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SURVEY AND SUMMARY

Mechanisms of damage tolerance and repair during DNA replication

Mohamed Elsaid Ashour and Nima Mosammaparast*

Department of Pathology & Immunology, Washington University in St. Louis School of Medicine, St. Louis, MO 63110, USA

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ABSTRACT

Accurate duplication of chromosomal DNA is essential for the transmission of genetic information. The DNA replication fork encounters template lesions, physical barriers, transcriptional machinery, and topological barriers that challenge the faithful completion of the replication process. The flexibility of replisomes coupled with tolerance and repair mechanisms counteract these replication fork obstacles. The cell possesses several universal mechanisms that may be activated in response to various replication fork impediments, but it has also evolved ways to counter specific obstacles. In this review, we will discuss these general and specific strategies to counteract different forms of replication associated damage to maintain genomic stability.

INTRODUCTION

The efficient duplication of genetic information demands the unimpeded progression of the replication fork. DNA lesions and physical barriers pose great threats to the faithful completion of replication. Thus, the cell has developed overlapping DNA damage repair and tolerance mechanisms to ensure that these obstructions do not result in genomic instability.

In most cases, lesions are not absolute impediments to replication fork progression. Replisomes can replicate over them, switch template strands, switch polymerases (Pols), reprime, or pause to give more time for repair. Moreover, replisome components are flexible, where functional uncoupling can occur between helicases and Pols, as well as leading and lagging strand Pols (1,2). Such dynamics built into the replication machinery represent the earliest tolerance mechanisms for template damage. However, due to the high number of challenges to replication, the eventual collapsing of the fork, defined as losing the capacity to continue DNA synthesis, is inevitable. This is where multiple DNA repair pathways are engaged based on the type of damage.

Some of the common types of lesions encountered by replication forks are ribonucleotides, base lesions, cyclobutene pyrimidine dimers (CPDs), interstrand crosslinks (ICLs), DNA–protein crosslinks (DPCs), and R-loops. These impediments represent a serious problem, particularly during replication, due to two reasons. First, although repair pathways may still remove the damage during replication, their uncontrolled action could be detrimental, since the DNA in the vicinity of replication fork is single-stranded DNA (ssDNA). Excising the lesion from ssDNA, as would be the case in base excision repair (BER), nucleotide excision repair (NER), and ribonucleotide excision repair (RER), would result in a DNA break and fork collapse. Second, the replication machinery itself potentially transforms the latent DNA damage into a more deleterious form. For example, ssDNA breaks are converted to double-stranded breaks (DSBs) upon encountering the progressing fork (3), and the collisions of replication machinery with trapped topoisomerases generates DSBs by 'replication run-off' (4). These replication-associated DSBs are single ended, requiring recombination for their repair.

The dynamics of replication, coupled with lesion skipping, translesion synthesis (TLS), template switching (TS), and fork reversal are shared strategies to avoid much of the replication associated DNA damage, ranging from small base lesion to large DPCs (Figure 1). Although these shared strategies are enough to counter most replication associated damage, specific types of damage still require further processing. In this review, we will first describe the general strategies to deal with established forms of replication associated damage. Then, specific requirements to deal with genomic ribonucleotides, R-loops, DPCs and ICLs during replication will be considered in greater detail.

*To whom correspondence should be addressed. Tel: +1 314 747 5472; Email: nima@wustl.edu

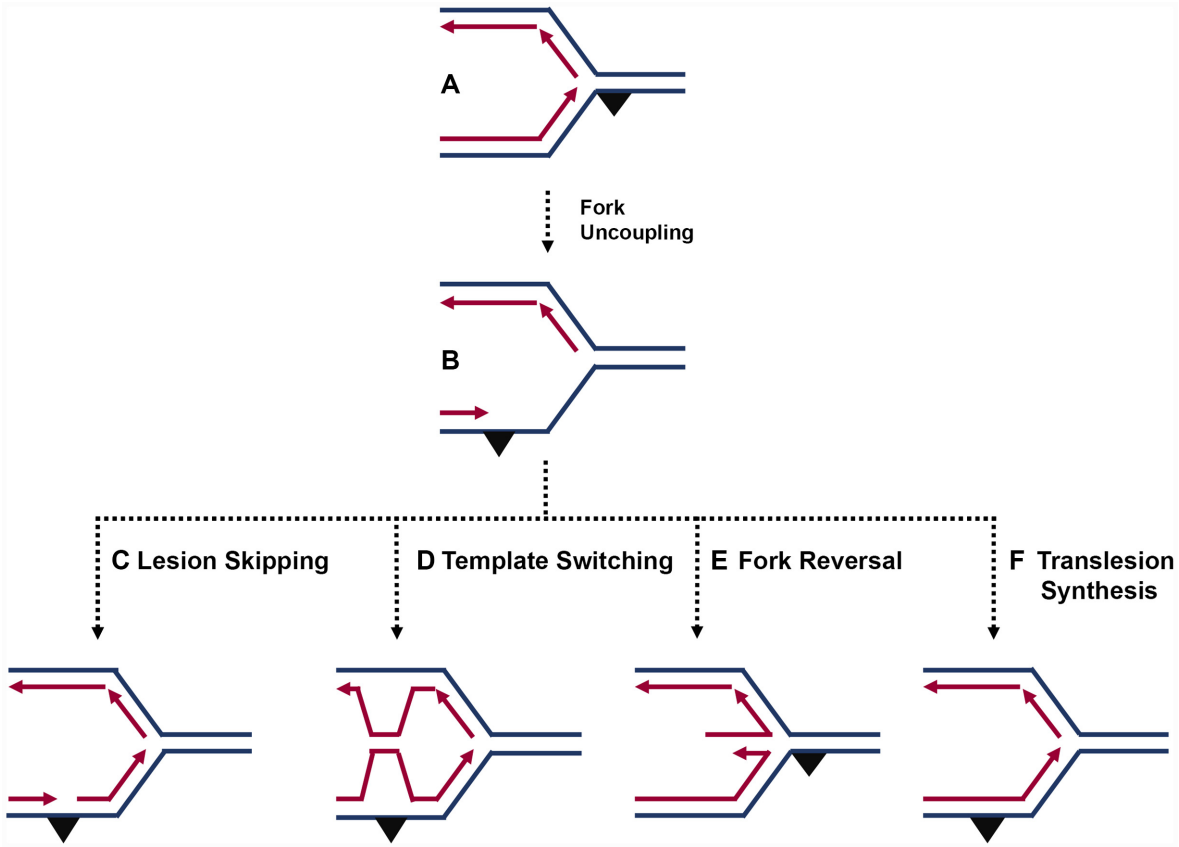

Figure 1. General strategies to bypass and repair DNA damage during replication. (**A**) A DNA lesion (black triangle) on the leading strand stops replication fork movement. (**B**) The DNA damage leads to the functional uncoupling of DNA polymerases and the replicative helicase, since the helicase can bypass the lesion without association with the polymerase. Several pathways are employed to bypass or repair the damage through (**C**) lesion skipping, where repriming occurs downstream the lesion; (**D**) template switching, where the newly synthesized DNA strand is used as the template; (**E**) fork reversal, where the nascent strands reanneal, giving the chance for DNA repair pathways to remove the damage without collapsing the replication fork; (**F**) or translesion synthesis, where specialized TLS polymerases temporarily replace the replicative polymerases to bypass the lesion.

