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SURVEY AND SUMMARY

DNA damage tolerance in stem cells, ageing, mutagenesis, disease and cancer therapy

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ABSTRACT

The DNA damage response network guards the stability of the genome from a plethora of exogenous and endogenous insults. An essential feature of the DNA damage response network is its capacity to tolerate DNA damage and structural impediments during DNA synthesis. This capacity, referred to as DNA damage tolerance (DDT), contributes to replication fork progression and stability in the presence of blocking structures or DNA lesions. Defective DDT can lead to a prolonged fork arrest and eventually cumulate in a fork collapse that involves the formation of DNA double strand breaks. Four principal modes of DDT have been distinguished: translesion synthesis, fork reversal, template switching and repriming. All DDT modes warrant continuation of replication through bypassing the fork stalling impediment or repriming downstream of the impediment in combination with filling of the single-stranded DNA gaps. In this way, DDT prevents secondary DNA damage and critically contributes to genome stability and cellular fitness. DDT plays a key role in mutagenesis, stem cell maintenance, ageing and the prevention of cancer. This review provides an overview of the role of DDT in these aspects.

INTRODUCTION

Prior to each cell division, DNA has to be faithfully replicated and chromosomes equally distributed over the daughter cells, as failure to maintain DNA integrity may lead to premature ageing and cancer (1). The DNA constantly suffers from lesions arising through endogenous as well as exogenous processes. Endogenous sources of DNA damage include spontaneous deamination, reactive oxygen species (ROS), S-adenosylmethionines, aldehydes and active enzymatic DNA processes like cytosine deamination by members of the AID/APOBEC family (2,3). Examples of exogenous sources of DNA damage include UV irradiation, γ-irradiation, tobacco smoke carcinogens, alcohol and DNA damaging chemotherapeutics. Thus, our genomes are constantly exposed to DNA damage and dedicated DNA damage response pathways protect the genome from DNA damages. The DNA damage response network can be divided into DNA damage signalling, DNA damage repair and DNA damage tolerance (DDT) pathways. Specific DNA repair pathways repair defined spectra of DNA lesions. These pathways include base excision repair, nucleotide excision repair, interstrand crosslink repair, mismatch repair, ribonucleotide removal pathway, direct damage reversal and several double strand break repair pathways (1,4). Next to DNA repair, the DDT system plays a key role during DNA replication, mainly in response to DNA damage. As not all lesions are repaired prior to DNA replication, replication forks may run into these lesions. When replication forks stall, lesions cannot be repaired canonically, as the two strands are separated and hence direct access to the repair-template is lacking.

Replicative polymerases δ and ε (POLδ and POLε) perform the bulk of DNA synthesis, operate on the lagging or leading strand, respectively (5–7), and are highly specialized in warranting precise and swift DNA synthesis (8). Furthermore, replicative polymerases POLδ and POLε have proofread capacity to prevent mis-incorporation of nucleotides. Due to these traits, lesions in the DNA can stall replicative polymerases, as these lesions generally do not base pair well with incoming nucleotides and therefore cannot be accommodated in the catalytic site of replicative polymerases, leading to replication fork stalling. Lesions that stall replicative polymerases include abasic sites, base adducts, intrastrand crosslinks, interstrand crosslinks, cyclobutane pyrimidine dimers (CPD) and (6–4) photoproducts (6-4PP). Furthermore, replicative polymerases can be

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halted by structures such as DNA–protein crosslinks, incorporated ribonucleotides, secondary DNA structures as G4 structures, fragile sites and R-loops (9–13). Replicative bypass of replication fork stalling structures and lesions requires DDT pathways. DDT is defined as the restarting of replication after replication fork stalling and filling in post replicative gaps generated upon repriming 3′ of the replication impediment. When replication forks stall, helicases and replicative polymerases are uncoupled, exposing an increased amount of single-stranded DNA. Single-stranded DNA is coated with replication protein A (RPA) protein, which activates DDT to prevent prolonged stalling and consequent fork collapse (14–17). Modes of DDT are translesion synthesis (TLS), fork reversal, template switching and repriming (18–23) (Figure 1). Repriming can be followed by post-replicative gap filling by TLS or template switching. TLS involves replicative bypass directly opposite the fork stalling entity by specialized TLS polymerases. During fork reversal, the replication fork reverts to a chicken foot like structure in order to use the nascent strand of the sister chromatid as a template to bypass fork stalling lesions. Repriming involves primer formation 3′ of the fork stalling lesion, allowing a quick restart of the fork but generating a post-replicative gap. Template switching uses the nascent sister chromatid as a template in post-replicative gap filling, in order to bypass the fork stalling lesion.

