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To skip or not to skip: choosing repriming to tolerate DNA damage

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Summary

Accurate DNA replication is constantly threatened by DNA lesions arising from endogenous and exogenous sources. Specialized DNA replication stress response pathways ensure replication fork progression in the presence of DNA lesions with minimal delay in fork elongation. These pathways broadly include translesion DNA synthesis, template switching, and replication fork repriming. Here, we discuss recent advances toward our understanding of the mechanisms that regulate the fine-tuned balance between these different replication stress response pathways. We also discuss the molecular pathways required to fill single-stranded DNA gaps that accumulate throughout the genome after repriming, and the biological consequences of using repriming instead of other DNA damage tolerance pathways on genome integrity and cell fitness.

Graphical Abstract

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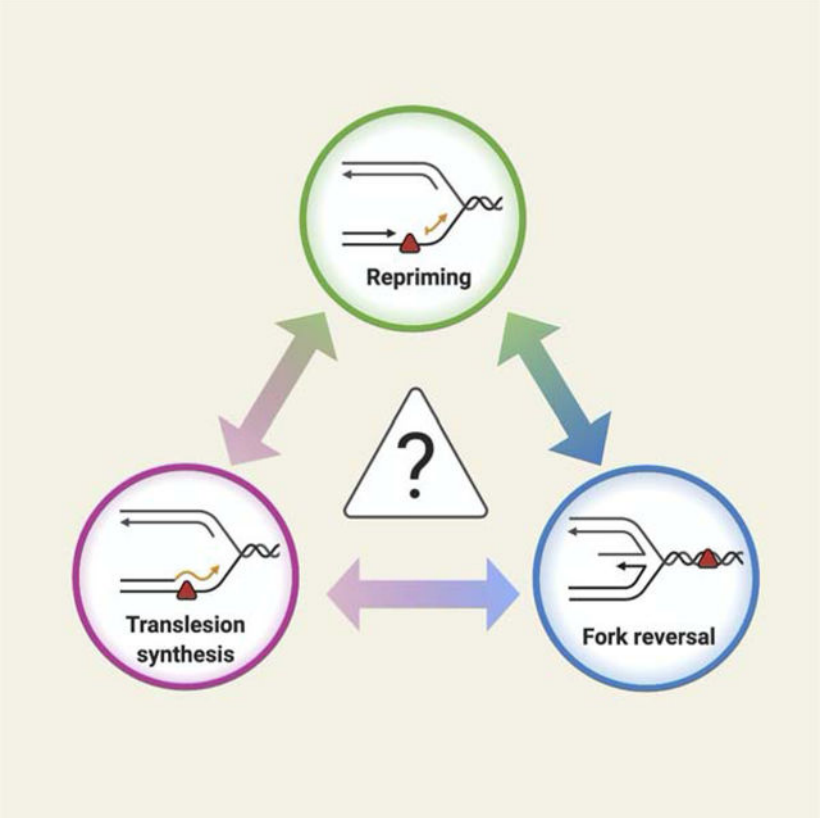

eTOC Blurb

Replication stress response pathways allow DNA replication to tolerate obstacles with minimal delay in fork elongation. In this review, Quinet et al. discuss the molecular contexts in which cells choose repriming over template switching and translesion synthesis to tolerate these obstacles, and the consequences of this choice on genome integrity.

High-fidelity DNA replication is constantly challenged by a diverse range of obstacles. These include DNA lesions created by endogenous and exogenous agents and intrinsic replication obstacles such as secondary structures in the DNA template, tightly-bound protein-DNA complexes, and conflicts with the transcription machinery. The transient slowing or stalling of replication forks in response to these challenges is termed “replication stress” (Berti et al., 2020). The accurate processing of stalled or damaged replication forks is central to preserve genome stability and ensure cell survival. Cells have evolved different molecular pathways aimed at preserving the stability of stalled replication forks and promoting their accurate restart. How cells choose between these pathways remains unclear.

DNA damage tolerance (DDT) mechanisms allow replication forks to overcome obstacles with a minimal effect in fork elongation. DDT broadly includes Translesion DNA Synthesis (TLS) and template switching (TS) pathways (Figure 1). A host of specialized polymerases drive TLS by direct bypass of DNA lesions. TLS polymerases (POL) include POL η, REV1, POL κ, POL ι, POL ζ, POL ν, and POL θ, all of which have the ability to replicate through a damaged template, albeit with lower fidelity (Sale, 2013; Vaisman and Woodgate, 2017).

TS is generally more accurate than TLS and involves the use of a homologous template, usually the newly synthesized daughter strand on the sister chromatid, to bypass DNA lesions (Adar et al., 2009; Izhar et al., 2013). One version of TS is replication fork reversal, in which replication forks reverse their course by annealing the two daughter strands, leading to the formation of four-way junction structures (Neelsen and Lopes, 2015). Remodeling of replication forks into reversed forks promotes bypass of damage by canonical TS mechanisms, or replication-coupled repair by repositioning the lesion in the double-stranded duplex ahead of the fork (Berti et al., 2020). Moreover, recent studies suggest that fork reversal also occurs at unchallenged forks as a global response to replication stress to hold replication forks in a “standby” mode until replication stress is resolved (Mutreja et al., 2018).

In addition to TLS and TS, a third mechanism, termed repriming, can be activated to overcome replication obstacles and ensure DNA replication progression (Figure 1). Repriming involves re-initiation of DNA synthesis beyond a DNA lesion, leaving unreplicated single-stranded DNA (ssDNA) gaps to be filled post-replicatively through either TLS or TS mechanisms. After a brief historical perspective on the repriming mechanisms, we discuss how cells choose between the repriming, TLS, and TS pathways. This has been a long-standing question in the field, and recent papers have provided important clues into how different factors favor one pathway over the other, including the nature of the DNA lesion, extent of DNA damage, PCNA post-translational modifications, as well as changes in the genetic background. Finally, we discuss how the ssDNA gaps that form upon repriming are repaired post-replicatively and the impact of employing repriming *versus* canonical DDT pathways on cell fitness and genome integrity.