COMMON STRATEGIES TO DEAL WITH REPLICATION BLOCKS

Replisome-intrinsic features

The presence of lesions on leading and lagging strands have different consequences for replication. In general, damage in the lagging-strand template is more tolerable due to the frequently initiated Okazaki fragments that delay the processing of the damage, leaving gaps behind that can be filled in post-replication (2,5). On the other hand, damage in the leading strand is more problematic because of its continuous nature of replication. This raises a question of how the cell coordinates between leading and lagging strand synthesis during repair occurring in one strand. It has been assumed that DNA synthesis on both strands must be coordinated to avoid strand uncoupling and ssDNA accumulation, yet this dogma has been recently challenged. Investigation of *Escherichia coli* DNA replication has revealed that polymerases act fully independently, and such coordination is absent (1). Similarly, human DNA polymerases have also been shown to function independently *in vivo*. After reducing the rate of lagging strand synthesis, cells sustain persistent levels of strand uncoupling and ssDNA accumulation without activation of replication checkpoint signaling (6). Therefore, this independent and stochastic behavior suggests that at any given time the leading strand synthesis could be slower or faster than the lagging-strand synthesis. Although the mechanisms behind this are not yet clear, these studies highlight the unexpected flexibility of the replication machinery to tolerate DNA damage.

In addition to leading-lagging polymerase uncoupling, leading strand lesions cause the replicative CMG (CDC45-MCM-GINS) helicase and DNA synthesis to become functionally uncoupled, because CMG can bypass the damage while the polymerase is paused. This event generates ssDNA as the leading-strand template is exposed (7–9) (Figure 1A and B). During polymerase pausing, DNA unwinding continues but at markedly reduced rate (~20% of normal), preventing CMG helicase from running far away from the polymerase and generating excessive ssDNA. This fail-safe mechanism is called the 'dead man's switch' (1). Consistently, a recent report has suggested that the act of leading strand polymerization by itself increases template unwinding rate by CMG (10). While the underlying mechanism is unknown, the nascent leading strand may prevent backtracking of the CMG. This provides an additional fail-safe to the 'dead man's switch' to limit excessive uncoupling that can lead to significant ssDNA accumulation.

Polymerase repriming, template switching, and fork reversal

After induction of polymerase-stalling damage such as UV-induced thymine dimers, discontinuous DNA synthesis occurs on both the leading and lagging strands in *Saccharomyces cerevisiae*, suggesting that repriming downstream of the lesions is a commonly used option during replication (7). The new primer allows DNA synthesis to resume, leaving behind a ssDNA gap (Figure 1C). *Escherichia coli* reinitiates leading strand synthesis downstream of the damage via DnaG-dependent repriming (11,12), while in metazoans, the repriming at the leading strand preferentially uses a specialized primase called PrimPol (13–16). The mechanisms responsible for activation of repriming are largely unknown, although a recent report has found that cells adapt to repeated cisplatin exposure by increased expression of PrimPol under certain conditions (16). PrimPol interacts directly with RPA, and it is possible that increased RPA-coated DNA, which would signal the presence of markedly damaged template, is the primary trigger to recruit Primpol (17,18). An alternative mechanism of avoiding a damaged DNA template is template switching (TS). This is an error-free mode of DNA synthesis that allows the stalled replication fork to use the newly synthesized strand as the template to avoid the lesion. TS activity is regulated by ubiquitination and SUMOylation of PCNA, and involves recombination between the nascent sister strands (19,20). Upon initiation of template switching, a DNA Pol extends the stalled nascent DNA along the nascent sister strands (Figure 1D). The paired nascent strands can be resolved by a dissolution mechanism akin to termination of homologous recombination (21). In budding yeast, it has been demonstrated that replicative helicase-coupled repriming by Polα/Primase facilitates TS. As a result, defects in Polα/Primase cause defects in strand annealing and reversed fork formation (22).

Another mechanism to deal with common template obstructions is fork reversal. Fork reversal results in reannealing of the nascent strands without further DNA Pol activity, leading to the formation of a 'chicken foot' structure (Figure 1E). Replication fork reversal (i) allows additional time for repair, (ii) protects ssDNA at the stalled fork, (iii) could serve as a mechanism of TS and (iv) avoids the collision of replication machinery with DNA lesions present ahead of the fork (23). It is estimated that about 15–30% of the forks are reversed after treatment with various genotoxic agents. This suggests that fork reversal is a global response to replication stress (24). Fork reversal is mediated by several proteins including ZRANB3, SMARCAL1, HLTF, FBH1, WRN, RAD54, FANCM, as well as RAD51 (25,26). It is largely unknown if they act in coordination with each other, or independently in different regions of the genome, or in different damage contexts. Once a reversed fork is formed, it creates a DSB that needs to be protected from nucleases, such as MRE11 and DNA2, which degrade and collapse the reversed fork. This protection is generally thought to be RAD51-dependent (27–29).

Besides its role in fork protection, RAD51 promotes fork reversal (24,29), and mediates the restart of stalled or collapsed forks (30). Surprisingly, a recent study demonstrated that RAD51 DNA binding activity alone is sufficient for both replication fork reversal and protection, and only the strand exchange enzymatic activity is required for replication fork restart (31). These roles of RAD51 at the fork could be positively or negatively regulated, and many of the canonical factors involved in Rad51-mediated homologous recombination (HR) are also involved in fork protection. For example, BRCA2 functions during HR by displacing the single-stranded DNA-binding protein, RPA, with RAD51 to promote the strand exchange reaction (32). Moreover, BRCA2 protects stalled replication forks from extensive nucleolytic degradation through stabilization of the RAD51 filament (28). Besides BRCA1 and BRCA2, which promote RAD51 recruitment, RAD51 paralogs, MMS22L–TONSL, and BOD1L also positively support RAD51 function in fork protection (33–35). The BRCA1 antagonist, 53BP1, has also been reported to play a role in fork protection, although this was not observed consistently between various groups (36–39). Recently, this contradiction was resolved by showing that the role of 53BP1 in fork protection is dependent on the chronic versus acute inactivation of 53BP1 (40). This suggested that distinct molecular pathways may exist for fork protection; indeed, the requirement of fork protection proteins depends on the pathway used in fork remodeling. Specifically, BRCA2, FANCD2, and ABRO1 protect forks generated by SMARCAL1, ZRANB3, and HLTF, whereas 53BP1, FANCA, FANCC, FANCG, BOD1L and VHL protect forks remodeled by FBH1 (40). Other proteins, including RADX, FBH1 and RAD52 prevent excessive fork reversal by negatively regulating RAD51 activity (41–43). This illustrates the importance of restricting RAD51 activity at forks, because RAD51 overexpression promotes excessive fork reversal, leading to fork degradation and DSBs (42,44).

In addition to the nucleases that degrade nascent DNA strands during fork reversal, other structure-specific nucleases may process stressed forks following prolonged stalling (45). Therefore, the requirement for the fork protection from nucleases is not limited to fork reversal. For example, cancer cells with microsatellite instability are synthetically lethal with defects in WRN helicase (46,47). Recently, Nussenzweig and colleagues have revealed that the TA repeats form secondary structures that stall replication forks and require unwinding by the WRN helicase. In the absence of WRN, the TA-dinucleotide repeats are cleaved by MUS81 nuclease, leading to double strand breaks, DNA end resection, RPA exhaustion, and cell death (48).

