

Mechanisms of Dealing with DNA Damage-Induced Replication Problems

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Abstract During every S phase, cells need to duplicate their genomes so that both daughter cells inherit complete copies of genetic information. Given the large size of mammalian genomes and the required precision of DNA replication, genome duplication requires highly fine-tuned corrective and quality control processes. A major threat to the accuracy and efficiency of DNA synthesis is the presence of DNA lesions, caused by both endogenous and exogenous damaging agents. Replicative DNA polymerases, which carry out the bulk of DNA synthesis, evolved to do their job extremely precisely and efficiently. However, they are unable to use damaged DNA as a template and, consequently, are stopped at most DNA lesions. Failure to restart such stalled replication forks can result in major chromosomal aberrations and lead to cell dysfunction or death. Therefore, a well-coordinated response to replication perturbation is essential for cell survival and fitness. Here we review how this response involves activating checkpoint signaling and the use of specialized pathways promoting replication restart. Checkpoint signaling adjusts cell cycle progression to the emergency situation and thus gives cells more time to deal with the damage. Replication restart is mediated by two pathways. Homologous recombination uses homologous DNA sequence to repair or bypass the lesion and is therefore mainly error free. Error-prone translesion synthesis

employs specialized, low fidelity polymerases to bypass the damage.

Keywords DNA damage · DNA replication restart · Homologous recombination · Translesion DNA synthesis

DNA Damage Checkpoints

DNA is constantly injured by a variety of damaging agents. The threat comes from inside a cell in the form of by-products of normal metabolism, such as reactive oxygen species and free radicals. DNA damage is also caused by exogenous sources, including UV light, ionizing radiation, and toxic chemicals. Additionally, DNA lesions arise spontaneously through hydrolysis reactions such as deamination of cytosines. Cells are equipped with a number of repair pathways that remove the damage and restore the intact DNA [1], but they are not sufficient to fully protect cells against DNA lesions. The efficient response to DNA damage requires the presence of signaling cascades called DNA damage checkpoints. They are activated by DNA lesions and subsequently regulate the activity of different components of the cell cycle machinery. The resulting delay or temporary arrest of the cell cycle progression gives the affected cells time to repair the damage and prevents transition to the next cell cycle phase in the presence of unrepaired DNA [2]. The damage signaling can stop the cell cycle at the G1/S and G2/M transitions and slow down progression through S phase (Fig. 1).

Replication of damaged DNA is likely to cause stalling of replication forks and accumulation of potentially dangerous mutations. The damage-induced G1/S checkpoint leads to cell cycle arrest before the onset of DNA synthesis, giving the cell time to repair the lesions in the DNA

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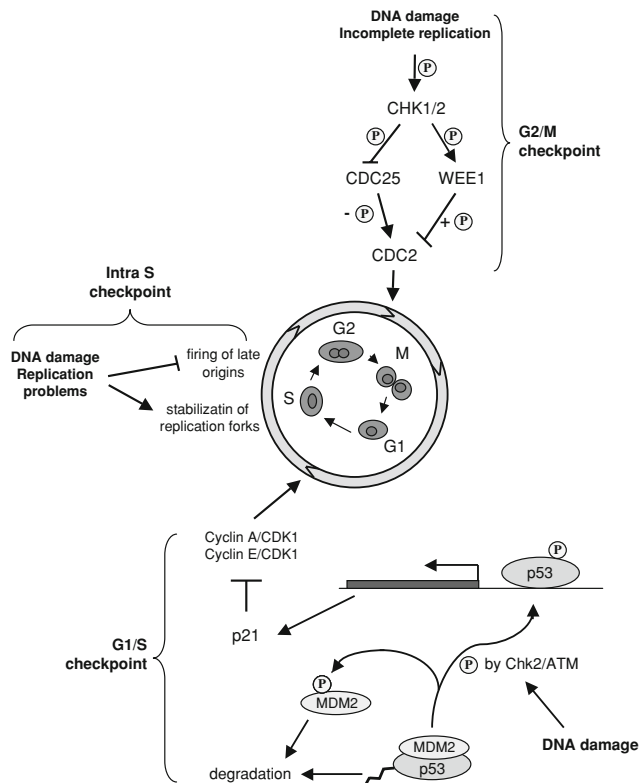


Fig. 1 Schematic representation of a number of cell cycle proteins and processes targeted by different DNA damage checkpoints. DNA damage can arrest cell cycle progression at the G1/S and G2/M transitions, and slow down S-phase progression. A main target of the G1/S checkpoint is the tumor suppressor protein p53. In undamaged cells, p53 forms a complex with ubiquitin ligase MDM2. The constitutive ubiquitination of p53 targets it for proteosomal degradation, ensuring rapid turnover of p53. DNA damage activates signaling cascades, which act to stabilize and activate p53 via multiple redundant mechanisms, including phosphorylation of p53 by CHK2 and ATM, and phosphorylation of MDM2 [109]. Stabilized p53 activates transcription of many genes including the gene encoding p21—an inhibitor of cyclin-dependent kinases (CDKs). Increase in p21 expression suppresses cyclin E and cyclin A-associated CDK activities, which are necessary for entering S phase. The S-phase checkpoint is activated by replication problems and promotes stabilization of replication forks and inhibition of late origin firing. The G2/M checkpoint prevents cell division in the presence of damaged or unreplicated DNA. The main cell cycle protein targeted by this checkpoint is CDC2, which controls entry into mitosis. CDC2 is regulated negatively by phosphorylation by the WEE1 kinase and positively by de-phosphorylation by CDC25 phosphatases. In response to DNA damage checkpoint, proteins CHK1 and/or CHK2 phosphorylate both WEE1 and CDC25. Phosphorylation activates WEE1, which in turn phosphorylates CDC2 and this results in inhibition of CDC2 and therefore prevents cell division. CDC25 is inhibited by phosphorylation and is no longer able to de-phosphorylate and thus can no longer activate CDC2

template. A main player of this checkpoint is the tumor suppressor protein p53. p53 is rapidly turned over in undamaged cells, but is stabilized and activated following DNA damage. Next, it activates transcription of genes encoding proteins involved in cell cycle control, including

p21, and thus prevents transition to S phase (Fig. 1) [3]. Alternatively, p53 can turn on expression of pro-apoptotic genes and induce apoptosis [4].

Despite the action of the G1/S checkpoint it is not possible to completely avoid the presence of damaged DNA templates during DNA replication as DNA damage can also be induced while cells are in S phase. Intra-S checkpoint signaling is critical during replication to protect cells encountering DNA damage or other problems such as low nucleotide pools. The main role of this checkpoint is to inhibit firing of late origins and to stabilize stalled replication forks [5, 6] (Fig. 1). The inhibition of late origin firing reduces the level of ongoing replication and leads to extended duration of S phase. This may provide additional time to restart stalled replication forks, i.e. forks with an intact replisome whose progression is blocked, or collapsed replication forks, i.e. forks with a disassembled replisome [7]. The stabilization of stalled replication forks helps to prevent accumulation of unusual DNA structures at the forks, which could lead to irreversible fork collapse and subsequent cell death.

