]. It will be imperative to determine if this interaction is also required to protect cells against replication stress, for example by measuring CD437 and MMC sensitivity as well as replication fork speed and γH2AX foci after Illudin S in ELOF1-KO cells complemented with an ELOF1 mutant (S72K/D73K) that is unable to interact with RNAPII [10].

One intriguing possibility is that ELOF1 plays a role in the removal of RNAPII upon TRC to prevent replication fork collapse. A similar function has been described for the PAF1 complex in yeast [64], with which ELOF1 genetically interacts in our genome-wide CRISPR screens [10]. The yeast PAF1 complex works together with the chromatin remodeling complex INO80 and Mec1-Ddc2

(ATR-ATRIP) to degrade RNAPII upon hydroxyurea (HU)-induced replication stress [64]. Given that ELOF1 facilitates RNAPII (RPB1-K1268) ubiquitylation by the $CRL4^{CSA}$ complex in response to UV irradiation [10, 11], ELOF1 might also promote RNAPII ubiquitylation and proteasomal degradation in response to replication stress, possibly in cooperation with PAF1-INO80-Mec1. It would be interesting to see whether cells that are impaired in RPB1-K1268 ubiquitylation (RPB1-K1268R) are sensitive to compounds that induce replication stress, and if so, is RPB1-K1268 ubiquitylated in an ELOF1-dependent manner. The RPB1 subunit of RNAPII is ubiquitylated on K48 in response to DSBs [50], which indicates that RPB1 is ubiquitylated on specific residues depending on the type of DNA damage. It is therefore possible that RPB1 is not ubiquitylated on K1268 in response to replication stress, thus it would be important to map RPB1 ubiquitylation sites in WT, CSB-KO, and ELOF1-KO cells upon treatment with compounds that induce replication stress.

It has been suggested that the PAF1-INO80-Mec1 pathway is not involved in the eviction of RNAPII upon UV irradiation [64]. However, it is possible that TCR-deficient cells, which are unable to ubiquitylate RNAPII in response to UV irradiation, rely on the PAF1-INO80-Mec1 to evict RNAPII upon TRC (Fig. 4a). Quantification of TCR kinetics revealed that CSB-deficient cells are more strongly impaired in the removal of DNA lesions from the transcribed strand than ELOF1-deficient cells [10], indicating that ELOF1-deficient cells are still able to repair some of the transcription-blocking DNA lesions. If ELOF1 is involved in the PAF1-INO80-Mec1 pathway, CSB/ELOF1-dKO cells would not only be completely impaired in TCR but also in the removal of RNAPII upon collisions with the replication machinery, which would explain why loss of CSB and ELOF1 together causes a higher degree of replication stress than either single KO [10].

ELOF1 is involved in preventing genomic instability by R-loops

Excessive formation of R-loops is a major source of genome instability [12, 26]. ELOF1-deficient cells accumulate R-loops at 3 h after UV irradiation, while CSB-deficient cells do not [10]. In addition, depletion of ELOF1 leads to an increase in chromosomal aberrations upon UV-induced DNA damage, which is accompanied by accumulation of FANCD2 foci in mitotic cells [11]. FANCD2 is part of the FA-pathway and is recruited to R-loops where it activates FANCM2 to resolve R-loops. If replication stress persists and resolution of R-loops is incomplete, FANCD2 remains on mitotic chromosomes [65, 66], providing an explanation for the UV-induced FANCD2 foci in ELOF1-depleted cells. It should be noted that at this time we do not know if FANCD2 accumulation in mitotic cells, as well as the increase in chromosomal aberrations, is due to a CSB-independent function of ELOF1 since it is currently unknown if loss of CSB also results in an UV-induced increase of mitotic FANCD2 foci and chromosomal aberrations. It would be of particular interest to test if the additional loss of ELOF1 in CSB-KO cells exacerbates the persistent FANCD2 foci formation in mitotic cells.