Translesion synthesis (TLS)

When replication forks stall, low fidelity TLS Pols transiently replace the replicative Pols to help bypass the lesion (Figure 1F). Mammalian cells have at least seven enzymes with TLS activity, including four Y-family Pols (η, ι, κ and REV1), one B-family Pol (ζ), and two A-family Pols (θ and ν). In general, TLS Pols have larger catalytic pockets that allow them to accommodate templates lesions. Furthermore, TLS Pols lack 3′-5′ proofreading domains (49). Therefore, TLS Pols confer damage tolerance at the cost of reduced replication accuracy. Although TLS Pols are inherently error prone, certain TLS Pols can mediate error-free repair of specific lesions. For example, TLS Polη accurately bypasses UV-induced CPD lesions (50). Accordingly, mutations in

the gene encoding Polη result in a genetic predisposition to skin cancer (51). Other TLS Pols, such as Polθ, Polι, Polκ and Polζ make errors while replicating UV-induced lesions (49). Due to the mutagenic potential of Polθ, it was suggested to contribute to skin-cancer development. Surprisingly, Prakash and colleagues have found that Polθ plays a protective role against skin cancer caused by UV. This is attributed to the role of Polθ in promoting replication fork progression in response to UV, which counteracts the formation of DSBs and genome rearrangements. This suggests that both error-prone and error-free TLS Pols may act as effective barriers to genomic instability and tumorigenesis (52). Similar to the mechanisms described above, PCNA monoubiquitination appears to be a key component regulating TLS polymerase recruitment (53), yet how the cell 'chooses' which TLS Pol is activated at any specific time is largely unclear.

How lesion skipping, TLS, TS and fork reversal are regulated, and how a replisome activates one mechanism over another when encountering a particular type of lesion is still largely unknown. Recent evidence suggests that fork reversal and repriming may be mutually exclusive pathways (16,54). In support of this, UV damage induces an increase in RAD51 loading onto chromatin in PrimPol depleted cells (13), whereas the same type of damage induces excessive elongation of nascent DNA by PrimPol in RAD51-depleted cells (55). More recently, HLTF has been shown to activate replication fork reversal and to prevent alternative tolerance mechanisms. Interestingly, while the loss of HLTF makes the cells rely on the PrimPol for unrestrained replication, defect in the HLTF DNA binding HIRAN domain makes cells rely on translesion synthesis (54). RAD52 could also play a role in regulating the switch between fork reversal and repriming (43) Together, these data strongly suggest an antagonistic interplay between the pathways involved in maintaining replication fork dynamics during damage.

Replication fork restart

Dormant origins are licensed origins (i.e. have loaded MCM helicase) that are not activated during replication. They serve as an important backup to restore replication when forks are stalled. The high number of dormant origins in mammalian cells reduces the necessity of replication fork restart (56). Moreover, a reversed fork could just simply merge with a converging fork. Nevertheless, fork restart does occur in mammalian cells. This restart could be achieved by repriming (discussed above), helicases, or break-induced replication (BIR).

Specific helicases, such as RECQ1, WRN and BLM, have important roles in reversed fork restart. RECQ1 promotes fork reversal restart, an activity that is negatively regulated by PARP1 to prevent unscheduled fork reversal restart to give more time for repair (23). Moreover, WRN and DNA2 induce the resection of regressed arms, leading to fork restart (57).

Alternatively, if a fork encounters a ssDNA break or other type of damaged template that collapses the fork, BIR, a unique type of HR mechanism, can be employed. Similar to other HR mechanisms, BIR requires extensive end processing to generate a 3′ ssDNA end that allows RAD51-ssDNA filament formation. RAD51 invades the homologous template to generate a displacement loop (D-loop) that allows replisome assembly and DNA synthesis. Unlike HR, which typically involves small regions of DNA synthesis, BIR engages in extensive DNA replication for many kilobases of DNA until the end of the chromosome (58). There are many differences between canonical replication forks and those established during BIR. First, unlike canonical replication, BIR involves a single ended DNA that acts independently, and progresses via a migrating bubble or D-loop (58). Second, BIR synthesis is asynchronous; the leading strand is synthesized as ssDNA, then the lagging strand uses the leading strand as the template (59–61). Third, BIR assembles a modified replisome, where an additional Polδ subunit, human POLD3, is added, and the DNA helicase PIF1 activity is required (60–62). In yeast, the Srs2 helicase is also required to prevent the formation of toxic structures during the invasion of leading strand into the DNA template (63). Fourth, compared to canonical DNA replication, BIR is associated with loss-of-heterozygosity, high mutation rates, and chromosomal rearrangements (58). This mutagenic synthesis is due at least in part to frequent dissociations of modified Polδ from the template (58,64,65), and the reduced efficiency of mismatch repair during BIR (66). BIR synthesis is either rescued by a replication fork coming from the opposite direction or terminated by MUS81 cleavage of the recombination intermediate (31,67,68). Although canonical BIR is RAD51-dependent, RAD51-independent BIR has also been identified. The exact mechanism of RAD51-independent BIR is unknown, and the relative contributions of RAD51-dependent and independent BIR to replication restart are currently unclear (58).

The above tolerance and repair strategies can be deployed to deal with virtually all types of DNA damage during replication. Yet, these are insufficient to maintain genome stability. In the next section, we will discuss the additional requirements to deal with specific types of damage during replication, which are commonly encountered during normal replication or during the presence of certain genotoxins.

GENOMIC RIBONUCLEOTIDES AND R-LOOPS

Ribonucleotide triphosphates (rNTPs) and deoxyribonucleotides triphosphates (dNTPs) are the precursors for RNA and DNA, respectively. The extra 2′-OH group in the ribose makes RNA relatively unstable, because it can potentially mediate nucleophilic attack of the sugar-phosphate backbone, generating a break. RNA is frequently embedded or annealed to genomic DNA, where it interferes with replication, inducing DNA damage and genomic instability. Paradoxically, DNA replication is the main source of genomic ribonucleotides, and ribonucleotide incorporation is the largest fraction of all endogenous DNA 'lesions' (Figure 2).

Ribonucleotide bypass by replicative and TLS polymerases

A major issue with ribonucleotides in DNA is that replicative Pols in yeast and human are inefficient at bypassing them when they are present in the template strand. More-