SSDNA GAP FORMATION AND REPRIMING

Early studies suggested that exposure to UV radiation causes minimal delay in DNA replication fork progression but leads to the accumulation of ssDNA discontinuities on the daughter strands in bacteria (Howard-Flanders et al., 1968), mouse (Lehmann and Kirk-Bell, 1972), and human cells (Meneghini, 1976). These daughter-strand ssDNA gaps accumulate on both the lagging and leading strands upon UV irradiation, as observed by electron microcopy in *Saccharomyces cerevisae* (Lopes et al., 2006). The same ssDNA gaps have been observed in mammalian cells upon exposure to a wide-range of DNA-damaging agents (Diamant et al., 2012; Elvers et al., 2011; Jansen et al., 2009; Quinet et al., 2016). However, the underlying mechanisms leading to the formation of these ssDNA gaps remained mechanistically ill-defined.

Generation of ssDNA gaps in the lagging strand after treatment with DNA-damaging agents can be explained by the discontinuous nature of lagging strand synthesis. In this scenario, synthesis of a new Okazaki fragment ensures replication fork restart and continued replication fork progression, despite blockage of the previous fragment by a DNA lesion. Pioneering studies in bacteria showed that the DnaG primase ensures replication fork restart downstream of a UV lesion in both the lagging and the leading strands (Heller and Marians, 2006) (Figure 2). These findings suggested that the replisome is able to reinitiate DNA synthesis downstream of leading strand lesions and that the repriming activity of DnaG leads

to the formation of a ssDNA gap between the lesion and the point where synthesis restarts. These early findings raised several new questions for the field: Is this repriming mechanism conserved in eukaryotes? Is there a human homolog of bacterial DnaG?

Following these early observations, several reports indicated that *Saccharomyces cerevisae* uses replication fork repriming to deal with different DNA-blocking lesions (Daigaku et al., 2010; Fumasoni et al., 2015; Karras and Jentsch, 2010; Wong et al., 2020). Repriming in budding yeast is ensured by Polymerase α (Polα)/Primase complex and Ctf4, a replisome factor that bridges the MCM (replicative minichromosome maintenance) helicase and the Polα/Primase complex (Fumasoni et al., 2015) (Figure 2).

The human Primase and DNA-directed Polymerase (PRIMPOL) is a recently discovered enzyme that possesses both primase and polymerase activities. PRIMPOL is a member of the archae-eukaryotic primase (AEP) superfamily (Iyer et al., 2005) and is emerging as a key player in replication stress response in mammalian cells. PRIMPOL synthesizes DNA with limited processivity, rarely incorporating more than four nucleotides on an undamaged template (Keen et al., 2014b). However, all the studies on PRIMPOL polymerase activity have been performed using synthetic DNA substrates and purified recombinant protein, raising the question of whether PRIMPOL polymerase activity has a physiologically relevant function *in vivo* (Bianchi et al., 2013; Garcia-Gomez et al., 2013; Mouron et al., 2013). While the role of PRIMPOL polymerase activity is still unclear, its primase activity is essential for many of the biologically relevant functions of PRIMPOL in the nucleus (Calvo et al., 2019; González-Acosta et al., 2020; Keen et al., 2014a; Kobayashi et al., 2016; Piberger et al., 2020; Quinet et al., 2020; Schiavone et al., 2016; Svikovic et al., 2019). The functional characterization of its unique primase activity provided the first clues for how repriming and ssDNA gap formation are regulated in mammalian cells (Bianchi et al., 2013; Garcia-Gomez et al., 2013; Mouron et al., 2013; Wan et al., 2013). Following these discoveries, the mechanisms that dictate the choice between the repriming, TLS, and TS pathways became the subject of intensive investigation.

CHOICE BETWEEN REPRIMING, TLS, and TS

Nature of DNA damage and extent of fork stalling.

Some agents challenge DNA replication without inducing DNA damage, such as hydroxyurea (HU) or aphidicolin, whereas others perturb fork progression by introducing a lesion in one or both DNA strands. The nature of the replication challenge is a key determinant of pathway choice (Figure 3). For example, PRIMPOL repriming is favored over TLS when the lesion present on the replication fork is too bulky to be bypassed by canonical TLS polymerases. Exposure to UV-C generates two types of pyrimidine dimers: cyclobutane pyrimidine dimer (CPD) and pyrimidine (6–4) pyrimidone (6–4PP) (Pfeifer et al., 2005). 6–4PPs cause a more pronounced distortion of the DNA double helix compared to CPDs. While CPDs are efficiently bypassed by the TLS polymerase POL η at the replication fork, formation of 6–4PPs leads to ssDNA gap accumulation behind replication forks in DNA repair-deficient mouse embryonic and human fibroblasts, suggesting that tolerance to UV-induced 6–4PPs involves replication fork repriming (Jansen et al., 2009; Quinet et al., 2018).

In agreement with the proposed role of repriming in the bypass of bulky 6–4PP, PRIMPOL binding to chromatin increases after treatment with UV-C, and PRIMPOL depletion, or loss of its primase activity, impairs replication fork restart upon UV-C irradiation (Mouron et al., 2013). Moreover, Primpol$^{-/-}$ DT-40 cells complemented with a primase-dead version of the protein are hypersensitive to UV radiation, as well as to treatment with methyl methanesulfonate (MMS) and cisplatin, broadening the spectrum of DNA lesions that can be ”skipped” by PRIMPOL (Kobayashi et al., 2016). Along the same lines, PRIMPOL-mediated repriming is required to rescue replication forks that have been stalled by cisplatin treatment in human cells (Quinet et al., 2020). Although cisplatin mainly generates intra-strand adducts (Poklar et al., 1996), approximately 5% of lesions are inter-strand crosslinks (ICLs) (Deans and West, 2011), which are generally considered an absolute block for replication fork progression. However, ICLs can be “traversed” in a reaction mediated by the FANCM/MHF DNA translocase (Huang et al., 2013). This mechanism relies on the primase activity of PRIMPOL, which leaves the ICL in the ssDNA gap behind the fork to be repaired post-replicatively (González-Acosta et al., 2020). Interestingly, PRIMPOL also plays a role in DNA replication stress response to HU treatment in human cells (Bai et al., 2020; Kobayashi et al., 2016; Mouron et al., 2013; Quinet et al., 2020). Moreover, HU treatment leads to ssDNA gap accumulation in budding yeast, suggesting that repriming is a general mechanism to deal with replication stress even when replication forks do not face DNA lesions (Gallo et al., 2019). These studies suggest that the reduction in the available nucleotide pool caused by HU treatment does not affect the primase activity of PRIMPOL and its ability to rescue stalled replication forks, at least at the HU concentrations used in these experiments. Importantly, PRIMPOL knock-out avian cells are not sensitive to treatment with camptothecin (CPT), γ-rays, or x-rays, suggesting that PRIMPOL is not involved in the repair of DNA breaks (Bianchi et al., 2013; Kobayashi et al., 2016).