The role of the G2/M checkpoint is to ensure that chromosomes are intact and ready for separation before cells enter mitosis (Fig. 1). This control point is very important for genomic stability, since an attempt to segregate partially replicated or damaged chromosomes can result in DNA breakage and lead to chromosomal aberrations and aneuploidy. However, it appears that the control mechanisms in G2 phase are not absolute and that cells might in fact enter mitosis with low levels of damaged, incompletely separated or incompletely replicated DNA. For instance, chromosomal aberrations observed in many repair-deficient cells, for example those defective in the structure-specific endonucleases Ercc1 or Mus81, seem to arise as a result of segregating unrepaired DNA [8, 9].

DNA Damage Checkpoints are Controlled by ATM and ATR

ATM and ATR are two main proteins that activate checkpoint signaling in response to DNA damage. They belong to a structurally conserved family. The members of this family contain catalytic domains resembling those found in phosphatidylinositol 3-kinase (PI3K), and are therefore called ‘PI3K-like protein kinases’ (PIKKs). Although related to a lipid kinase, they phosphorylate proteins on serine and threonine residues. Their preferred substrates contain SQ/TQ motifs, in which the serine or threonine is directly followed by glutamine [10]. Such motifs are often found in clusters, and SQ/TQ-rich domains are especially common in proteins involved in checkpoint signaling and DNA repair, e.g. in BRCA1 and CHK1.

ATM and ATR have distinct, although partially overlapping functions. They share many substrates, and accumulating evidence shows that there is crosstalk between ATR- and ATM-controlled pathways. However, the different phenotypes of ATM- and ATR-deficient mice and cells indicate that these kinases are not completely redundant.

ATM is specifically activated by DSBs *in vivo*. Consistent with its central position in the signaling cascade, ATM responds very quickly to DSB induction. Within seconds after irradiation, ATM kinase activity rapidly increases. However, the actual stimulus required to initiate the signaling cascade, the exact requirements for the initiation, and the precise order of the events leading to ATM activation remain uncertain. It is clear that at least three events are important for ATM activation: (1) ATM auto-phosphorylation, (2) the recruitment of ATM to the sites of DSBs and its interaction with the MRE11/RAD50/NBS1 (MRN) complex, and (3) the action of acetyltransferases, which cause DSB-induced changes of chromatin structure and acetylation of ATM itself. The number of ATM substrates identified so far indicates that ATM controls a remarkably broad range of cellular responses to DSBs. It regulates cell cycle progression at G1/S transition, in S phase, and at the G2/M transition. ATM deficiency causes significant defects in all of these checkpoints and extreme sensitivity to ionizing radiation and DSB-inducing agents.

The ATR protein kinase plays a central role in the cellular response to replication stress and a wide range of DNA damaging agents, including UV light, alkylating agents, and DSBs. In human cells, ATR exists in a stable complex with its indispensable partner ATRIP (ATR-interacting protein). ATR participates in an elaborate, step-wise accumulation of the checkpoint signaling machinery at the DNA lesions that leads to activation of the downstream effector proteins, the main one being CHK1 (Fig. 2). The ATR-dependent branch of damage signaling is essential for cell viability. ATR-deficient mice die early during embryogenesis, and ATR^{-/-} cells accumulate chromosomal breaks prior to apoptosis. Deletion of other proteins involved in the ATR pathway, e.g. RAD17, RAD1, and CHK1, also results in embryonic lethality in mice [11–14]. This indicates that this branch of checkpoint signaling is required not only to protect cells against external DNA damage, but also to deal with spontaneous lesions, such as oxidative base damage or abnormal DNA structures arising during DNA replication.

ATR is activated by RPA-coated single-stranded (ss) DNA, a common intermediate produced at stalled replication forks and by DNA repair reactions [15]. Since RPA stimulates binding of ATRIP to ssDNA *in vitro*, it has been proposed that interaction between ATRIP and the RPA-coated ssDNA brings ATR/ATRIP complex to the sites of

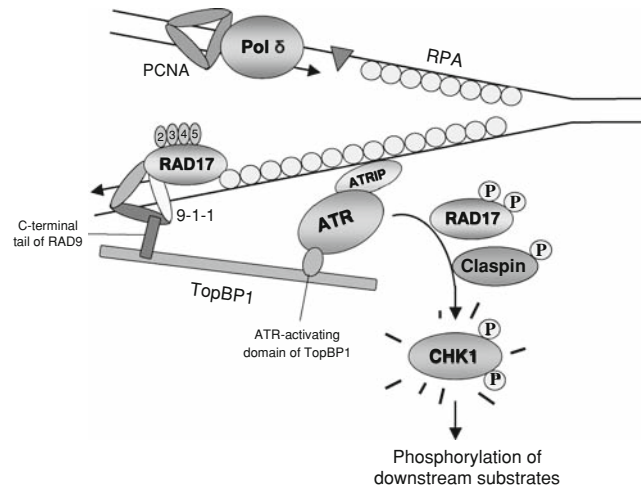


Fig. 2 A model for activation of the ATR-signaling pathway. The assembly of distinct protein complexes at the site of damage is required for activation of ATR-dependent signaling. RPA-coated ssDNA produced at the lesion site serves to recruit ATR/ATRIP and RAD17. RAD17, complexed with RFC2-5, in turn loads the 9-1-1 complex onto 5' junctions. Next, binding of TopBP1 to RAD9, mediated by the C-terminal part of RAD9, places TopBP1 in the proximity of ATR and allows it to activate ATR kinase activity. ATR then phosphorylates RAD17. Claspin localizes at the replication forks and, together with phosphorylated RAD17, is required for ATR-mediated CHK1 phosphorylation and activation

damage (Fig. 2). This presumably places ATR in the vicinity of its substrates and other essential components of the checkpoint cascade, and it is necessary, but not sufficient for the activation of checkpoint signaling. Another required event involves RAD17-mediated loading of the RAD1-RAD9-HUS1 (9-1-1) complex onto damaged DNA. RAD1, RAD9, and HUS1 share structural similarity with PCNA and are predicted to form a PCNA-like heterotrimeric ring in undamaged cells [16–19]. RAD17 is homologous to RFC1, a subunit of the RFC1-5 complex that loads PCNA onto primer/template junctions during replication. RAD17 is recruited to the double-stranded (ds)/ssDNA transitions at the damage sites independently of the ATR/ATRIP complex by means of its interaction with RPA-coated ssDNA [20–22]. Next, RAD17-RFC2-5 catalyzes loading of the 9-1-1 complex onto these transitions (Fig. 2) in a reaction similar to RFC1-5-mediated loading of PCNA [16, 23, 24]. The chromatin-bound 9-1-1 complex in turn recruits TopBP1 (topoisomerase IIβ binding protein 1) via direct interaction between RAD9 and TopBP1. This brings TopBP1 close to the damage sites, enabling it to directly interact with ATR. This interaction induces a large increase in the kinase activity of ATR and is critical for ATR activation [25]. Activated ATR mediates damage-induced phosphorylation of RAD17. Phosphorylated RAD17 interacts with another protein required for activation of ATR-dependent signaling, named

Claspin, and promotes Claspin phosphorylation. This phosphorylation is essential for the direct Claspin-CHK1 interaction, which subsequently enables ATR to phosphorylate and activate CHK1—the main effector kinase (Fig. 2). Activated CHK1 turns on intra-S checkpoint signaling, i.e. stabilizes arrested replication forks and slows down S-phase progression by inhibiting firing of late origins. CHK1 also prohibits the G2/M transition as long as damaged or incompletely replicated DNA is present in a cell.