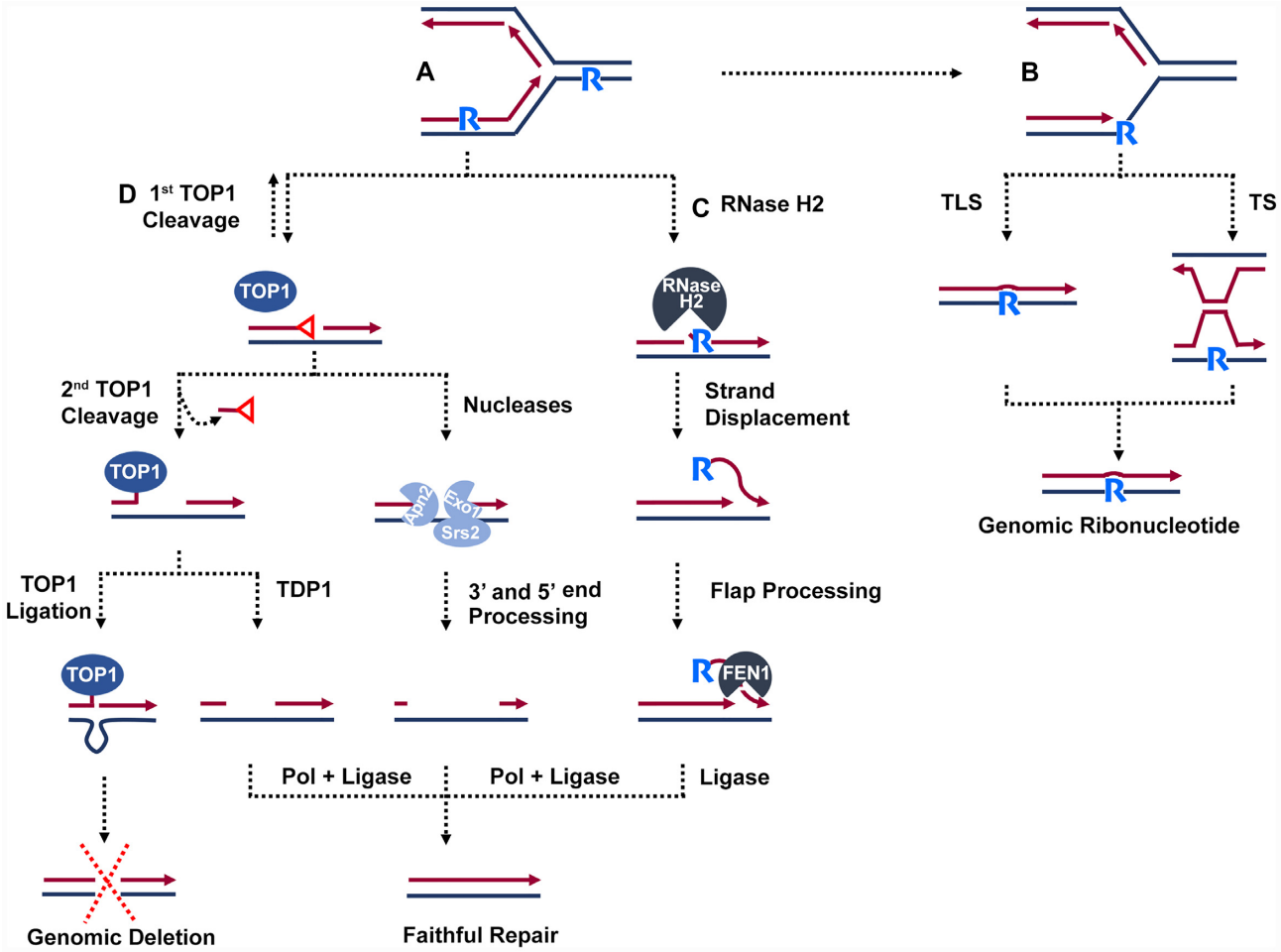

Figure 2. Genomic ribonucleotide repair and bypass during replication. (**A**) DNA polymerases incorporate ribonucleotides (blue 'R') during replication which can be bypassed, removed by ribonucleotide excision repair (RER), or processed by topoisomerase I (TOP1). (**B**) Left unrepaired, a replication fork would encounter a ribonucleotide in the template strand. The replicative DNA polymerases are inefficient in bypassing template ribonucleotides, leading to replication stress. In this case, translesion synthesis (TLS) or template switching (TS) are activated to bypass the ribonucleotides to complete replication. (**C**) During RER, RNase H2 incises 5′ to the embedded ribonucleotide, DNA polymerase δ generates a flap which is nucleolytically processed by FEN1, followed by ligation, leading to error-free repair. (**D**) In the absence of RER, TOP1 mediates the removal of genomic ribonucleotides. TOP1 incises 3′ to the embedded ribonucleotide. Then, nucleophilic attack by the 2′-OH group on the ribose generates a 2′,3′-cyclic phosphate and releases TOP1. The 2′,3′-cyclic phosphate can be reversed by TOP1 or removed by a second TOP1 cleavage, or by various nucleases. Alternatively, the trapped TOP1 can be removed in a manner which may lead to a small deletion, or via TDP1 in an error-free manner.

over, the bypassing capability decreases as the number of consecutive template ribonucleotides increases. For example, the efficiency decreases from 70% to 35% for Polδ and from 66% to 3% for Polε as the number of ribonucleotides increases from 1 to 3 (69–71). Similarly, physiological levels of rNTPs inhibit mtDNA synthesis by Polγ (72). Therefore, genomic ribonucleotide accumulation induces replication stress, leading to DNA breaks and genomic instability, which has been observed in yeast and human cells (73–79). The replication stress could also be induced indirectly by DNA breaks generated by spontaneous hydrolysis or by TOP1-mediated ribonucleotide cleavage, which will be described below.

TLS and TS are important means of bypassing template ribonucleotides (Figure 2) and are activated in RNase H1/2 depleted cells (74,76). The TLS polymerase Polζ can bypass ribonucleotide-containing DNA and efficiently copies DNA templates containing four consecutive ribonucleotides (76). Moreover, oxidized ribonucleotides, like 8-oxorG, in DNA could be more problematic for replication, because they strongly block primer extension by Polα. Interestingly, TLS polη can bypasses both undamaged and damaged ribonucleotides (80). These results suggest that TLS Pols may act as genomic ribonucleotide tolerance mechanisms. On the other hand, TLS Pols can use rNTPs as a substrate to bypass certain lesions. Polι was reported to bypass abasic sites and 8-oxo-G lesions using rNTPs as substrates (81). Moreover, under limited dNTP pools triggered by hydroxyurea (HU), robust ribonucleotide incorporation is mediated by human TLS Polη during TLS of CPD, 8-oxo-G, 8-met-G and a cisplatin intrastrand guanine crosslink. These results suggest that Polη can contribute to the accumulation of genomic ribonucleotides (82,83). In the presence of RNase H, this may be an acceptable compromise. However, in the absence of RNase H activity, Polη activity becomes increasingly cytotoxic. Consistently, the deletion

of Polη rescues the HU sensitivity of RNase H deficient cells (84).

Ribonucleotide excision repair (RER)

RER, which is considered to be error free, is the primary pathway for the removal of genomic ribonucleotides, and is mediated by RNase H (Figure 2). The eukaryotic RNase H family consists of the monomeric RNase H1 and the trimeric RNase H2 (A, B and C subunits) (85). RNase H1 requires at least a stretch of four consecutive rNMPs to cleave an RNA-DNA hybrid. While it is not required during canonical RER, its activity is essential for mtDNA replication and R-loop removal (86,87). On the other hand, RNase H2 can act both on stretches or single incorporated rNMPs in the genome (87). RNase H2 incises the DNA on the 5′ side of the ribonucleotide. Then, Polδ (or, less efficiently Polε) performs strand displacement synthesis, creating a flap that is subsequently removed by FEN1 or EXO1. The nick is sealed by DNA ligase (87). Mammalian RNase H2 is recruited to replication forks through the interaction of RNASEH2B and PCNA as well as the catalytic site of RNASEH2A (88,89). Both yeast and mammalian cells lacking RNase H2 activate replication stress signaling and have higher genomic instability (73–79). Moreover, cells lacking RNase H2 accumulate high levels of micronuclei that lead to cGAS-STING-mediated inflammatory signaling (90). Due to the role of ribonucleotides in inducing replication stress, ATR inhibition is synthetically lethal in RNase H2 deficient cells (91), highlighting one of the many essential functions of ATR in response to replication stress.