The yeast orthologue of ELOF1, ELF1, cooperates with SPT4/5 and the FACT complex during transcription elongation [67, 68]. SPT4/5 binds to RNAPIIo close to the upstream DNA that has just been transcribed where they prevent the formation of R-loops by physically separating the DNA from the nascent RNA

[68]. Although ELOF1 binds to the front of RNAPII, where the DNA enters, and SPT4/5 binds to the back of RNAPII, where the DNA exits, SPT5 and ELOF1 are adjacent to one another [68]. It is important to note that the yeast orthologue of CSB, RAD26, interacts with RNAPII at the same position as SPT4/5 [69]. Therefore, following stalling of RNAPIIo at a lesion, SPT4/5 will dissociate and CSB will bind to RNAPII. One intriguing possibility would be that ELOF1 is somehow required for the prevention of R-loops by SPT4/5. In this scenario, loss of ELOF1 would result in the formation of R-loops in the presence and absence of DNA damage. In line with this, loss of ELOF1 also causes increased R-loop formation in the absence of DNA damage (unpublished data by us). Alternatively, ELOF1 may regulate the resolution of R-loops through other mechanism. Most notably, the putative helicase DHX35 was one of the top hits in the ELOF1 genetic-interaction map and caused synthetic lethality in combination with loss of ELOF1. Whether DHX35 has a role in resolving R-loops in a ELOF1-dependent manner remains to be determined.

During co-directional collisions the replisome can resolve R-loops, however, upon head-on encounters, the replisome cannot resolve R-loops and they might even increase due to torsional stress. Research showed that in the absence of DNA damage knockout of ELOF1 results in reduced transcription towards the end of longer genes and around the TTS [10], which have a higher occurrence of head-on collisions [24]. Furthermore, collisions between the transcription and replication machinery are more likely to occur in long highly transcribed genes [17]. It is therefore possible that loss of ELOF1 causes reduced transcription towards the end of longer genes due to excessive R-loop formation at head-on collisions, resulting in replication fork collapse. In addition, we noted in our nascent RNA sequencing experiments (unpublished data) that ELOF1-KO cells tend to display read-through beyond the TTS following UV irradiation, which was not observed in WT cells, or earlier in CSB-deficient cells [70]. It is conceivable that RNAPIIo molecules that do not terminate properly in ELOF1-KO cells may cause collisions with replisome moving in the opposite direction.

In the human genome, many genes are transcribed and replicated in the same direction [23], which decreases the likelihood of head-on collisions and subsequent replication stress. However, knockout of either CSB or ELOF1 result in the UV-induced upregulation of Cyclin E [10], this can lead to unscheduled origin firing which can increase the occurrence of head-on collisions [57]. We propose that in ELOF1-deficient cells this increase in head-on collisions results in the excessive formation of R-loops and R-loop-associated DNA damage, while in CSB-deficient cells the formation of R-loops is prevented or quickly resolved (Fig. 4a, b). A testable prediction of this model would be that R-loop resolution by RNase H1 overexpression would suppress UV-induced genome instability in ELOF1-deficient cells.

Loss of OTUD5 alleviates the Illudin S sensitive phenotype of ELOF1-KO cells, while it causes additive sensitivity in CSB-KO cells [10]. OTUD5 is a deubiquitylase that represses the FACT complex and was suggested to arrest RNAPII upon UV irradiation [71]. Live-cell imaging experiments showed that the SPT16 subunit of the FACT complex is required for the recruitment of UVSSA to sites of UV-induced DNA damage and the restart of transcription following repair [72, 73]. Interestingly, it has also been shown that FACT depletion results

in R-loop accumulation, DNA breaks, and hyperrecombination in a transcription and replication-dependent manner [74]. Furthermore, even though knockdown of FACT resulted in genome instability upon collisions in both orientations, the effects were more pronounced upon head-on collisions. R-loop resolution by RNase H1 overexpression suppressed genome instability, indicating that genome instability upon FACT depletion is R-loop dependent [74]. It is interesting to note, that depletion of OTUD5, which regulates the FACT complex, also results in replication stress [71]. Therefore, it is likely that misregulation of the FACT complex can lead to genomic instability upon the induction of DNA damage. One interesting possibility is that ELOF1, SPT4/5 and FACT cooperate to prevent the formation of R-loops and maintain genome integrity, which becomes particularly important upon head-on TRC (Fig. 4b).