It is not fully understood how phosphorylation activates CHK1. Several studies indicate that phosphorylated CHK1 has increased kinase activity [26, 27]. The underlying molecular mechanism might involve the C-terminal part of CHK1. It has been suggested that the C-terminus may play an inhibitory role and that phosphorylation may release this inhibition [28, 29]. It is also possible that the phosphorylation enables CHK1 to interact with its substrates and/or other proteins regulating its activity. CHK1 associates with chromatin, and phosphorylation releases this association [30]. This suggests that phosphorylation might help CHK1 to spread the damage signal throughout the nucleus and reach substrates, e.g. cell cycle proteins, that are likely not present at the damage site.

Repair of Stalled Replication Forks

Maintaining genomic integrity requires extremely precise and efficient DNA replication. The replicative DNA polymerases insert correct bases in the growing DNA molecule, making a mistake as rarely as 1 per every 10^6 nucleotides copied. However, the presence of DNA lesions as well as proteins bound to DNA interferes with the progression of replication forks because, in most cases, replicative DNA polymerases cannot replicate over unusual DNA structures. To ensure efficient and faithful genome duplication, it would be ideal to repair the damaged template before starting to replicate it. To achieve this, cells are equipped with a number of checkpoint mechanisms, discussed above, as well as DNA repair pathways but it is impossible to completely avoid the presence of DNA lesions during S phase. First, DNA damage can be introduced during S phase. Second, some types of DNA lesions, e.g. interstrand DNA crosslinks, often escape detection until they interfere with replication [31]. Other lesions are inherent to the replication process, such as DSBs formed at fragile replication sites. Moreover, natural replication pause sites caused by convergent transcription or DNA secondary structures can lead to replication fork stalling. It has been estimated that as many as 15–20% of all replication forks stall or collapse in one generation of a single *Escherichia coli* cell [32]. Therefore,

cells must have mechanisms to resume blocked replication, not only to avoid mutations and chromosomal aberrations, but even to complete DNA replication and to divide. Two main pathways, homologous recombination (HR) and translesion synthesis (TLS), are responsible for dealing with replication problems. During TLS specialized, low-fidelity DNA polymerases temporarily gain access to the primer terminus and insert nucleotides opposite the lesion. Therefore, TLS polymerases allow bypassing a damaged site, but, at the same time, they often introduce mutations. HR uses the intact sister chromatid as a template for repair, and is therefore essentially error free. Which of these two pathways will deal with a given arrested fork likely depends on the type of lesion and the structure created at the fork. In addition, the two pathways might also cooperate in the repair of certain lesions.

Homologous Recombination

Homologous recombination (HR) is the process of exchange of genetic information between homologous DNA molecules. A classical HR model describes repair of a two-ended DSB, which can be created by ionizing radiation and specific endonucleases, including HO and Spo11 during mating type switching in *Saccharomyces cerevisiae* cells and meiotic recombination, respectively [33, 34]. However, it appears that in somatic mammalian cells the majority of two-ended breaks are repaired by non-homologous end joining. Although it is an error-prone pathway, it is presumably faster than HR and does not require the presence of homologous DNA sequence. Consequently, HR might be dispensable for repair of two-ended DSBs, but might be important for dealing with replication-associated DNA damage, e.g. ssDNA gaps and one-ended DSBs. The vital role of HR is evidenced by embryonic lethality caused by deletion of several recombination genes (e.g. *RAD51*, *RAD51D* and *XRCC2*) [35–38] and increased cancer predisposition associated with mutations in *BRCA2* and *RecQ* helicases, which are involved in recombination processes [39, 40].

The first step of HR is the resection of the blunt end of a DSB to produce a 3' ssDNA overhang (Fig. 3a). The resection requires the MRN complex and the CtIP protein [41, 42]. CtIP physically interacts with the MRN complex and affects its enzymatic activity, suggesting that CtIP might regulate and/or activate the DSB resection involving the MRN complex. However, the details of the reaction remain unknown and it is even unclear whether the nuclease activity of MRE11 is directly responsible for generating the overhang, or whether MRN is indirectly involved in this reaction [43, 44]. In the next step of HR, *RAD51* protomers assemble on the ssDNA to form a

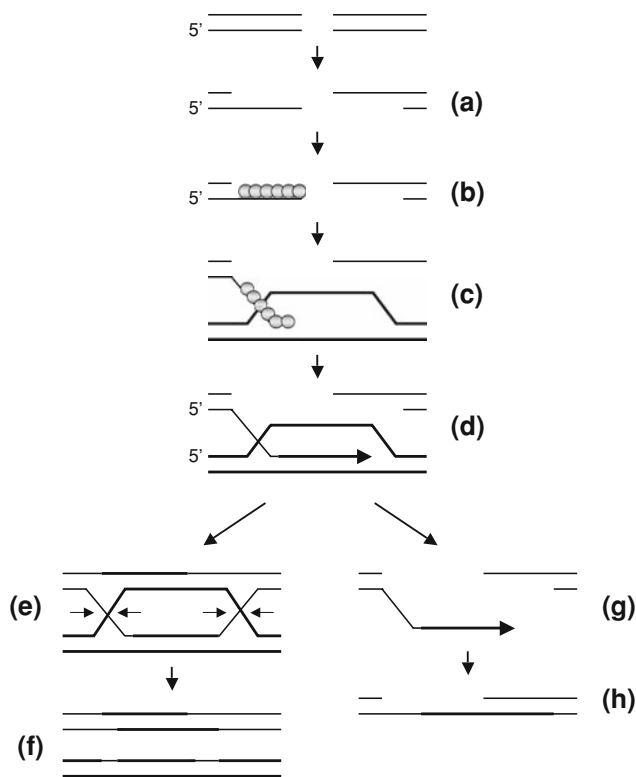


Fig. 3 A model of DSB repair by homologous recombination. A DSB is resected to produce a 3' ssDNA overhang (a), which is subsequently bound by RAD51 (b). RAD51 catalyzes homology search and the invasion of the first end into the homologous DNA duplex, which leads to the formation of a joint molecule (c). The 3' end then serves as a primer for DNA synthesis (d). It is unknown whether RAD51 dissociates after strand invasion; for clarity RAD51 is not shown in (d). After the extension of the invading end the reaction can be channeled into different pathways. The second end may also invade the homologous template in a RAD51-mediated reaction, or it can simply anneal to the displaced strand of the homologous duplex. This process is promoted by single strand annealing activity of Rad52. Engagement of the second end leads to the formation of a double Holliday Junction (e), which in somatic cells is predominantly resolved to produce non-crossovers (f). Alternatively, the first end extended by a DNA polymerase can be displaced from the joint molecule (g) and re-anneal with the second end of the break (h). In a process called synthesis-dependent strand annealing (SDSA), the information lost at the site of the break is recovered, and the resulting gap can be filled to restore the intact DNA molecule