In the absence of RER, topoisomerase 1 (TOP1) mediates ribonucleotide cleavage, creating mutagenic 2′,3′-cyclic phosphate ends (Figure 2). TOP1 cleaves the DNA at the 3′-side of a ribonucleotide, generating a covalent TOP1-DNA complex, which is often referred to as the topoisomerase cleavage complex (TOP1cc). Subsequently, the 2′-OH of the ribose sugar attacks the phosphotyrosyl linkages of TOP1cc. The resulting nick flanked by the 2′,3′-cyclic phosphate and 5′-OH is not capable of ligation (92,93). Several scenarios have been suggested for the processing of this nick. First, the reversible re-ligation of the nick by TOP1 allows a second attempt of the excision repair (94,95). Second, TOP1 can initiate a second cut on DNA two base pairs upstream of the nick, releasing rNMP-dNMP dinucleotide. In this case, the TOP1cc could be released by TDP1, leaving behind a gap that can be repaired in an error-free manner (95). Alternately, TOP1 may re-ligate the DNA backbone, causing deletions (92,94,95). On the other hand, processing of the 5′-OH via Srs2 helicase and Exo1 nuclease, and the 2′,3′-cyclic phosphate by the abasic endonuclease Apn2 creates a gap that inhibits TOP1-mediated ligation, thereby reducing the risk of mutations (96,97). Third, TOP1 can cut the DNA strand opposing the rNMP, creating a DSB (98). Thus, it is clear that TOP1-dependent ribonucleotide excision is potentially highly mutagenic, leading to gene deletions and chromosomal rearrangements (78,92,94,98). Interestingly, in the absence of RNase H2, ribonucleotide cleavage by TOP1 results in lesions that trap PARP1 by PARP inhibitors, leading to cell killing. This highlights the potential therapeutic value of PARP1 inhibitors in the treatment of metastatic prostate cancer and chronic lymphocytic leukemia where RNase H2 is frequently deleted (79).

Replication block by R-Loops and its repair mechanisms

During transcription, newly transcribed RNA can hybridize with the transiently accessible template strand downstream of the transcriptional machinery, forming a structure called an R-loop. Although R-loops play essential physiological functions, they contribute to genome instability, particularly when replication forks collide with the transcription machinery. Thus, tight R-loop regulation must be maintained. Factors that function in the transcription process are essential in preventing excessive R-loops formation. For example, SRSF1 and THO/TREX complex, which mediate mRNA processing, prevent R-loop formation (99,100). Topoisomerases relax negative supercoils formed behind the transcriptional bubble to reduce the access of mRNA to template DNA, reducing R-loop accumulation (101). Once formed, different factors act to remove R-loops, including RNase H1 and H2, which degrade the hybridized RNA with DNA, and helicases like DHX9, Aquarius, senataxin, and PIF1 which resolve R-loops by unwinding the RNA component away from the template (102,103). In addition, several components of canonical DNA repair pathways, including HR (BRCA1, BRCA2), the Fanconi anemia pathway, and transcription-coupled NER (XPG and XPF) are also implicated in R-loop suppression (104–107).

Transcription and replication necessarily share the same DNA template. When replication forks encounter the transcription machinery, the resulting transcription-replication collisions (TRCs) can cause potentially lethal DNA damage and genomic instability. Transcription of human genes that are larger than 800 kb spans more than one complete cell cycle, so TRCs are inevitable (108). TRCs can occur in head-on or co-directional orientation, with the former scenario having more deleterious effect than the latter (109). Head-on TRCs promote the formation of R-loops that block replication fork progression (109,110). Importantly, R-loops, but not normal transcription complexes, induce DSBs during TRCs (109). How a replication fork blocked by an R-loop can restart remains elusive. Recent studies have shown that R-loop induced replication fork stalling is an active process involving cycle of fork reversal, cleavage and re-ligation. Mechanistically, after RECQ1 mediates reverse branch migration, RECQ5 disrupts RAD51 filaments to prevent a new round of fork reversal and to facilitate fork cleavage by MUS81. RAD52 and LIG4/XRCC4 help to catalyze fork re-ligation. Interestingly, this cycle allows the movement of the replication machinery through oppositely transcribed DNA regions without disrupting transcription (111). In support of this model, a recent study has shown that ATR-Chk1 is activated by R-loops through the action of MUS81 on reversed forks. Activated ATR prevents TRCs as well as excessive cleavage by MUS81, and enforces cell cycle arrest (112). Taken together, it is likely that R-loops may function as a signal for potential TRCs to activate the fork cleavage and re-ligation cycle to resume the transcription of associated genes.

DNA–PROTEIN CROSSLINKS

Proteins can be covalently and irreversibly linked with DNA, forming DNA–protein crosslinks (DPCs). DPCs are highly detrimental to cells because they constitute large adducts that potently block DNA replication (Figure 3). Various chemotherapeutics are used in the clinic which induce DPCs. As mentioned earlier, topoisomerases form covalently-bound TOPccs as part of their catalytic cycle. Topoisomerase poisons such as irinotecan and etoposide selectively trap TOP1 and TOP2, respectively, on DNA by stabilizing the TOPcc (113,114). Moreover, pyridostatin, a compound that stabilizes G-quadruplex (G4) sequences has been recently shown to induce TOP2cc formation (115). Similarly, 5-aza-dC is a cytosine analogue that is incorporated in DNA and acts as a pseudosubstrate for DNA methyltransferase 1 (DNMT1), resulting in its covalent trapping (116). Platinum derivatives, such as cisplatin, induce nonspecific crosslinking of chromatin-interacting proteins to DNA (117), and PARP inhibitors, such as Olaparib, induce PARP trapping on DNA. Although not crosslinked covalently, PARP is tightly bound to DNA, resulting in a DPC-like lesion (118). Exogenous sources, like ionizing radiation (IR) and UV, can also cause DPCs (119). Finally, abasic sites, which can occur spontaneously or during base excision repair, have intrinsic protein-crosslinking properties, leading to covalent trapping of histones and the newly discovered HMCES protein (120).

Due to the large size of DPCs, it was initially thought that they block both replicative Pols and CMG helicase. Surprisingly, recent reports revealed that DPCs do not block CMG, and moreover DPC repair does not require CMG unloading (discussed below). A lagging strand DPC can be immediately bypassed by CMG, perhaps with the help of the accessory protein MCM10, to ensure lagging strand DPCs do not interfere with CMG movement (121). Unexpectedly, Walter and colleagues have shown that CMG can also bypass an intact leading strand DPC before proteolysis. The DNA helicase RTEL1 facilitates the bypass by generating ssDNA beyond the DPC. This allows the CMG to bypass the DPC, which then triggers DPC proteolysis (9,122). CMG slows after bypass, likely due to uncoupling from DNA synthesis as previously mentioned (1,9). This further reinforces the remarkable capacity of CMG to overcome obstacles, and the flexibility of the replisome.

DPC repair mechanisms

The diversity of the potential components of a DPC likely served as the basis for the evolution of multiple mechanisms to deal with these lesions. For example, Topccs may be flanked by a SSB or a DSB in the case of TOP1cc and TOP2cc, respectively. Regardless, the collision of replication machinery with TOP-DPCs could lead to DSB formation. In addition to the general repair strategies (Figure 1), TOP-DPCs have dedicated enzymes for their removal, called tyrosyl-DNA phosphodiesterases, or TDPs. TDP1 and TDP2 hydrolyze the phosphodiester bond between DNA and the tyrosine residue of TOP1 and TOP2, respectively (Figure 3B). TDP1 activity produces 3′ phosphate ends, which require further processing by PNKP, followed by the canonical SSB repair to seal the nick. On the other hand, TDP2 produces a clean DSB which is directly processed by non-homologous end joining (NHEJ) (123). TDP1 and TDP2 are incapable of removing the intact TOP1cc and TOP2cc and require their pre-proteolysis (see below) (124,125). A recent report has discovered that TEX264–p97 complex facilitates the proteolysis of TOP1cc via SPRTN upstream of TDP1 during DNA replication (126). Interestingly, the SUMO ligase ZATT (ZNF451) may mediate direct resolution of the TOP2cc by TDP2 without the need for TOP2cc pre-proteolysis (127). Thus, the cell has evolved multiple possible mechanisms to deal with just one particular group of DPCs.