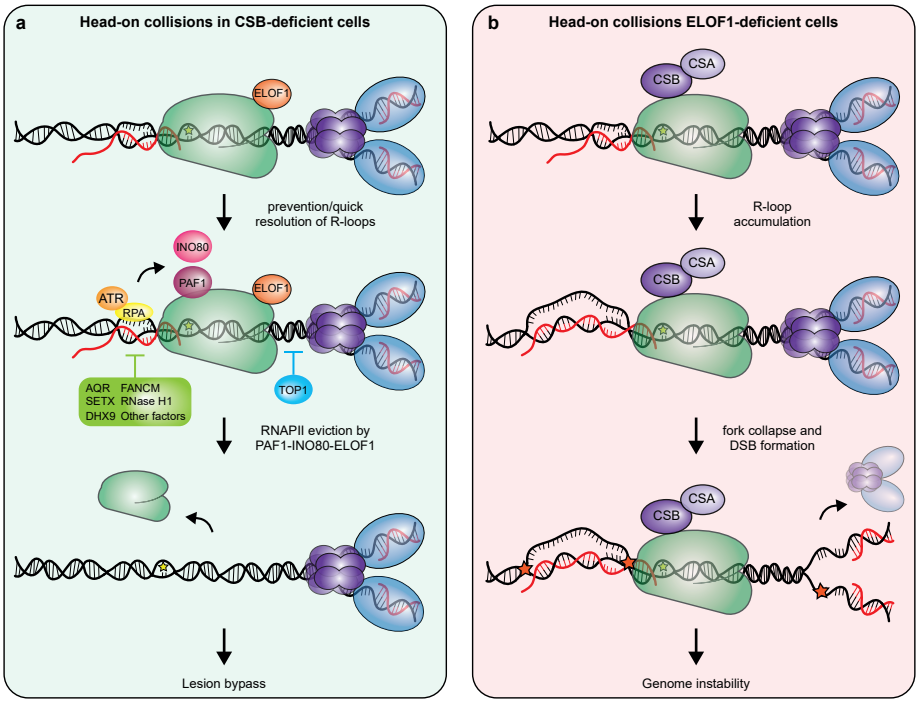


Fig. 4: Hypothetical model of the CSB-independent role for ELOF1. (**a**) In the absence of CSB, the replication fork transiently stalls upon collision with persistently stalled RNAPII at DNA lesions. Excessive accumulation of positive supercoils and R-loops is prevented by the combined actions of TOP1, activation of the ATR pathway, and helicases. Activation of the ATR pathway signals to PAF1, INO80 and ELOF1 for the removal of RNAPII, after which the replication machinery can bypass the lesion and resume replication. (**b**) In the absence of ELOF1, R-loops will accumulate in the absence and presence of DNA damage. Head-on collisions between the replication machinery and persistently stalled RNAPII at DNA lesions results in negative supercoiling behind RNAPII, which is sensitive for reannealing of the nascent transcript, resulting in excessive accumulation of R-loops. Even if cells are able to remove the R-loops, the replication fork will still collapse since ELOF1 is required for eviction of RNAPII by the PAF1-INO80 pathway.

Concluding remarks

Our knowledge of the molecular mechanism underlying TCR has grown dramatically in recent years, largely due to the development of new techniques that allowed us to assess the global transcriptional response to DNA damage and identify novel TCR proteins, including ELOF1. Recent studies demonstrated that an essential step in the repair of transcription-blocking DNA lesions is the ubiquitylation of RNAPII (RPB1-K1268) in a CSB, CSA, and ELOF1-dependent manner [5, 10, 11]. Even though mounting evidence suggests that defects in RNAPII processing can have serious consequences not only for transcription but also for replication, we are only just beginning to understand the links between transcription-stress and replication-stress. Important goals for future research are to investigate if collisions between the transcription- and replication machinery in CSB, CSA, and ELOF1-deficient cells lead to replication fork collapse or if cells employ alternative pathways to remove RNAPIIo from the DNA allowing replication to continue. If cells employ alternative pathways to remove RNAPIIo from the DNA it would be imperative to identify the full repertoire of proteins involved in these pathways. Considering that ELOF1 functions in a second pathway that deals with DNA damage in a transcription and replication-dependent manner, an intriguing possibility would be that ELOF1 is somehow involved in the removal of RNAPIIo to prevent replication fork collapse upon collisions between the transcription and replication machinery. Undoubtedly, the coming years will bring answers to these important questions and will provide much needed insights into the mechanisms that mitigate transcription-replication collisions.

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