Previous findings showed that reversed replication forks accumulate in human cells treated with a variety of genotoxic agents including UV-C, MMS, mitomycin C (MMC), cisplatin, CPT, hydrogen peroxide, and HU (Zellweger et al., 2015). This work raises the question of the frequency of fork reversal events relative to repriming or other replication stress response pathways. Unfortunately, a straightforward answer to this question is complicated by the limitations in the approach used to study this process. Electron microscopy, used to detect reversed forks, only takes a snapshot of this reaction by “freezing” the replication intermediates with the cross-linking step (Vindigni and Lopes, 2017), likely leading to an underestimation of the actual number of replication intermediates that have undergone fork reversal upon drug treatment.

In addition to the type of replication challenge, the extent of fork stalling caused by different concentrations of the same genotoxic agent might affect the equilibrium between different replication stress response pathways. For example, prolonged treatment of *BRCA1*- or *BRCA2*-deficient cells with HU concentrations ranging between 2 and 5 mM for 2 to 5 hours leads to reversed fork degradation (Kolinjivadi et al., 2017; Lemacon et al., 2017; Mijic et al., 2017; Taglialatela et al., 2017). This is consistent with the emerging role of BRCA proteins in reversed fork protection. However, treatment of BRCA-deficient cells with lower HU concentrations no longer promotes fork degradation, but leads to the accumulation of ssDNA gaps (Lim et al., 2018; Panzarino et al., 2019). In particular,

treatment of BRCA1-null cells with 1 mM HU for 3 hours induces fork degradation only upon depletion of the Ubiquitin Specific Peptidase 1 (USP1), suggesting that USP1 protects forks from degradation at this lower HU concentration (Lim et al., 2018). Moreover, USP1 is not needed for fork protection when BRCA1-deficient cells are treated with an even lower HU concentration (0.5 mM for 2 hours), which instead promotes the accumulation of ssDNA gaps. Along the same lines, Panzarino et al. showed that treatment of *BRCA1/2*-deficient cells with the same HU concentration (0.5 mM for 2 hours) leads to unrestricted replication fork progression and accumulation of ssDNA gaps on the replicating DNA, without causing fork degradation (Panzarino et al., 2019). These studies show that pathway choice is dictated not only by the type of replication challenge, but also by the concentration of replication inhibitor. In particular, they suggest that repriming is favored over reversal when BRCA-deficient cells are treated with mild HU concentrations. However, future experiments are necessary to determine whether the ssDNA gaps that accumulate in *BRCA*-deficient cells treated with mild HU concentrations are indeed a consequence of PRIMPOL repriming.

Why would BRCA-deficient cells favor reversal over repriming at higher HU concentrations? First, increasing the HU concentration might cause a drop in the dNTP pool concentration below a threshold needed for efficient PRIMPOL repriming. Second, higher HU doses would increase the extent of fork uncoupling, possibly leading to more RPA bound to ssDNA. On the basis of previous findings that high concentrations of RPA inhibit PRIMPOL recruitment to DNA *in vitro* (Guilliam et al., 2017; Martinez-Jimenez et al., 2017), we speculate that higher levels of RPA bound to ssDNA might limit replication fork repriming and favor alternative replication stress response pathways, such as fork reversal. Third, increased RPA binding would lead to hyper-activation of the ATR pathway, and ATR activity was recently shown to promote global fork reversal in BRCA-proficient cells in response to ICLs (Mutreja et al., 2018). However, the role of ATR in fork reversal is still controversial because ATR signaling is not necessarily detected in response to all genotoxic agents that induce fork reversal (Zellweger et al., 2015). Moreover, previous findings suggested that ATR activity counteracts, rather than promotes, fork reversal by restraining SMARCAL1 function in reversed fork formation in HU-treated cells (Couch et al., 2013). A possible reason for these contradictory results could be related to the different types of replication challenges used in these studies. A more systematic analysis using different types and concentrations of replication inhibitors would be important to properly address how the extent of replication stress or DNA damage load dictates the choice between replication fork reversal and repriming, as well as the role of ATR in this process. Moreover, whether the function of ATR signaling in fork reversal changes in BRCA-proficient *versus* -deficient cells remains unknown. As several of these inhibitors are used for the treatment of BRCA-proficient and -deficient tumors, these studies are also crucial to understand the impact of clinically relevant doses of replication inhibitors on pathway choice.

Finally, it is worth mentioning that prolonged fork stalling or replication fork de-protection caused by the loss of BRCA proteins can lead to fork breakage and formation of one-ended double-stranded breaks (DSBs), which require specialized break-induced replication (BIR) pathways to be repaired (Scully et al., 2019). Specific HR factors such as RAD51 are required for reversed fork formation and protection (Berti et al., 2020), but their requirement

for BIR is still debated and may reflect mechanistic differences between yeast and human cells (Kramara et al., 2018). Of note, RAD51 function in fork reversal is different from its potential function in BIR because it does not require its strand exchange activity. Moreover, the formation of reversed forks does not require stable RAD51 filaments, which are instead required to protect the already formed reversed forks from nucleolytic processing. As for fork reversal and repriming, the concentrations and timing of drug treatment that promote fork breakage and activate a BIR pathway likely vary as a function of the specific genetic background and type of replication challenge.

Changes in the genetic background.