Beyond these specific mechanisms, the MRN complex (Mre11–Rad50–NBS1) is a highly conserved nuclease which may function to remove DPCs (Figure 3B). MRN is unaffected by the identity of the protein adduct and both the enzymatic and non-enzymatic DPCs are potential targets for MRN (128–131). Moreover, MRN can remove protein adducts at the 5′ end or the 3′ end of DSBs. Such endonucleolytic activity produces a clean DSB, which can be repaired by canonical DSB repair mechanisms (130). The CtIP nuclease (Sae2 in *S. cerevisiae*), stimulates the endonucleolytic cleavage of DPCs by MRN (130,132). Additional nucleolytic activities are likely involved in excising TOP1ccs.

The proteasome participates in DPC removal (Figure 3), and proteasome inhibitors impair DPCs removal and sensitize cells to DPC inducing agents (125). The proteasome degradation requires DPC polyubiquitylation, which is partially dependent on the E3 ligase TRAIP (122). In recent years, a more specific proteolytic pathway has been identified with the finding of Wss1 in yeast cells and its orthologs SPRTN in mammalian cells (133). Wss1/SPRTN can cleave almost every type of DPC, including TOPcc, high mobility group protein 1, histones, and itself in a DNA-dependent manner (133–138). SPRTN can also degrade nonubiquitylated DPCs (122). Spartan is recruited by binding ubiquitinated PCNA via its UBZ domain and a PIP box (139,140). As expected, the protease activity of SPRTN is tightly regulated via its DNA binding, ubiquitination and autocleavage (133–138).

Even after DPCs proteolysis by Wss1/SPRTN or the proteasome, they are not fully removed from DNA. Small peptides are left covalently bound to DNA, which in turn can block replicative Pols. The peptide-DNA can be processed by TDPs in case of TOPcc (126). In case of non-enzymatic DPCs, the small peptides that remain can be removed by NER (Figure 3C), which is only capable of excising DPCs up to a certain size (8–10 kDa) (141,142). However, it seems that the action of NER is limited to G1 phase prior to replication (133). In addition, bypass of peptide-DNA may rely on the TLS pathway (143). Finally, if a DPC is left unprocessed, the permanently stalled replication fork might be cleaved by endonucleases, resulting in a single-ended DSB. This situation may therefore trigger break-induced replication (133). More recently, genetic screening using tdp1/wss1 double-mutant yeast has identified the aspartate protease Ddi1 to act as alternative to Wss1 and 26S proteasome to resolve DPCs (144). Thus, the cell has evolved multiple complex mechanisms by which DPCs can be processed, highlighting the importance of dealing with this type of lesion.

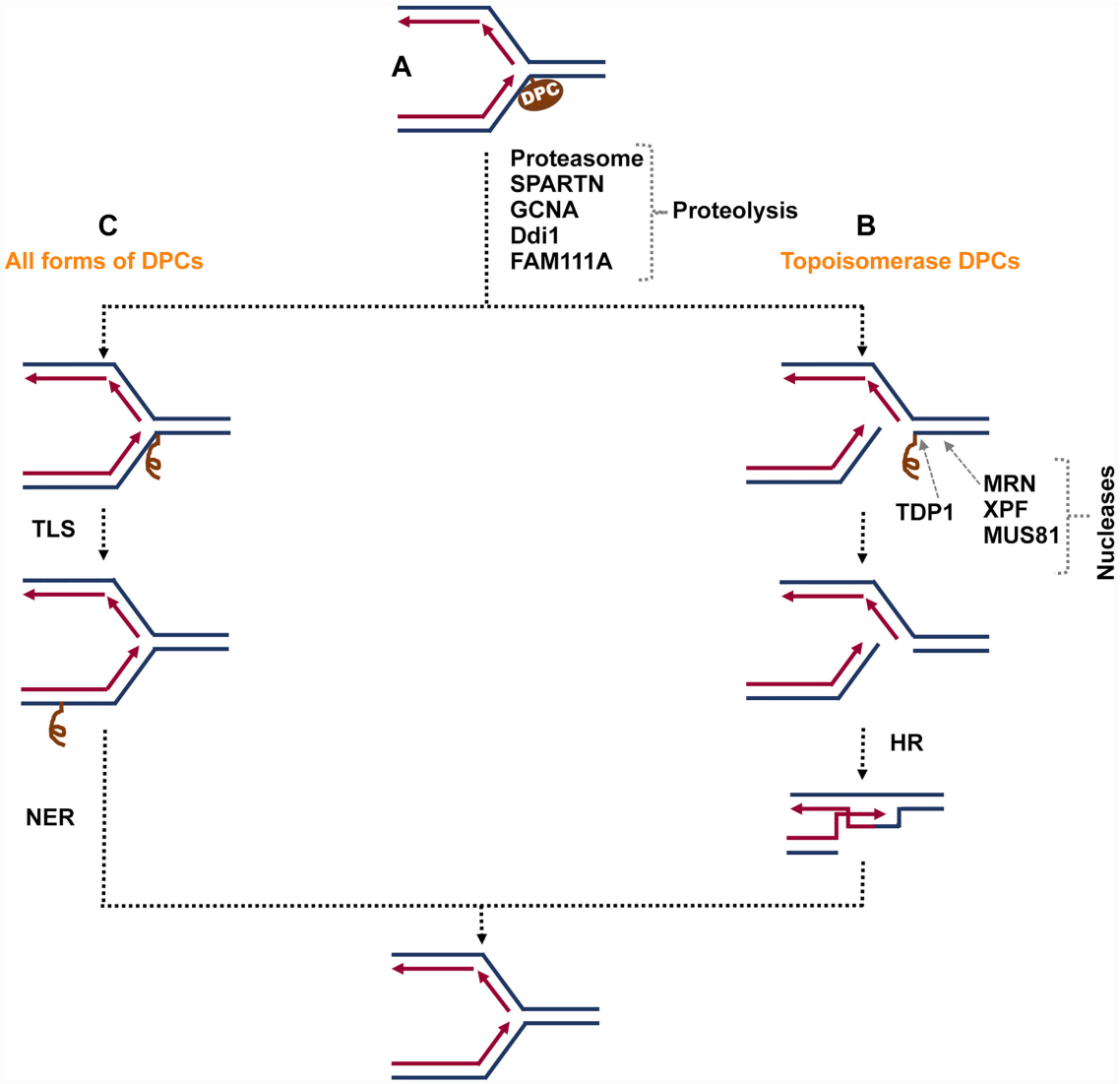

Figure 3. DNA–protein crosslink (DPC) repair and bypass during replication. (**A**) A DPC blocks replication fork progression. The repair of the DPC begins with proteolysis of the DPC by various proteases. (**B**) The repair of topoisomerase induced DPCs is a special case, because TOP-DPCs are flanked by a single strand break (SSB) or double strand break (DSB), in case of topoisomerase I and II, respectively. The collision of the replication fork with trapped topoisomerases will eventually lead to a DSB by 'replication run-off'. For simplicity, only the collision of replication fork with topoisomerase I-induced DPC is depicted here. After proteolysis, the peptide-DNA crosslink can be removed by TDP1 or nucleases. The resulting DSB is repaired by homologous recombination (HR) to induce fork restart and progression. Although not depicted here, the replication fork can avoid the collision with trapped topoisomerases and the generation of DSB by inducing fork reversal ahead of the DPC. (**C**) For all forms of DPCs, the remaining peptide–DNA crosslink can be bypassed by translesion synthesis (TLS), and subsequently removed by nucleotide excision repair.