nucleoprotein filament (Fig. 3b). This structure is responsible for catalyzing invasion of the ssDNA into dsDNA, searching for homology and for driving strand exchange between homologous DNA molecules. The human RAD51 protein belongs to the RecA-like family of recombinases, which includes bacterial RecA, archeal Rada, *S. cerevisiae* Rad51, and human DMC1 [45]. All of them are DNA-dependent ATPases and require ATP binding, but not hydrolysis, for filament formation and catalyzing strand exchange [46–48]. Although they are considered structural and functional homologs, their amino acid sequence

conservation is limited to the core ATP-binding domain [49, 50]. Despite limited sequence homology, the recombinases form structurally similar nucleoprotein filaments, as visualized by electron microscopy [51]. Biochemical and biophysical experiments, as well as single molecule imaging techniques, indicate that nucleoprotein filaments are dynamic structures, with monomers being exchanged, redistributed, and forming discontinuous patches [52–54]. The properties of the filaments may also change depending on whether they are formed on ss or dsDNA [55]. The dynamic nature of the filaments is likely to be critical for performing complex DNA transactions required during homology search and strand exchange.

Strand invasion results in the formation of central intermediate of HR—a joint molecule, also called a displacement loop (D-loop) or a single-end invasion (SEI) intermediate (Fig. 3c). The invading 3' end of the D-loop serves as a primer for DNA synthesis (Fig. 3d) and the information lost at the site of the break is retrieved using the homologous DNA molecule as a template. Both the way and the timing of RAD51 dissociation from the invaded strand are unclear. It is unknown whether RAD51 filament has any (stimulatory or inhibitory) role in D-loop extension, i.e. whether it has to dissociate from the invaded strand before the replication proteins can assemble, or whether it can attract the replication machinery to the SEI intermediate. Dissociation of RAD51 from DNA *in vitro* is triggered by ATP hydrolysis by RAD51, but it is uncertain whether this reaction alone is sufficient for RAD51 displacement *in vivo*, and whether ATP hydrolysis occurs spontaneously or is regulated by other proteins. At least one protein, *S. cerevisiae* Rad54, can promote the displacement of Rad51 from dsDNA apparently making the SEI intermediate accessible to further DNA transactions [56, 57].

Structurally, the SEI intermediate (Fig. 3d) is different from a regular primer-template junction. Therefore, the normal replicative DNA polymerases Pol δ and Pol ϵ might not be able to extend it. Indeed, fractionation of human cell extracts revealed that the D-loop extension activity comigrated with Pol η . Since Pol η is a TLS polymerase, it is likely to tolerate unusual DNA structures. On the other hand, it is surprising that an error-prone polymerase is involved in an essentially error-free HR pathway. It is likely that Pol η inserts only a few nucleotides and then is replaced with Pol δ or Pol ϵ , in a mechanism similar to polymerase switch during TLS (see below). This would reduce the chance of introducing mutations. Additionally, the discrepancy between the biochemical data and the cellular phenotypes caused by Pol η dysfunction raises a question whether Pol η is the only or even the main polymerase responsible for D-loop extension. First, Pol η -deficient cells are viable and, second, these cells do not

show any obvious defects in HR. Since the inability to extend D-loop would result in a recombination defect similar to the one caused by *RAD51* deletion, it is likely that in vivo other polymerases can fulfill this function. Pol ζ is a promising candidate because its genetic deletion is lethal [58]. Moreover, studies in chicken DT40 cells revealed that Pol ζ plays a role in HR-dependent DSB repair, and that deletion of Pol ζ resulted in decreased gene targeting efficiency [59].

The recombination reaction can proceed via alternative pathways leading to a repaired intact duplex [60]. The second ssDNA tail, derived from the other end of the DSB, may also invade the dsDNA (second-strand invasion). It can also anneal to the displaced DNA strand that is produced by DNA synthesis from the 3' end of the first ssDNA (second end capture). These pathways can eventually produce cross-stranded structures, called double Holliday Junctions (HJs) (Fig. 3e). Branch migration and resolution of HJs occur during late stages of HR (Fig. 3f) [61]. In bacteria, branch migration and HJ resolution are catalyzed by the RuvABC complex [62]. RuvAB dimer specifically binds HJs and drives branch migration by “pumping out” the DNA in the opposite directions. RuvC resolves the HJs by endonucleolytic cleavage, producing nicked duplexes that are repaired by DNA ligase. The activities similar to those of the RuvABC enzyme have been observed in fractionated human cell extracts [63], but despite extensive search, the identity of the enzyme/enzymes responsible for cleaving HJs in eukaryotic cells remains unknown. The extended joint molecules can also be processed by the BLM helicase and topoisomerase III α [64]. In an alternative pathway, called synthesis-dependent strand annealing (SDSA) (Fig. 3g, h), the 3' invading end extended by a DNA polymerase can be displaced from the joint molecule and re-anneal with the complementary strand of the second end. SDSA is an error-free mechanism [65].

RAD51 is the main protein catalyzing strand exchange reactions. However, it does not act alone. Cells are equipped with numerous accessory proteins, or mediators, which influence and control the action of *RAD51* at every step of HR. The main role of early recombination factors, e.g. RPA and BRCA2, is to facilitate assembly of *RAD51* filaments on ssDNA [66, 67]. The accessory proteins acting at later stages, such as *RAD51AP1*, BLM, and *RAD54*, assist in the formation of a joint molecule, affect its stability, and regulate its subsequent processing, e.g. branch migration [39, 68–70]. The action of mediators is critical to ensure that HR occurs properly. This is of vital importance for cell survival and well-being because uncontrolled or incorrect recombination can cause deletions, gene amplifications, and loss of heterozygosity, all of which contribute to genomic instability and increase the risk of

malignant transformation. The mediators appear to be the key to understanding how HR works and how it is regulated, and therefore unraveling molecular mechanisms of their action will likely continue to be an important part of research in the genome stability field.