Recent studies in budding yeast showed that repriming restrains extensive replication fork uncoupling and reversal and that aberrant reversed replication forks accumulate in repriming-deficient Polα/Primase/Ctf4 yeast mutants (Fumasoni et al., 2015). Differently from budding yeast, replication fork reversal is a frequent and physiologically important mechanism used by mammalian cells to cope with replication challenges (Zellweger et al., 2015). This notion is supported by the discovery that different members of the SWI/SNF translocase family, including SMARCAL1, ZRANB3, and HTLF, are capable of converting a three-way junction DNA replication fork into a four-way junction reversed fork in mammalian cells (Bai et al., 2020; Betous et al., 2012; Kile et al., 2015; Vujanovic et al., 2017). Suppression of fork reversal by depletion of SMARCAL1 leads to accumulation of ssDNA gaps in human cells treated with cisplatin, as detected by DNA fiber assay using the ssDNA-specific S1 nuclease (Quinet et al., 2020). In this context, PRIMPOL depletion renders replication tracts insensitive to S1 nuclease cleavage, indicating that the ssDNA gaps formed upon SMARCAL1 loss are PRIMPOL-dependent (Quinet et al., 2020). Along the same line, loss of HLTF promotes daughter-strand ssDNA gap accumulation in human cells treated with low doses of HU (50 or 500 μM) relative to HLTF-proficient cells (Bai et al., 2020; Peng et al., 2018). In addition, depletion of PRIMPOL in HLTF KO cells prevents ssDNA gap accumulation following HU treatment indicating that PRIMPOL repriming is responsible for the observed phenotype (Bai et al., 2020). These data suggest that PRIMPOL-mediated repriming is activated in both SMARCAL1- and HLTF-deficient cells treated with agents that challenge DNA replication by drastically different means (Figure 3). Although both fork remodelers are implicated in fork reversal and their loss promotes repriming, there is also a notable difference because loss of HLTF, but not SMARCAL1, leads to unrestrained fork progression, in addition to promoting ssDNA gap accumulation (Bai et al., 2020; Peng et al., 2018; Quinet et al., 2020). This difference might be related to the distinct genotoxic agents used to challenge DNA replication or to distinct, and yet to be defined, roles of HLTF and SMARCAL1 in replication fork remodeling.

Interestingly, the BRCA1-associated helicase FANCJ (BACH1/BRIP1) is required for unrestrained replication fork progression in HLTF-deficient cells treated with mild doses of HU (Peng et al., 2018). FANCJ is a hereditary breast/ovarian cancer and Fanconi anemia gene functioning in homologous recombination (HR) and replication fork protection (Levitus et al., 2005; Litman et al., 2005). In addition, FANCJ travels with the elongating forks to counteract replication perturbations (Alabert et al., 2014; Sirbu et al., 2011). Combined, the recent findings on the roles of FANCJ (Peng et al., 2018) and PRIMPOL (Bai

et al., 2020) in HLTF-deficient cells point to a potential link between FANCJ activity and PRIMPOL repriming in promoting unrestrained fork progression and ssDNA gap formation in this genetic background.

In addition to suppressing fork reversal, loss of another fork remodeler, ZRANB3, leads to unrestrained fork progression upon treatment with different genotoxic agents including CPT, MMC, and UV-C, suggesting that the observed unrestrained fork progression phenotype is independent of the particular kind of replication challenge, at least in the case of ZRANB3-depleted cells (Vujanovic et al., 2017). However, these studies did not determine whether the unrestrained fork progression phenotype of ZRANB3-depleted cells is associated with PRIMPOL-dependent ssDNA gap accumulation, as observed in HTLF-depleted cells.

The notion that suppression of replication fork reversal favors PRIMPOL-dependent repriming is strengthened by the observation that depletion of the central recombinase RAD51, which is required for replication fork reversal (Zellweger et al., 2015), promotes PRIMPOL repriming following UV radiation (Vallerga et al., 2015) or cisplatin treatment (Quinet et al., 2020). Similarly, loss of RAD51 generates daughter-strand ssDNA gaps in *Xenopus laevis* extracts treated with MMS (Hashimoto et al., 2010). Moreover, preventing reversed fork accumulation by inhibiting PARP activity also leads to PRIMPOL-dependent ssDNA gaps, further supporting the model that preventing replication fork reversal favors repriming and consequent ssDNA gap formation (Quinet et al., 2020). Furthermore, increasing PRIMPOL expression is sufficient to promote replication fork repriming and ssDNA gap formation (Quinet et al., 2020) (Figure 3).

Interestingly, replication forks challenged with cisplatin are still able to progress upon combined loss of PRIMPOL and SMARCAL1, RAD51, or PARP1, at least in the one-hour time window monitored by DNA fiber analysis (Quinet et al., 2020). On the basis of these results, it is tempting to speculate that cells might use an alternative TLS pathway when repriming and reversal are both suppressed, thereby ensuring replication fork progression and damage bypass. However, this does not seem to be the case in HTLF-depleted cells where the suppression of PRIMPOL leads to significantly shorter tracts (Bai et al., 2020), reinforcing the notion that there are important differences between HLTF and SMARCAL1. Interestingly, the HIRAN domain of HLTF is required to restrain fork progression (Kile et al., 2015). Recently, Bai et al. found that cells expressing an HLTF-HIRAN mutant behave differently from HLTF-deficient cells because the unrestrained replication phenotype of the HLTF-HIRAN mutant is not linked to PRIMPOL-dependent ssDNA gap accumulation, but rather relies on TLS activity of the REV1 polymerase (Bai et al., 2020). Similarly, cells expressing FANCJ^{S990A}, a FANCJ mutant that promotes POL η-dependent TLS (Xie et al., 2010), display unrestrained replication fork progression upon HU treatment but no accumulation of ssDNA gaps behind the forks (Nayak et al., 2020). Moreover, replication fork reversal was not observed in cells expressing FANCJ^{S990A}, suggesting that increased TLS activity restricts fork reversal (Nayak et al., 2020). Altogether these findings point to a finely tuned regulation between repriming, fork reversal, and TLS pathways, which can be affected by changes in the genetic background.

PCNA post-translational modifications.

PCNA monoubiquitination and polyubiquitination are crucial players in the choice between different DDT pathways (Figure 3) (Kanao and Masutani, 2017; Ulrich, 2009). PCNA monoubiquitination facilitates recruitment of specific TLS polymerases through a polymerase switching mechanism (Kannouche et al., 2004), whereas PCNA polyubiquitination has been associated with TS mechanisms, including fork reversal (Hoege et al., 2002; Vujanovic et al., 2017; Xiao et al., 2000). In particular, the ZRANB3 translocase interacts with polyubiquitinated PCNA to promote fork reversal (Ciccia et al., 2012; Vujanovic et al., 2017). More recently, the E3 ubiquitin ligase HLTF has also been shown to be required for replication fork reversal *in vivo* (Bai et al., 2020; Kile et al., 2015). However, the contribution of HLTF-dependent PCNA polyubiqutination in fork reversal remains unclear, because HLTF fork reversal activity is strictly dependent on its motor ATPase activity and HIRAN domain (Bai et al., 2020; Kile et al., 2015). In this regard, Kile et al. suggested that HLTF-dependent PCNA polyubiquitination might be required to promote efficient recruitment of ZRANB3, which in turn associates with polyubiquitinated PCNA to promote fork reversal (Kile et al., 2015). This model would argue that HLTF acts upstream of ZRANB3 in a common fork reversal pathway mediated by PCNA polyubiquitination.