While it is widely accepted that DPCs challenge the integrity of the genome during replication, Cortez *et al.* recently suggested a role for DPCs during faithful replication. An evolutionarily ancient protein called HMCES was found to be a sensor of abasic sites in ssDNA during replication (120). Strikingly, it generates a DPC with the ring open form of the abasic site deoxyribose to shield these lesions from error-prone processing by AP endonucleases and TLS Pols. How the HMCES-DNA is eventually resolved is unknown. In contrast to the above model, a recent study has shown that AP endonucleases may act in concert with HMCES for the resolution of AP sites. This study suggested that the HMCES-DPC may help to recruit AP endonuclease to channel these lesions to the proper repair pathway (145). In support of this, AP sites are known to react with several proteins such as histones, KU heterodimer, human ribosomal protein S3, nucleoside diphosphate kinase, PARP1, and several others (146–152). It is possible that HMCES may function in these two distinct manners in a cell-cycle dependent fashion; that is, it may promote AP endonuclease function outside of S-phase yet protect from the deleterious effects of AP endonuclease during replication, which could potentially induce a DSB. Certainly, further studies are needed to clarify the role of the HMCES-DPC and its regulation.

Several critical questions regarding the repair of DPCs during replication remain. Specifically, why the cell has invested in several distinct proteases that perform the same or similar function in DPC removal is unknown. The fact

that removal of each of these factors has some independent phenotype suggests that their functions are not completely overlapping. Moreover, the discovery of HMCES suggests that cells may have adapted to utilizing physiological DPCs potentially for modulating repair pathway choice.

INTERSTRAND CROSSLINKS

Unlike the lesions described above, ICLs are toxic lesions that block DNA replication by physically preventing DNA strand separation (Figure 4). Chemotherapeutic agents such as mitomycin C and platinum compounds (e.g. cisplatin and carboplatin) induce ICLs. Metabolic processes such as histone demethylation, lipid peroxidation, and alcohol metabolism produce aldehydes which can also induce ICLs (153–157). These physiological sources of ICLs likely explain the various mechanisms that have evolved to repair these lesions.

Replication block by interstrand crosslinks and their repair mechanisms

ICLs that occur outside of S-phase are believed to be repaired by the NER pathway (158). However, in S-phase, ICL repair starts with the formation of an X shaped DNA structure that occurs upon convergence of two replication forks at the ICL site (159,160) (Figure 4A). Unhooking the crosslink is essential for the completion of DNA synthesis. This is achieved through incision-dependent or incision independent pathways. TRAIP (E3 ubiquitin ligase) acts as a switch between these different repair pathways by controlling CMG ubiquitination. Short ubiquitin chains recruit NEIL3 glycosylase, while excessive ubiquitination activates the Fanconi anemia (FA) pathway (161). In turn, this ubiquitination regulates the downstream mode of ICL repair.

Higher levels of TRAIP-mediated ubiquitination promotes CMG unloading at the ICL by the AAA+ ATPase p97, which functions as an essential step to initiate the Fanconi anemia (FA) pathway (161) (Figure 4B). The FA pathway involves an intricate process that is abrogated in a genetic disease characterized by congenital abnormalities, bone marrow failure, and cancer predisposition. To date, 22 FA genes have been identified, which are grouped into FA core complex (encoding an E3 ligase complex), the FANCI/FANCD2 (ID) complex, and the downstream effector proteins. When DNA replication is blocked by ICL, the FA core complex (FANCA, B, C, E, F, G, L and M) monoubiquitinates the ID complex (157). Monoubiquitinated ID complex constitutes an essential gateway for ICL repair by recruiting downstream factors to initiate excision, TLS and HR. The mechanism by which the ID complex is targeted to ICL is not clear, but it has been suggested that the recruitment to a stalled fork precedes the monoubiquitination event (162). In support of this, a recent cryo-EM study has revealed that monoubiquitination of the ID complex causes a loss of preference for the ICL lesion and remodels the complex to become a sliding DNA clamp that functions to coordinate the downstream repair reaction (163). Removal of the ICL starts with nucleolytic incisions of the ICL. SLX4/FANCP and its associated 3′-flap structure-specific endonuclease XPF(FANCQ)/ERCC1, generate nucleolytic incisions and unhook the ICL (164,165). Other nucleases, MUS81/EME1 and FAN1, have been also implicated in ICL incision (157,166). Following unhooking, the ICL remnant is bypassed by TLS polymerases REV1 and Polζ, a heterodimeric complex of REV3 and REV7 (167). The other DNA duplex with a DSB will be repaired by canonical HR, using the restored nascent strand as a template for strand invasion. FA pathway deficiency not only causes reduction in HR efficiency, but also increases deleterious repair through NHEJ, which induces genomic instability. Accordingly, ICL sensitivity can be rescued by inhibition of specific NHEJ factors (168–170). Lastly, FANCD2 is deubiquitinated by USP1–UAF1 complex to terminate the FA pathway (171,172). Interestingly, D'Andrea and colleagues have recently identified TRIP13 ATPase as a negative regulator of REV7, which links REV1 and REV3 Polζ complex. This work suggests that TRIP13 has a role in disassembling the REV7–REV3 TLS complex, serving to regulate another component of the FA pathway (173). Since TLS has other functions outside of the Fanconi pathway, it's likely that these negative regulators evolved separately, yet function in a cooperative manner to shut off the pathway in the context of ICL repair.

Unlike FA activation, shorter TRAIP-mediated ubiquitination events on CMG recruits NEIL3 (Figure 4C), a DNA glycosylase that has functions outside of BER (161). NEIL3 specifically unhooks psoralen-ICL and AP-ICL (formed by abasic site and an adenosine) during DNA replication in *Xenopus* egg extracts. Other forms of ICLs are generally not substrates for NEIL3. Unhooking ICLs by NEIL3 does not nick the DNA backbone, and accordingly does not generate DSBs (174). The same result has been confirmed in human cells, where NEIL3 is also the major pathway for psoralen-ICLs repair. Interestingly, NEIL3 recruitment to a psoralen-ICL is strictly PARP-dependent (175). NEIL3 generates an abasic site which in turn is bypassed by TLS Pols (174). Thus, the NEIL3 pathway is potentially mutagenic but does not require complex processing of a DSB, which may serve to protect the genome.

Recent work on the repair mechanism of acetaldehyde (AA) ICLs have suggested that an alternative pathway may exist for these lesions. Consistent with previous animal models and genetic studies, work in *Xenopus* egg extracts have discovered that AA-ICL repair requires an active FA pathway (153–156). Unexpectedly, however, about half of the acetaldehyde-induced ICLs are repaired by a second, faster mechanism (Figure 4D). This second mechanism is independent of the FA pathway, or any glycosylases, including NEIL3. Moreover, in this rapid route, abasic sites are not generated, nor are any cuts made to the DNA strands. Instead, the ICL is cut within the crosslink, leaving the adduct on one of the DNA strands that is bypassed by TLS polymerases REV1 and Polζ (155). The unhooking of the AA-ICL by the new mechanism was suggested to be mediated non-enzymatically by the mechanical force of fork conversion or enzymatically by an unknown protein.