Repair of Stalled Replication Forks by Homologous Recombination

Promoting restart of blocked replication forks appears to be an essential role of HR. Depending on the type of lesion and the DNA strand, affected different structures can be created at the replication fork and different HR sub-pathways might be needed to deal with such arrested forks. Bulky lesions, such as the ones induced by polycyclic hydrocarbons and UV light (e.g. 6-4 photoproducts and pyrimidine dimers), will halt replicative DNA polymerases, whose active sites are designed to fit regular DNA bases and would not accommodate bigger elements. If the lesion is located in the lagging strand, the next Okazaki fragment downstream can be used to restart DNA synthesis. This will leave a ssDNA gap behind the fork. The presence of such a lesion in the leading strand (Fig. 4a) poses a potentially more serious threat to the continuity of DNA synthesis. However, experiments with *E. coli* proteins show that DNA replication can also be restarted downstream of the lesion (Fig. 4b) [71]. In contrast to the lagging strand, this is not a default pathway and requires a specialized set of proteins. But similarly to the lagging strand this will leave a ssDNA gap behind the fork, although the gaps might differ in length. The ssDNA gap (Fig. 4b) can be filled by the combined action of TLS polymerases and replicative polymerases (Fig. 4c, d) or by recombination-based mechanisms, such as template switching (Fig. 4l) [65].

DNA replication might not be restarted downstream the lesion in the leading strand, but this will likely not affect the movement of replicative helicase, which is coupled to the lagging strand polymerase. The helicase can continue to unwind dsDNA, and DNA replication can proceed, at least for some distance, on the lagging strand template (Fig. 4e). It is possible that such a fork regresses and the nascent strands re-anneal. The resulting four-way junction is called ‘chicken foot’ (Fig. 4f). Forming a chicken foot places the lesion site back in the dsDNA region. Consequently, the homologous strand is then available as a template for repair of the damage. After the repair is completed, reverse branch migration could lead to a structure from which replication can be resumed (Fig. 4k). In another scenario, the longer nascent DNA strand, synthesized using the undamaged DNA strand, can be used as the alternative template for the nascent strand that was

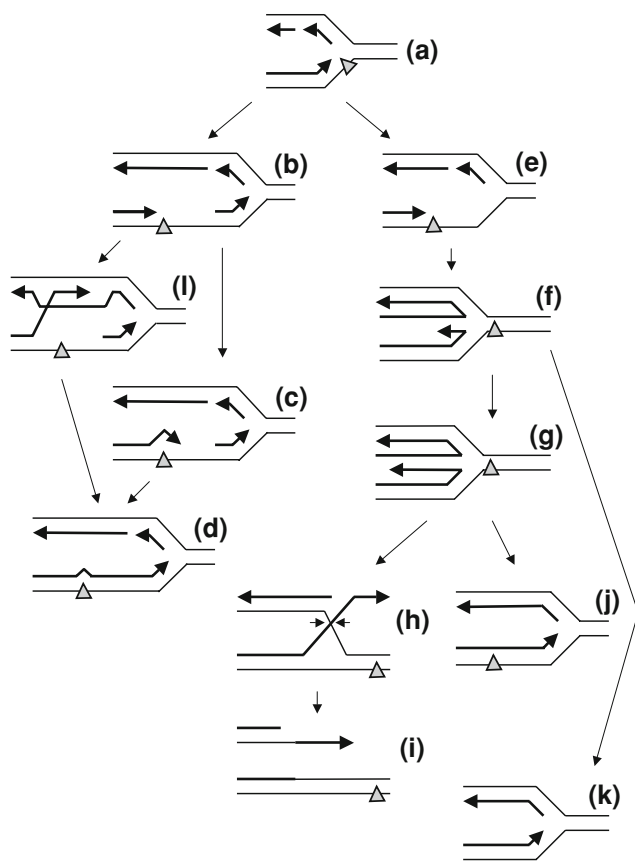


Fig. 4 Pathways of restarting stalled replication forks. Stalling of a replication fork at a lesion in the DNA template (a) in most cases does not stop replicative helicase, which continues to unwind DNA (b, e). Often, DNA synthesis is re-initiated downstream of the lesion, leaving a gap behind (b). The gap can be filled by the combined action of a translesion polymerase, which inserts nucleotides opposite the lesion (c), and a replicative polymerase, which extends the DNA fragment (d). The gap can also be repaired by HR-mediated template switch mechanism (l). The replication fork stalled at the lesion (e) can also regress to form a four-way junction often referred to as a “chicken foot” (f). This places the lesion back in dsDNA (f, g). Therefore, the homologous DNA strand can serve as a template for repairing the damage. After the damage is removed and the nascent strands re-anneal with their original template strands, the active replication fork can be restored (k). The chicken foot structure also allows the nascent DNA strand, synthesized at the other site of the stalled fork, to serve as an alternative template for the strand whose synthesis was blocked by the lesion (f, g). If the replicated DNA fragment is long enough, it will cover the lesion site once the fork adapts its original conformation (j). This will result in an error-free damage bypass. Alternatively, the chicken foot can be cut by structure-specific endonucleases (h) to generate a one-ended DSB (i), which, after being coated with RAD51, can invade the homologous DNA molecule. If the damage has been removed from the DNA before the invasion (not shown), this pathway will restore the intact replication fork. If the damage is still present (i), the restored fork will face the same obstacle that halted it originally and will require other pathways to be restarted

blocked by the lesion (Fig. 4f, g). DNA synthesis using this new template strand and subsequent branch migration will result in error-free bypassing of the lesion (Fig. 4j). This

scenario is very similar to the template switch mechanism, during which the undamaged sister chromatid, instead of the lesion-containing DNA strand, is used as a template for replicating DNA (Fig. 4l). Alternatively, a chicken foot could be cut by a structure-specific endonuclease. The resulting one-ended DSB can invade the homologous DNA duplex. If the damage has been repaired by this time, the invasion will re-establish the active replication fork (Fig. 4h, i). If not, this DNA gymnastics will be profitless. Both the formation of a chicken foot and the template switch are enigmatic processes whose frequencies and mechanisms remain largely unknown.

Another way of dealing with arrested replication involves the formation of a DSB at, or close to, a stalled replication fork. DSBs will inevitably be created once an advancing replication fork encounters a ssDNA break in the template (Fig. 5a). Also, the long ssDNA regions, generated as a result of uncoupling the replicative polymerase and helicase (Fig. 4e), may be intrinsically fragile. Their spontaneous breakage could create a one-ended DSB at the stalled fork. In addition to this ‘passive’ fork collapse, DSBs can be actively generated by cleavage of a stalled replication fork by structure-specific endonucleases. Replication forks stalled by lesions that affect only one DNA strand, by inhibitors of DNA polymerases, or by depleting cellular dNTP pools can be converted to DSBs, although it is difficult to estimate which fraction of the affected forks is processed in this way, and which fraction is rescued via other pathways. In contrast, replication forks stalled at interstrand DNA crosslinks appear to require processing into DSBs in order to be restarted. Irrespective of the way they were created at the fork, such one-ended DSBs are substrates for HR machinery. Initiation of recombination reaction requires the presence of ssDNA overhang, which in some cases might already be present at the fork due to DNA unwinding by replicative DNA helicase. The ssDNA-RAD51 complex can then invade the intact homologous DNA molecule to re-establish the replication fork (Fig. 5b–f).