As already discussed, loss of either ZRANB3 or HLTF leads to unrestrained replication fork progression, and the longer replication tracts of HLTF-deficient cells are characterized by the presence of PRIMPOL-dependent ssDNA gaps behind the advancing replication forks. However, these studies did not establish whether there is a direct link between reduced PCNA polyubiquitination and increased PRIMPOL-dependent repriming because at least two E3 ubiquitin ligases, HLTF and SHPRH, contribute to PCNA polyubiquitination, and polyubiquitination is still observed upon the loss of both proteins (Krijger et al., 2011). Moreover, the connection between PCNA ubiquitination and fork reversal is further complicated by recent studies suggesting that fork reversal still occurs in the PCNA-K164R ubiquitination-defective cells (Thakar et al., 2020). These studies argue that PCNA ubiquitination is required for Okazaki fragment maturation and that reversed forks that accumulate in PCNA-K164R cells contain abnormally long Okazaki fragments.

Loss of USP1, which is required to remove ubiquitin from monoubiquitinated PCNA (Huang et al., 2006) leads to increased levels of monoubiquitinated PCNA and accumulation of ssDNA gaps in BRCA1-deficient cells treated with low HU doses (Lim et al., 2018). How increased levels of monoubiquitinated PCNA lead to ssDNA gap accumulation upon the combined loss of BRCA1 and USP1 remains unclear. Interestingly, depletion of the TLS polymerases POL κ and REV1 suppresses the ssDNA gaps accumulation phenotype observed upon USP1 loss, suggesting that the formation of these gaps is somehow dependent on these TLS enzymes (Lim et al., 2018). An important question for future studies is whether these ssDNA gaps are PRIMPOL-dependent and how POL κ and REV1 are involved in this process. Human FANCD2 and RAD51 also support PCNA monoubiquitination and TLS (Chen et al., 2017). Another key objective for future research will be to investigate whether the proposed roles of these Fanconi anemia and HR proteins in regulating PCNA monoubiquitination affect other replication stress response pathways and

whether other signaling events, in addition to the changes in the PCNA ubiquitination status, affect the equilibrium between repriming, TLS, and TS.

ssDNA GAP-FILLING

The ssDNA gaps left behind advancing replication forks as a consequence of repriming need to be properly filled by gap-filling or post-replicative repair mechanisms. As for DDT at stalled replication forks, TLS or TS are the two universal strategies to tolerate DNA lesions opposite to ssDNA gaps and thereby fill in these gaps. The concept of post-replicative repair was proposed by Lehmann and Kirk-Bell studying the effect of UV irradiation in mouse cells (Lehmann and Kirk-Bell, 1972) and by the Prakash group studying post-replicative repair in budding yeast (Prakash, 1981). TS appears to be the main pathway of gap-filling in *E. coli* (Berdichevsky et al., 2002; Laureti et al., 2015). Early work suggested that TLS predominantly mediates gap filling in budding yeast (Daigaku et al., 2010; Gallo et al., 2019; Karras and Jentsch, 2010). However, this model was challenged by later studies showing that the ssDNA gaps generated upon repriming by the Polα-primase complex are filled by TS or by an alternative HR salvage pathway in *S. cerevisiae* (Fumasoni et al., 2015; Gonzalez-Huici et al., 2014; Karras et al., 2013). This apparent discrepancy might be due to the difference in the concentration and type of DNA-damaging agent used to promote gap formation (Wong et al., 2020).

Several studies suggest that TLS is the main pathway of ssDNA gap-filling in mammalian cells. The contribution of selected TLS polymerases to this process is still debated. For example, the TLS polymerases REV1 and POL ζ are essential for gap-filling in mammalian cells exposed to UV radiation (Diamant et al., 2012; Jansen et al., 2009). However, the notion that REV1 is required for gap filling in higher eukaryotes is not supported in other studies (Edmunds et al., 2008; Quinet et al., 2016). Along the same lines, Elvers et al. suggested that POL η plays a role in ssDNA gap repair (Elvers et al., 2011), whereas other studies suggested that POL η is primarily involved in TLS at the replication fork (Despras et al., 2010; Quinet et al., 2014; Vallerga et al., 2015). The interpretation of these results may be complicated by the redundancy that exists among TLS polymerases, combined with the fact that most TLS polymerases can act both at the stalled replication forks and at ssDNA gaps.

Defining the contribution of TS in gap-filling in mammalian cells is more challenging due to lack of direct methodologies to investigate homology-mediated mechanisms that do not involve strand transfer. Adar et al. used a gapped plasmid repair assay to directly assess gap-filling in mouse and human cells (Adar et al., 2009). They found that, in addition to TLS, an alternative HR pathway efficiently fills in gaps opposite of a synthetic abasic site or bulky adducts formed upon treatment with benzo[a]pyrene-diol-epoxide (BPDE). Interestingly, RAD51 and NBS1 proteins are required for HR-dependent gap-filling (Adar et al., 2009). These findings agree with a recent report describing a RAD51-dependent HR pathway to repair PRIMPOL-dependent ssDNA gaps opposite to BPDE-induced adducts in human cells (Piberger et al., 2020).

The relative gap filling contribution of TLS *versus* TS and HR in the human genome remains largely unknown. As for DDT at stalled replication forks, the nature of the DNA damage likely plays an important role in the choice between different gap-filling pathways. The results discussed above suggest that TLS is required to fill ssDNA gaps induced by UV radiation, whereas RAD51-dependent HR mediates post-replicative repair of bulky BPDE-induced adducts (Piberger et al., 2020). Moreover, there is evidence of cross-talk between factors involved in TLS and TS/HR. For example, the TLS polymerases REV1 and POL η were shown to play a role in HR-mediated DSB repair (McIlwraith et al., 2005; Sharma et al., 2012). Conversely, depletion of RAD51 decreases efficiency of both HR and TLS in repairing ssDNA gaps in the plasmid assay (Adar et al., 2009). Along the same line, BRCA1 was proposed to modulate TLS, although whether BRCA1 promotes or inhibits TLS remains unclear (Pathania et al., 2011; Tian et al., 2013).