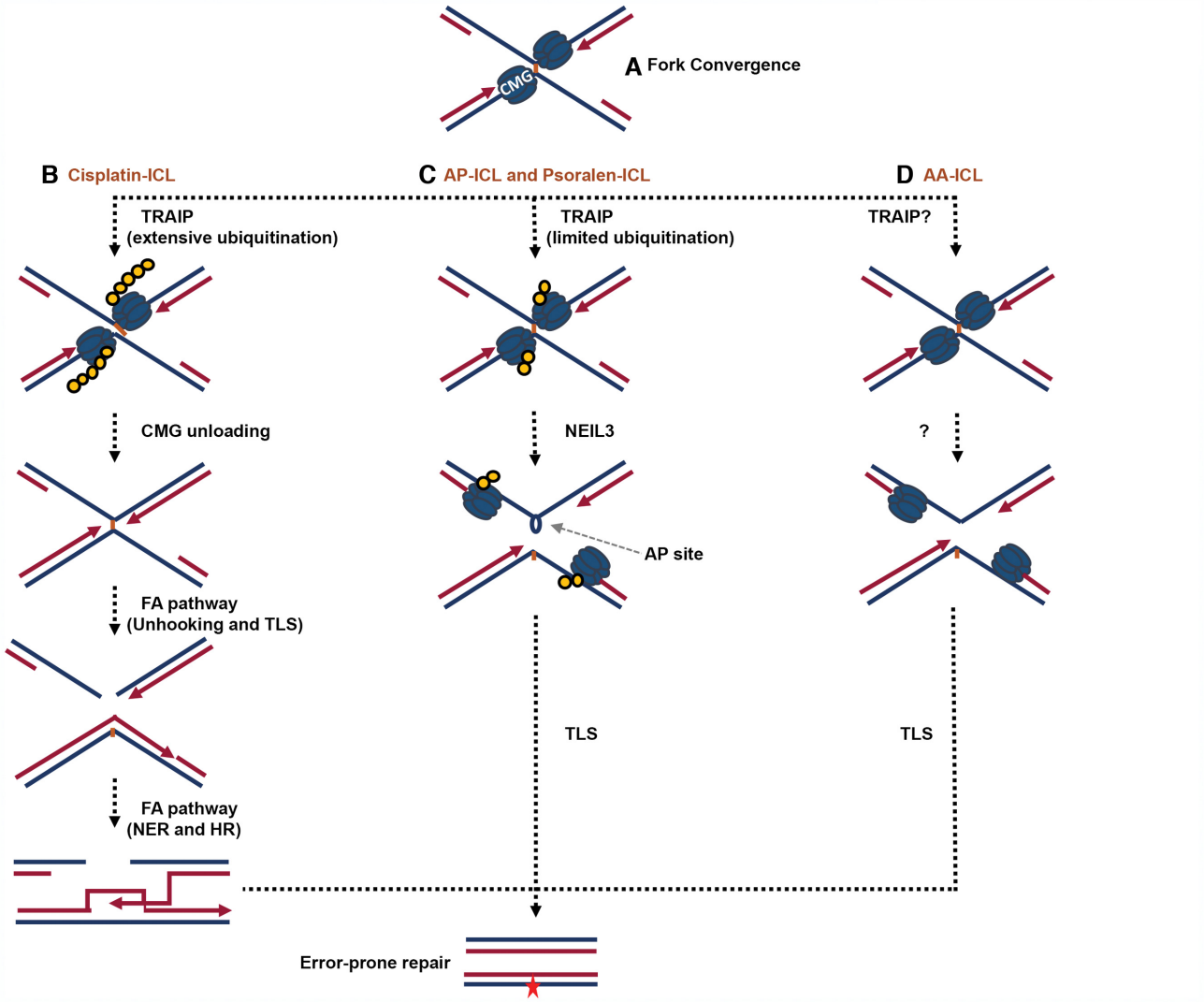

Figure 4. Interstrand crosslink (ICL) repair during replication. (**A**) The mechanism of ICLs repair depends on the type of ICL. While the Fanconi anemia (FA) pathway is the general mechanism to repair most forms of ICLs during replication, other repair mechanisms have evolved to repair specific types of ICLs. All ICL repair mechanisms require the conversion of replication forks. The E3 ligase, TRAIP, functions to regulate different repair pathways by controlling CMG ubiquitination. (**B**) Extensive ubiquitination of CMG by TRAIP lead to the CMG unloading, which is an essential step to activate the FA pathway. FA pathway regulates the incision of ICLs by nucleases, then activates translesion synthesis (TLS) to bypass the adduct, which is subsequently removed by nucleotide excision repair. The double strand break (DSB) at one strand is repaired by homologous recombination (HR), using the other strand as the template. (**C**) In the case of abasic (AP)-ICL and psoralen-ICL, TRAIP adds short ubiquitin chains on CMG that channels the damage to NEIL3, which incises the ICL without inducing a DSB. TLS bypasses the adduct and the AP site. (**D**) In case of acetaldehyde-ICL (AA-ICL), another mechanism has been recently identified that acts to bypass this lesion. The role of TRAIP or specific enzymes that incise this type of ICL is unknown. This repair pathway does not induce DSB or an AP site intermediate. All ICL repair converges through TLS bypass of the adduct to complete the repair in an error-prone manner.

Taken together, these relatively recent results suggest that while the FA pathway can remove virtually all forms of ICLs, the induced DSB by this mechanism is a potential source of genomic instability. This may explain the development of other repair pathways for specific types of ICLs, such as NEIL3, which limit the use of the FA pathway. The choice between FA activation and NEIL3 mediated bypass through the degree of CMG ubiquitination by TRAIP permits a stalled replisome to use the latter pathway first; yet upon its failure, the FA pathway can be activated in its place, making the DSB as a mechanism of last resort for ICL repair.

CONCLUSIONS AND OUTLOOK

The various tolerance and repair pathways help to guarantee the completion of the replication process. While many of the molecular mechanisms of these individual pathways are well understood, their coordinate regulation remains obscure and represents a major gap in our knowledge. For example, how an individual replisome preferentially selects a specific pathway upon encountering a specific type of damage, whether general or lesion-specific, is not clear. Reminiscent of DSB pathway choice, which involves a commitment to HR or NHEJ, many of these replication-associated

pathways of repair are antagonistic. Similar to DSB pathway regulation, it is likely that post-translational modifications are in play which function to favor one pathway over another. Yet, beyond PCNA monoubiquitination versus polyubiquitination, or TRAIP-mediated ubiquitination of CMG, how the cell selects one particular pathway over another is far from clear. Second, it is not clear why the cell developed multiple pathways to fix the same or very similar types of damage during replication. The extreme case is the presence of largely redundant DPC proteases. It is possible that different types of DPCs require their presence, or different chromatin contexts may require specific pathways. Third, how the cell avoids the excision of ssDNA in the vicinity of the replication fork by excision repair pathways (e.g. RER or BER) during replication is an unanswered question. One strategy is the bypassing of the damage by TLS or TS to allocate the lesion in the context of a double-stranded template, which would allow the faithful action of the incision step. Another strategy involves fork reversal, which also helps maintain the lesion in a double-stranded context. In virtually all of these examples, the precise regulatory steps are also almost completely unexplored. Future work will undoubtedly shed light on these key aspects of repair during DNA replication.

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