DSBs created at the stalled forks are dangerous DNA lesions, but their formation may be beneficial. In bacteria, such DSBs are suggested to recruit replication proteins to recombination intermediates, and therefore promote origin-independent replication restart [72]. Induction of DSBs in response to DNA damaging agents has been also observed in eukaryotic cells and may serve similar purposes, as well as facilitate subsequent repair processes [9, 73]. The mammalian structure-specific endonuclease Mus81-Eme1 is involved in generating such DSBs. In vitro Mus81-Eme1 cleaves branched DNA structures, e.g. 3' flaps and replication fork-like structures. Although the mechanism of action of Mus81 in vivo remains unclear, such substrate preference suggests two possible roles of Mus81 in damage

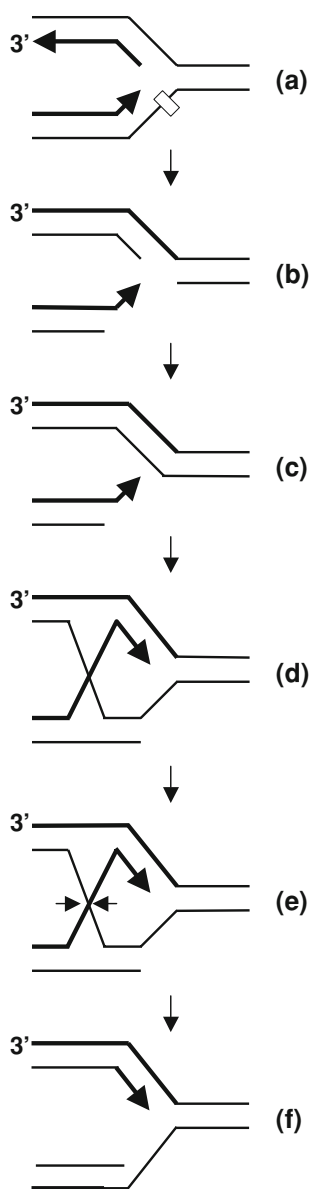


Fig. 5 A pathway of restarting collapsed replication forks. Introducing a DSB at a stalled fork converts it to a collapsed fork. The DSB can be made by a structure-specific endonuclease (not shown), or can be passively formed when the replication fork encounters a ssDNA break in the template (a). Initiation of HR requires the presence of ssDNA overhang. Depending on the lesion, the structure and the amount of ssDNA at the stalled fork, the overhang can be already present or has to be produced by end resection (b). After the intact template molecule is restored (c), it can be invaded by the broken DNA fragment (d). The invasion leads to the re-establishment of the active fork and leaves a single Holliday Junction behind (e), which can be resolved by a structure-specific endonuclease (f)

response: Mus81 might directly cleave stalled replication forks and generate DSBs that will be subsequently processed by the HR machinery. Alternatively, Mus81 could act upon recombination intermediates created at the arrested forks and thus help to resolve these intermediates.

It is worth noting that the recombination subpathways described above do not always remove the damage that caused fork stalling. Only in some cases, e.g. when a ssDNA break is encountered, or when the progression of the fork is hindered by shortage of dNTPs or presence of replication inhibitors, can the recombination machinery restore the intact, undamaged DNA molecule. When the fork is halted by an obstacle in the DNA template, e.g. benzo[a]pyrene adduct, or interstrand crosslink, HR can help to re-establish the fork, and, depending on the pathway employed, can bypass the damage, but will not remove it. Therefore, restarting of the stalled forks may in some cases require support of other DNA repair pathways.

Translesion Synthesis

Translesion synthesis (TLS) employs a specialized set of DNA polymerases to replicate over damaged DNA [74, 75]. The common characteristics of TLS polymerases are more open active sites which allow them to accommodate irregular template structures [76], and the lack of 3'→5' exonuclease proofreading activity [77]. These features allow the TLS polymerases to copy lesion-containing DNA, but, at the same time, make them intrinsically error-prone. TLS polymerases frequently introduce mutations by incorporating incorrect nucleotides on both damaged and undamaged templates, and therefore have to be precisely controlled.

Replication of damaged DNA is regulated by the genes in the *RAD6* epistasis group in *S. cerevisiae* [78]. Some members of this group encode enzymes involved in ubiquitin conjugation: Rad6 is an E2 ubiquitin conjugating enzyme, which acts together with Rad18, a RING finger-containing E3 ubiquitin ligase [79]. Another pair of E2/E3 enzymes consists of Mms2/Ubc13 (a ubiquitin-conjugating enzyme) and Rad5 (a RING finger ubiquitin ligase). This complex is unusual in that the poly-ubiquitin chains it forms are linked via internal lysine K63 of ubiquitin, rather than the more common K48 used for targeting proteins for proteasomal degradation [80, 81].

Recent discoveries have shown that the main target of these enzymes in the damage tolerance pathway is the replication processivity factor PCNA [79]. In response to DNA damage, Rad6 and Rad18 catalyze binding of a single ubiquitin moiety to the conserved K164 of PCNA. Multiple ubiquitin molecules can be subsequently added, forming a K63-linked poly-ubiquitin chain, in a reaction catalyzed by Rad5 and Mms2/Ubc13. Interestingly, the two modifications have different outcomes [82]. Poly-ubiquitination promotes error-free, recombination-mediated rescue of stalled replication forks. This process and the mechanisms of its regulation by PCNA poly-ubiquitination are poorly

understood. In contrast, PCNA mono-ubiquitination activates TLS. TLS polymerases pol η and pol ι preferentially interact with the mono-ubiquitinated form of PCNA via two evolutionary conserved ubiquitin-binding domains. Such domains are present in all Y-family TLS polymerases (REV1, Pol η , Pol ι and Pol κ) [83]. The increased affinity likely serves to recruit the TLS polymerases to the stalled replication forks [84, 85], although the molecular details of exactly how their recruitment to the template DNA occurs are still unclear. TLS polymerases could be constitutively associated with the replication machinery at the moving replication fork. The ability of TLS polymerases to bind PCNA, as well as co-localization studies showing that some TLS polymerases are present in replication factories in undamaged cells, are consistent with this possibility [86]. Mono-ubiquitination of PCNA at a stalled fork would increase its affinity for the TLS polymerases, which could induce protein rearrangements at the fork, resulting in a TLS polymerase gaining access to the 3' OH primer terminus. Alternatively, mono-ubiquitinated PCNA could also attract free TLS polymerases to the replication machinery after encountering a lesion in the DNA template. The absence of Pol η foci in Rad18-deficient cells favors this possibility [85].