Two elegant studies showed that PCNA ubiquitination is required for post-replicative ssDNA gap repair in budding yeast (Daigaku et al., 2010; Karras and Jentsch, 2010). However, the role of PCNA ubiquitination in gap-filling in higher eukaryotes remains controversial (Edmunds et al., 2008; Temviriyanukul et al., 2012). Avian DT40 cells expressing the ubiquitination-deficient K164R-PCNA mutant are impaired in gap-filling in response to UV-C irradiation (Edmunds et al., 2008). Conversely, post-replicative repair of ssDNA gaps induced by UV-C is unaffected in mouse embryonic fibroblasts harboring the same K164R-PCNA mutation (Temviriyanukul et al., 2012). Interestingly, avian cells lack any ortholog of HLTF, and Rad5, the HLTF homolog in yeast, promotes ssDNA gap repair by recruiting TLS polymerases to ssDNA gaps (Gallo et al., 2019). These studies open the tantalizing scenario that HLTF might be required for gap-filling in mammalian cells and could explain the discrepancy between the results obtained in chicken and mouse cells.

The cell cycle phase could also influence the choice between different gap-filling pathways. Recent studies proposed that gap-filling by TS is facilitated by PCNA polyubiquitination during the S phase in budding yeast (Branzei and Szakal, 2016; Gonzalez-Huici et al., 2014; Karras et al., 2013). Conversely, TLS at ssDNA gaps is promoted by PCNA monoubiquitination, while counteracted by PCNA polyubiquitination, and may occur preferentially in G2. Branzei and Szakal also proposed that TS error-free pathways act first during the S-phase, whereas the more error-prone mechanisms, such as TLS and salvage HR, preferentially act during G2/M, if the gaps cannot be properly filled during the S-phase (Branzei and Szakal, 2016). Supporting this model, different studies suggested that TLS-dependent gap-filling takes place during the late S and G2 phases in the human genome (Diamant et al., 2012; Elvers et al., 2011; Quinet et al., 2016; Temviriyanukul et al., 2012). Moreover, Adar et al. suggested that HR, facilitated by PCNA SUMOylation and independent of PCNA ubiquitination, preferentially occurs in the G2 phase in mammalian cells (Adar et al., 2009; Branzei and Szakal, 2016). Of note, recombination in G2 would not necessarily be restricted to the sister chromatid, potentially leading to strand transfer and genomic rearrangements. An important topic for future research would be to determine the exact relationship between the TLS, TS, and HR mechanisms of gap filling as a function of the different cell cycle phase.

BIOLOGICAL CONSEQUENCES OF ALTERING THE BALANCE BETWEEN REPRIMING, TLS, AND TS

What are the consequences of employing repriming *versus* TLS or TS on genome integrity and cell survival? PRIMPOL-knockout mice or human cell lines are viable, suggesting that PRIMPOL is not essential for cell survival (Bailey et al., 2019; Bianchi et al., 2013; Mouron et al., 2013; Quinet et al., 2020). However, human and avian DT40 cells lacking PRIMPOL display increased cellular sensitivity to different DNA-damaging agents, including UV-C, cisplatin, HU, 4NQO, BPDE, MMS, chain-terminating nucleoside analogs, MMC, and trimethyl psoralen activated with UV-A (TMP-UVA) (Bai et al., 2020; Bianchi et al., 2013; Keen et al., 2014a; Kobayashi et al., 2016; Olivieri et al., 2020). Similarly to human cells, PRIMPOL-KO mice are also hypersensitive to MMC and display a significant impairment of bone marrow cell proliferation when treated with this drug (González-Acosta et al., 2020). These studies suggest that PRIMPOL mediates the cellular response to an array of DNA-damaging agents that perturb replication fork progression. Suppprting this notion, loss of PRIMPOL causes defects in replication fork progression and restart, increased sister chromatid exchanges, increased mutagenesis, and micronuclei formation following UV-C radiation and treatment with ICL-inducing agents (Bailey et al., 2019; González-Acosta et al., 2020; Mouron et al., 2013). However, TLS pathways can partially mitigate the replication defects associated with PRIMPOL loss. For example, loss of PRIMPOL significantly affects proliferation and viability in avian DT40 cells only when PRIMPOL is depleted in combination with the TLS polymerases POL η and POL ζ, suggesting that these two TLS polymerases partially compensate for PRIMPOL loss (Kobayashi et al., 2016). Along the same lines, loss of PRIMPOL does not seem to sensitize human cells to UV-C, unless POL η is co-depleted (Bailey et al., 2019), supporting the notion that POL η and PRIMPOL might have complementary roles in response to UV damage. Interestingly, POL κ and POL ζ appear to slow replication forks during the S-phase (Jones et al., 2012; Mehta et al., 2020), suggesting that they might engage on replication forks to prevent faster pathways such as PRIMPOL-mediated repriming from acting in the first place.

Similar to repriming, fork reversal is a physiologically important mechanism to deal with different kinds of replication challenges (Zellweger et al., 2015). However, there are instances when replication fork reversal is deleterious for genome integrity. For example, reversed forks are extensively degraded by nucleases if they are not adequately protected by BRCA proteins (Kolinjivadi et al., 2017; Lemacon et al., 2017; Mijic et al., 2017; Taglialatela et al., 2017). Treatment with multiple cisplatin doses suppresses replication fork reversal and promotes repriming by PRIMPOL in BRCA1-deficient cells (Quinet et al., 2020). These studies suggest that in the absence of BRCA proteins, cells adapt to treatment with multiple cisplatin doses by suppressing fork reversal and upregulating PRIMPOL-mediated repriming as an alternative strategy to cope with cisplatin-induced lesions and prevent pathological reversed fork degradation. Of note, the ATR pathway is a key regulator of the PRIMPOL-dependent adaptive response to cisplatin treatment (Quinet et al., 2020). Moreover, PRIMPOL overexpression decreases BRCA1-deficient cell sensitivity to co-treatments with cisplatin and ATR inhibitors (Quinet et al., 2020), suggesting that the

PRIMPOL pathway regulates chemotherapy response to combinatorial treatments with ATR inhibitors, which are currently in clinical trials (NCI-2016–00355).

As already discussed, PRIMPOL-mediated repriming can be more generally activated also in BRCA-proficient cells under conditions of impaired fork reversal, such as loss of the SMARCAL1 or HLTF translocases (Bai et al., 2020; Quinet et al., 2020). Although loss of either SMARCAL1 or HTLF promotes repriming, there are some notable difference among the cellular phenotypes of SMARCAL1 and HLTF-depleted cells. Loss of HLTF increases resistance to HU and MMC (Bai et al., 2020), whereas loss of SMARCAL1 increases cellular sensitivity to HU, CPT, and the DNA polymerase inhibitor aphidicolin (Bansbach et al., 2009). Moreover, loss of HLTF but not SMARCAL1 leads to unrestrained fork progression in a PRIMPOL-dependent manner, as already discussed. The different effects observed upon HLTF and SMARCAL1 loss once again point to important differences between these fork reversal factors, which deserve further investigation.

As fork reversal becomes pathological when reversed forks cannot be adequately protected, PRIMPOL repriming likely leads to increased genomic instability when the ssDNA gaps that form as a consequence of PRIMPOL activity cannot be properly repaired. Indeed, loss of factors involved in gap-filling, such as TLS POL ζ or REV1, significantly increases genomic instability and cell sensitivity to UV-C irradiation (Quinet et al., 2016; Temviriyanukul et al., 2012). When left unrepaired, ssDNA gaps can collapse into DSBs (Elvers et al., 2011; Quinet et al., 2016; Saxena et al., 2019). At least a fraction of these DSBs can be repaired by a HR-mediated mechanism dependent on RAD51, ATR, and the RAD51-paralog XRCC3 (Elvers et al., 2011; Saxena et al., 2019). In the absence of XRCC3, DSBs are not repaired, thereby compromising cell viability (Saxena et al., 2019). In addition, ssDNA gaps activate the ATR/CHK1 pathway in human cells (Jansen et al., 2009; Quinet et al., 2014), and ATR inhibition or depletion increases cell sensitivity to genotoxic agents under conditions of ssDNA gap accumulation (Quinet et al., 2014; Saxena et al., 2019). Moreover, under-replicated DNA that proceeds into mitosis can be converted to DNA lesions, presumably DSBs, and shielded by 53BP1 nuclear bodies in the following G1 phase (Harrigan et al., 2011; Lukas et al., 2011). We speculate that a similar mechanism applies to persistent unrepaired ssDNA gaps and that protection by 53BP1 nuclear bodies would provide a tolerance mechanism until the lesions are repaired.

The idea that ssDNA gaps become toxic intermediates if they are not repaired in a timely fashion is supported by recent studies showing that ssDNA gap accumulation correlates with chemotherapy response in BRCA-deficient tumors. In particular, Cong et al. found that PARP inhibition promotes accumulation of ssDNA gaps in BRCA-deficient cells and that ssDNA gaps are no longer present when the same cells acquire PARP inhibitor resistance (Cong et al., 2019). Along the same lines, Panzarino et al. found that loss of the chromatin remodeling enzyme CHD4 as well as its interaction partners PARP1, EZH2, FEN1, and ZFHX3 suppresses the accumulation of ssDNA gaps in BRCA2-deficient cells while conferring chemoresistance (Panzarino et al., 2019). Moreover, they found an interesting correlation between gap suppression and chemoresistance using BRCA1-null breast cancer patient-derived xenografts with differential sensitivities to cisplatin (Panzarino et al., 2019). The underlying mechanisms of defective gap-filling and the ensuing chemosensitivity in

these genetic backgrounds are unknown. In addition, how exactly loss of CHD4 or its interacting partners leads to gap suppression and enhanced cell resistance in BRCA-null cells remains unclear.

The studies on the mechanisms that regulate the choice between repriming, TLS, and TS are central to understand how replicating cells mediate lesion tolerance while maintaining genome stability and also raise several outstanding questions to be addressed in the future: Are there other factors or signaling pathways that influence the balance between repriming, TLS, and TS mechanisms? What is the relative contribution of TLS *versus* TS and HR to gap filling in the human genome? How does activation of PRIMPOL and the ensuing accumulation of ssDNA gaps correlate with genome instability and cell viability in response to chemotherapeutics? Can we target factors involved in gap-filling to improve DNA-damaging chemotherapy response? For example, overexpression of the REV1 and POL ζ polymerases has been associated with chemotherapy resistance in a variety of cancers such as glioma, cervical cancer, and ovarian carcinoma (Rocha et al., 2018), paving the way to the proposal of inhibition of ssDNA gap filling by TLS as a novel strategy for targeted cancer therapy (Yamanaka et al., 2017). A key question for future studies is to identify other factors involved in gap-filling that could potentially be targeted in cancer therapy. Answering these pressing questions is mandatory to fully define the molecular contexts in which cells choose repriming over TS and TLS activity, as well as to develop efficient strategies to target these pathways in cancer therapy.

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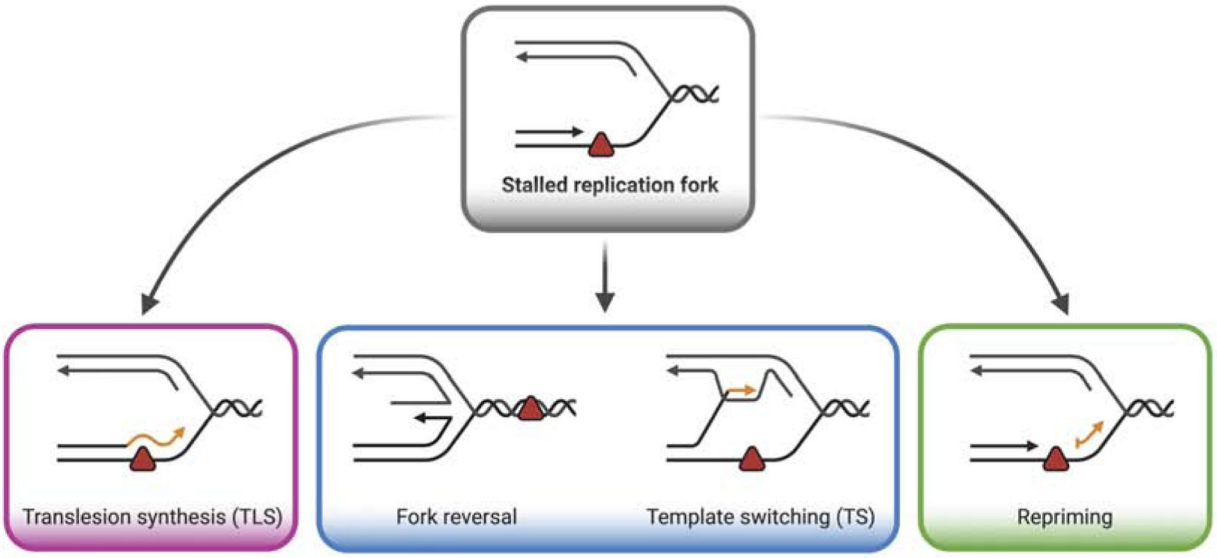

Figure 1. DNA replication stress response mechanisms.