Although the precise mechanism of polymerase switch is unknown, it is clear that the main event triggering it is the ubiquitination of PCNA. Ubiquitination is induced by UV light and benzo[a]pyrene [84, 87], which generate replication-stalling DNA lesions. Ubiquitination is also induced by hydroxyurea, which hinders replication by

depleting nucleotide pools, and not by introducing any physical damage to the DNA template [84]. This indicates that PCNA mono-ubiquitination is primarily activated by the presence of stalled replication forks. Levels of mono-ubiquitinated PCNA are also regulated by a de-ubiquitinating enzyme USP1. UV light-induced auto-cleavage of USP1 leads to the accumulation of modified PCNA and activates TLS [88]. TLS is an inherently error-prone process, and its rigorous control is critical to prevent unwanted mutations. Therefore, it is important to determine whether PCNA ubiquitination is actively regulated, for example by damage signaling. At present, there is no evidence for the regulation of TLS activity by checkpoint pathways. PCNA mono-ubiquitination is unaffected in cells deficient in Rad3 (*Schizosaccharomyces pombe* homolog of human ATR) and checkpoint signaling is normally activated in cells expressing PCNA K164R mutant [89]. It also remains unknown how UV light triggers inactivation of USP1. Neither ATM- and ATR-controlled damage response pathways, nor the nucleotide excision repair proteins seem to be involved in this process [88]. It is also possible that PCNA ubiquitination is a direct and passive consequence of arresting replication forks. In *S. cerevisiae* Rad18, the ubiquitin ligase catalyzing mono-ubiquitination of PCNA interacts not only with Rad6 and PCNA, but also with ssDNA [90]. As increased amount of ssDNA is present at a stalled replication fork, this interaction might be involved in recruiting Rad18 and Rad6 to its PCNA target.

Another yet unanswered question concerns the choice of the TLS polymerase that will bypass a specific lesion.

Table 1 Summary of properties of TLS polymerases present in higher eukaryotes

Y family

- | | |
|--------------|--|
| Pol η | Efficiently inserts correct nucleotides opposite UV light-induced cyclobutane thymine dimers (but not 6-4 photoproducts (6-4 PP)), and is responsible for their accurate bypass in vivo [110]. Its deficiency results in the variant form of xeroderma pigmentosum, characterized by extreme sensitivity to sunlight and increased incidence of skin cancer [111]. Pol η is constitutively localized in replication factories [112]. It also plays a role in HR by extending the invading strand in a D-loop structure [113, 114] |
| Pol ι | Has very low processivity and very high error rate. It inserts bases opposite some types of damage, e.g. 6-4 PP and abasic sites, but is unable to extend from the inserted base [115]. Pol ι is found in replication factories [86]. Its precise function is unknown |
| Pol κ | Can extend terminal mismatches on undamaged templates and bypass benzo[a]pyrene adducts [116, 117] and has been proposed to function as an extender in bypass of UV light-induced lesions [91]. Pol κ -deficient cells are sensitive to benzo[a]pyrene and methyl methanesulfonate [118]. Pol κ is localized to replication factories only in a fraction of S-phase cells [119] |
| Rev1 | Is a dCMP transferase [120]. It inserts dCMPs opposite either guanines or abasic sites, and is able to bypass 6-4 PP and abasic sites. REV1 protein, but not its catalytic activity, is required for damage-induced mutagenesis. Via its C-terminal region REV1 interacts with other Y-family polymerases and with REV7 [94–96] |

B family

- | | |
|-------------|--|
| Pol ζ | Is composed of REV3 (catalytic) and REV7 (regulatory) subunits [121]. It is required for damage-induced mutagenesis and for bypass of 6-4 PP and abasic sites. It is efficient in extending terminally mismatched primers or primer termini opposite lesions, and therefore is supposed to be involved in the extension step of TLS [91] |
|-------------|--|

In addition to polymerases listed here, other, yet less characterized TLS polymerases (Pol θ , Pol ν , Pol λ , and Pol μ) could also be involved in lesion bypass

Eukaryotic cells express a number of TLS polymerases, which have different properties and different substrate preferences (summarized in Table 1). The current “trial and error” model predicts that they simply compete for binding to ubiquitinated PCNA and either insert nucleotides or fall off if they cannot cope with the damage, and leave the stage for another candidate [74]. The choice of the polymerase is even more complicated by the fact that cooperation of two different enzymes is often needed to overcome certain lesions. The two-step model of lesion bypass comprises incorporation of a limited number of nucleotides directly opposite the damage by the first TLS polymerase, followed by extension from the inserted nucleotides by the second TLS polymerase [91, 92]. Because the damage is present in the template DNA, the first insertion will likely generate a mismatched or distorted primer terminus. Replicative DNA polymerases are not able to extend such a structure and will degrade it using their 3′→5′ exonuclease activity. Therefore, extension by a second TLS polymerase, which lacks the exonuclease activity and can tolerate abnormal primer termini, is probably critical for efficient TLS. However, this scenario poses another question: Whether or how are the polymerases programmed to act in a specific order? The ways of controlling their reorganizations remain to be discovered, but it is possible that ubiquitination of TLS polymerases themselves [83, 93] could be involved in this process. Ubiquitinated PCNA might serve to attract the first TLS polymerase, which will insert nucleotides opposite the damage site. The subsequent ubiquitination of this polymerase would create the binding site for the ubiquitin-binding domain of the second TLS polymerase, which could then extend the DNA molecule past the lesion. REV1, a dCMP transferase belonging to the Y-family of TLS polymerases, could also be involved in controlling and/or facilitating polymerase switching. REV1 physically interacts with all main TLS polymerases: Pol η , Pol ι , Pol κ , and the REV7 subunit of Pol ζ , and therefore it was proposed to form a platform for recruiting and/or rearranging the polymerases at the stalled forks. Consistently, REV1, but not its catalytic activity, is required for damage-induced mutagenesis [94–96].

Once the DNA fragment opposite the lesion has been extended enough not to be susceptible to removal by exonucleolytic proofreading, the high-fidelity replicative polymerases should take over DNA synthesis (Fig. 6). This raises another important question of how the action of a TLS polymerase at the replication fork is terminated, and how replicative polymerases regain access to the 3′ OH end of the growing DNA molecule. Since TLS is activated by ubiquitination of PCNA, it could be expected that it is switched off by removing ubiquitin from PCNA. However, levels of ubiquitinated PCNA increase after UV light