Replication obstacles (represented by the red triangle) transiently stall fork progression. Replication obstacles can be "tolerated" by three distinct pathways to allow resumption of replication fork progression: translesion synthesis (left), template switching or fork reversal (middle), and repriming (right).

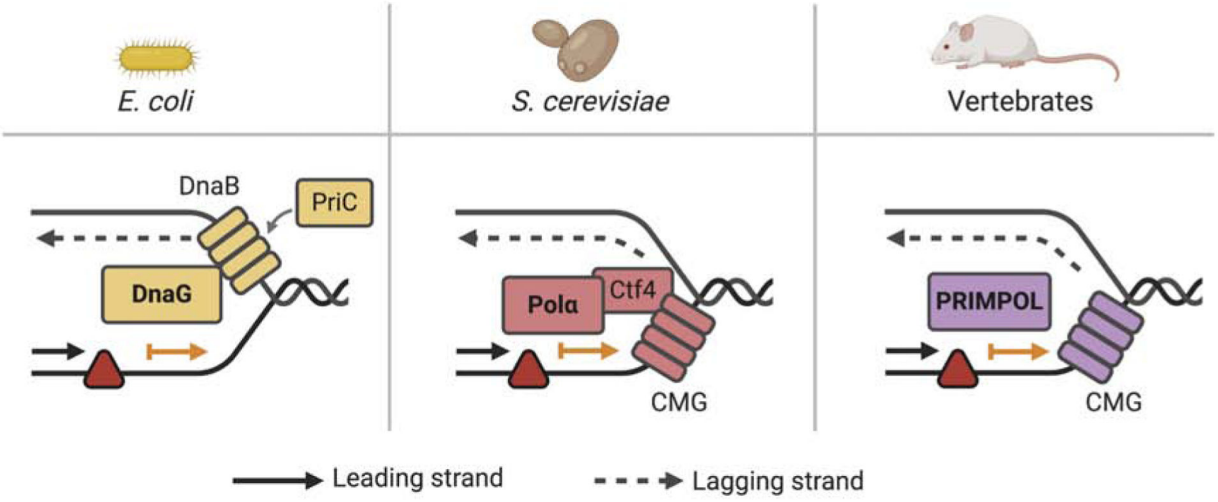

Figure 2. Mechanisms of repriming and ssDNA gap formation in different organisms.

In *E. coli*, the DnaG primase, as part of the PriC system, interacts with DnaB and promotes repriming in both the leading and lagging strand (left). In budding yeast, repriming is promoted by the Polα/Primase complex and Ctf4, a replisome factor that bridges the MCM component of the CMG helicase and the Polα/Primase complex (middle). In vertebrates, repriming is ensured by PRIMPOL. How recoupling of leading strand synthesis and the CMG helicase occurs after PRIMPOL-mediated repriming *in vivo* is unknown.

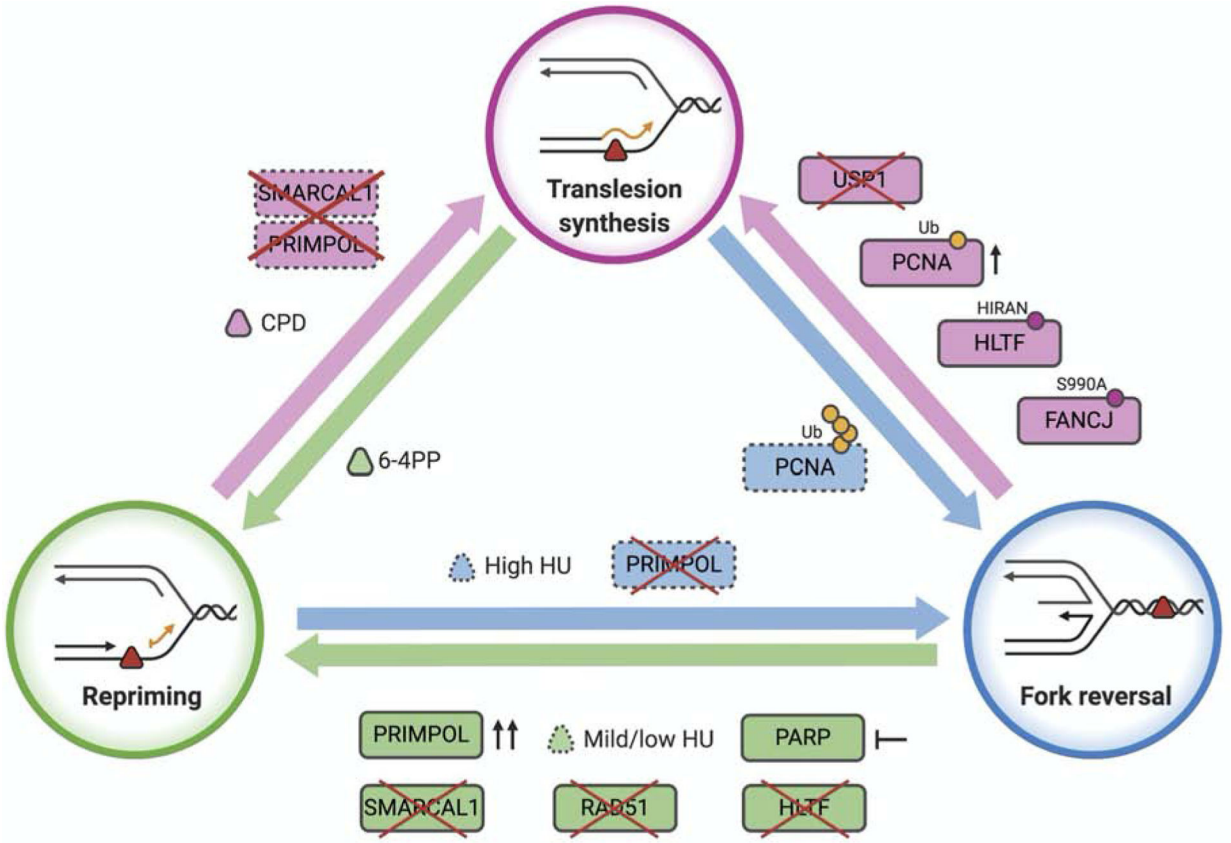

Figure 3. Factors influencing the choice between the TLS, fork reversal and repriming.
Dashed lines indicate factors that need more investigation. See text for details.