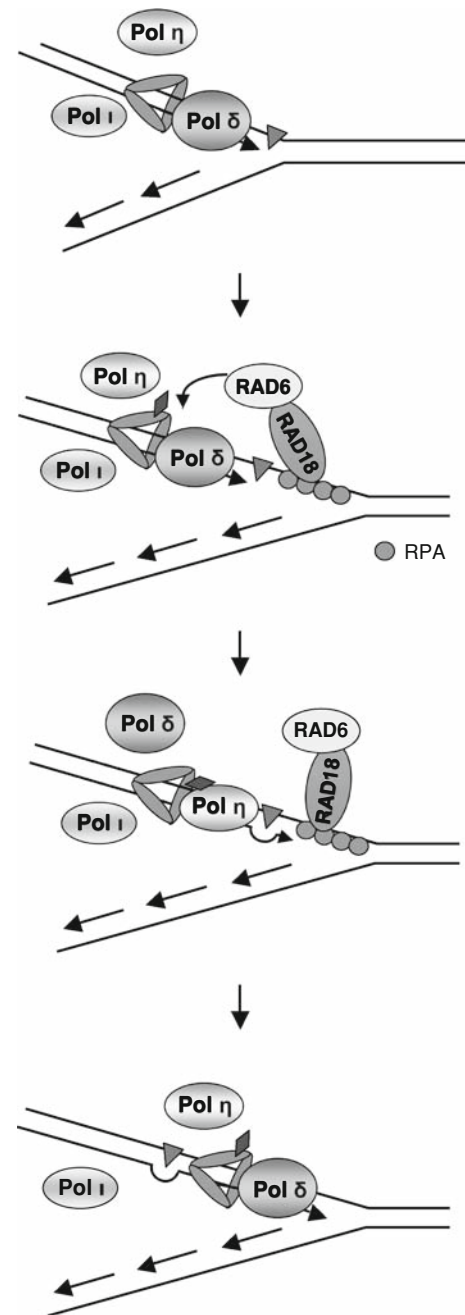


Fig. 6 Model of translesion synthesis. A replicative polymerase (e.g. Pol δ) stalls at a lesion in the DNA template. The RPA-coated ssDNA generated at the damage site might help to recruit Rad18/Rad6, which in turn ubiquitinate PCNA. PCNA ubiquitination induces polymerase switch, as a result of which a TLS polymerase (e.g. Pol η) gains access to the template and synthesizes a short fragment of DNA opposite the lesion. The second polymerase switch places a replicative polymerase at the primer terminus and allows it to continue high fidelity DNA synthesis

irradiation, and stay elevated for more than 24 h [84]. It is currently unknown whether this reflects the duration of PCNA modification at an individual fork, or whether this represents the equilibrium between de-ubiquitination at the

already restarted forks and ubiquitination at the forks that have just encountered a lesion. The latter possibility is perhaps more likely, since it might be dangerous to maintain the modification that attracts error-prone polymerases to the template. It is also possible that TLS polymerases dissociate from the template because of their inherently poor processivity. TLS polymerases generally synthesize short DNA fragments, e.g. pol η inserts only 1–8 nucleotides on any template [97]. So, after synthesizing such a short piece, they could simply fall off the DNA. It is unclear though how the “falling off” happens—Do the TLS polymerases dissociate, or are they still attached to PCNA, and only re-group within the replication complex? It is also unknown what happens to the replicative polymerases during the TLS process. As yet there is no direct evidence whether or not their affinity for PCNA is affected by its mono-ubiquitination.

Translesion DNA Synthesis Versus Homologous Recombination

The damage tolerance systems consist of error-prone TLS and error-free recombination-mediated pathways, activated by mono- and poly-ubiquitination, respectively. While the components and activities of these two pathways are relatively conserved, their relative contributions to overcoming replication blocks differ significantly among eukaryotes. In yeast both mono- and poly-ubiquitination are readily detectable. In mammalian cells, mono-ubiquitination is the main UV light-induced PCNA modification. Poly-ubiquitination is detectable at significantly lower levels, but, as in yeast, poly-ubiquitination is involved in the error-free pathway of lesion bypass [98, 99]. It might be surprising that mammalian cells seem to favor the error-prone option of damage bypass. However, HR also potentially poses a threat to genome stability. It does not induce point mutations, but, if misregulated, can cause more dangerous types of damage, e.g. DSBs, deletions, duplications, and translocations. Since mammalian genomes are much larger than those of bacteria or yeast, and contain many more repetitive sequences, HR in mammalian cells might be associated with relatively higher risk and therefore might be much more limited.

RecA-mediated HR is the main pathway dealing with stalled replication forks in bacteria. However, in addition to its mechanistic role during homology search, RecA also plays a crucial role in inducing a global damage response, termed the SOS response, which includes TLS-mediated mutagenesis. The presence of damage-induced RecA filaments assembled on ssDNA is the main trigger for autodigestion of the transcription repressor LexA. Inactivation of LexA results in the upregulation of more than 40

genes involved in DNA repair and regulation of the cell cycle, the so-called SOS response [100]. UmuD and UmuC, whose expression is thus induced, form a UmuD₂C complex. Its RecA-induced autocleavage leads to the formation of a catalytically active UmuD₂'C, which is the main bacterial TLS polymerase—Pol V [101]. Strikingly, not only the formation of active Pol V enzyme depends on RecA. Both genetic and biochemical experiments proved that the presence of RecA is required for lesion bypass activity of Pol V [102–104]. The finding that overexpression of UmuD₂'C inhibits RecA-catalyzed recombination suggests that the dependence might be mutual, and that the interaction between Pol V and RecA could act to favor the TLS pathway [105, 106]. Interestingly, RecA expression is induced very rapidly, while UmuD₂'C levels increase relatively late during the SOS response [107]. This difference in timing suggests an elegant way of regulating the order in which TLS and HR act: the RecA-mediated recombination is the preferred mechanism, and is active early during the SOS response. In case it cannot cope with the damage, TLS is subsequently activated and, by inhibiting HR, it takes over the task of rescuing the stalled forks.

Similar links between HR and TLS probably exist in eukaryotic cells. However, despite significant progress in understanding how both pathways work, many questions remain about how they are interconnected or interdependent. Some replication-blocking lesions can be bypassed by both HR and TLS, but it is unknown what determines which pathway will be used in a given situation and whether or not there is a specific order in which these pathways are employed. Mono-ubiquitination by Rad6/Rad18 proceeds poly-ubiquitination by Rad5/Mms2/Ubc13, but does this reflect the order TLS and HR might act? It is also unclear whether RAD51 or assembled RAD51 filament is required for lesion bypass activity of eukaryotic TLS polymerases. Thus, there are many important and interesting challenges waiting to be solved to get a more complete grasp of how the mutual interplay among checkpoint pathways, TLS and HR leads to accuracy in genome duplication.

Future Perspective

A significant percentage of replication forks experiences stalling or collapse during every S phase. Efficient response to such problems involves activating DNA damage checkpoints that signal the presence of unusual DNA structures, and mechanisms promoting fork restart: translesion synthesis and homologous recombination. Despite great progress in understanding how cells respond to and solve DNA damage-induced replication problems, many molecular details of the mechanisms promoting replication restart

remain unknown. Likewise, the interplay between different restart pathways and factors that influence which pathway will be employed in a given situation await discovery. Recent advances in life cell imaging and single molecule techniques [108] might make it possible to follow events at a individual replication forks both in vivo and vitro and the synthesis of the results obtained through these diverse approaches should take our understanding of DNA damage-induced replication restart pathways to a new level.

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