TRANSLESION SYNTHESIS (TLS)

TLS is performed by specialized DNA polymerases, which insert DNA opposite of a damaged template or replication impediment. These specialized TLS polymerases generally have larger active sites and lack proofread-activity, which allows non-Watson Crick base pairs to accommodate within the catalytic site (24). Both features enable TLS polymerases to tolerate lesions that stall replicative polymerases: POLδ and POLε. Mammalian DNA polymerases with TLS capabilities include REV1, POLH (POLη), POLI (POLι), POLK (POLκ), POLN (POL*ν*), POLQ (POLθ) and POLζ (consisting of catalytic subunit REV3/accessory subunit REV7) and PRIMPOL (8,18–20,25). POLH, POLI, POLK and REV1 belong to the Y-family of DNA polymerases. Most TLS polymerases use the lesion as template, REV1 on the other hand is a DNA template independent dCMP-transferase that uses its own arginine residue 324 as protein template (26). The TLS polymerase complex POLζ catalytic subunit REV3 is a B-family polymerase and can extend from mismatches left by other TLS polymerases. The A-family of DNA polymerases comprising POLQ and POLN also have TLS capability, though the role of POLN in DDT remains unknown (8,25,27). POLQ plays a role in alternative-end joining, a DNA double strand break repair pathway (28,29). In addition, POLQ is also involved in TLS (30).

Regulation of TLS by PCNA monoubiquitination

Central to the regulation of DDT is proliferating cell nuclear antigen (PCNA). PCNA is a homotrimeric DNA clamp that acts as a key processivity factor for many DNA polymerases and central hub regulating a myriad of processes during DNA replication, tolerance and repair (16,31). Regulation of TLS occurs through site-specific PCNA monoubiquitination (PCNA-Ub) on lysine 164 (K164), which promotes polymerase switching from one of the replicative POLδ or POLε to a TLS polymerase (Figure 2) (32–34). PCNA is monoubiquitinated by the E2/E3 ubiquitin ligase complex RAD6/RAD18. Other E3 ligases targeting PCNA K164 have been revealed, such as $CRL4^{Cdt2}$ and RNF8 (35–38). Additionally, spliceosome-associated factor 3 (SART3) has also been reported to affect PCNA monoubiquitination and POLH recruitment into foci (39). Furthermore, PCNA-associated factor 15 (PAF15) is double monoubiquitinated and regulates polymerase switching through its interaction with PCNA (40,41). PAF15 is deubiquitinated upon fork stalling, this releases PCNA and facilitates interaction with TLS polymerases. *Paf15* depletion through shRNA-mediated knockdown leads to increased mutagenesis upon UV exposure, likely due to an increased error-prone TLS activity. *In vitro* analysis using purified PCNA, POLH and PAF15 revealed an inhibition of POLH TLS when PAF15 is present (42).

E3 ubiquitin ligase HECT, UBA And WWE Domain Containing 1 (HUWE1) has been shown to interact with PCNA, which is important for suppressing replication stress, though the exact mechanism remains unclear (43). FANCD2-associated nuclease 1 (FAN1) has an PCNA interaction peptide (PIP) through which it binds to PCNA-Ub (44). The FAN1 PIP is required for FAN1 foci formation and prevents fork collapse upon replication fork stalling induced by G4 stabilizing compounds. This function of FAN1 is likely independent of its function in interstrand crosslink repair, as FAN1 foci still form upon interstrand crosslink inducer MMC in cells expressing FAN1 PIP mutant.

Factors that regulate PCNA and RAD18 interaction includes BCRA1. BRCA1 is a key factor in double strand break and interstrand crosslink repair, though also regulates RAD18 (45). BRCA1 deficiency impairs RAD18 recruitment and PCNA K164 ubiquitination. Next to BRCA1, SIVA1 apoptosis inducing factor (SIVA1) was found to direct RAD6/RAD18 to ubiquitinate PCNA (46). In addition, SprT-like domain-containing protein SPRTN is involved in a feed forward loop with PCNA-Ub, as SPRTN is recruited by PCNA-Ub, and also involved in recruitment of RAD18 and TLS polymerases that requires the DNA-binding domain of SPRTN (47–52). Whether this relates to a direct effect on recruitment of TLS polymerases or through stabilization of PCNA-Ub remains to be established. Additionally, NIBRIN (NBN) that is mutated in Nijmegen breakage syndrome also affects PCNA-Ub formation through interaction with RAD18. Knockdown of NBN leads to decreased number of POLH foci, UV sensitivity and increased mutagenesis (53).

Deubiquitinase USP1 is the most investigated deubiquitinating enzyme of PCNA-Ub, surprisingly USP1 is degraded through autocleavage upon DNA damage induction by UV, but not mitomycin C (MMC), methyl methanesulfonate (MMS) or hydroxylurea (HU) (54,55). The degradation of USP1 upon UV is counterintuitive, considering that the PCNA-Ub trimer should be deubiquitinated to dissociate from the TLS polymerase once the TLS step is completed. This suggests that USP1 activity keeps PCNA deubiquiti-

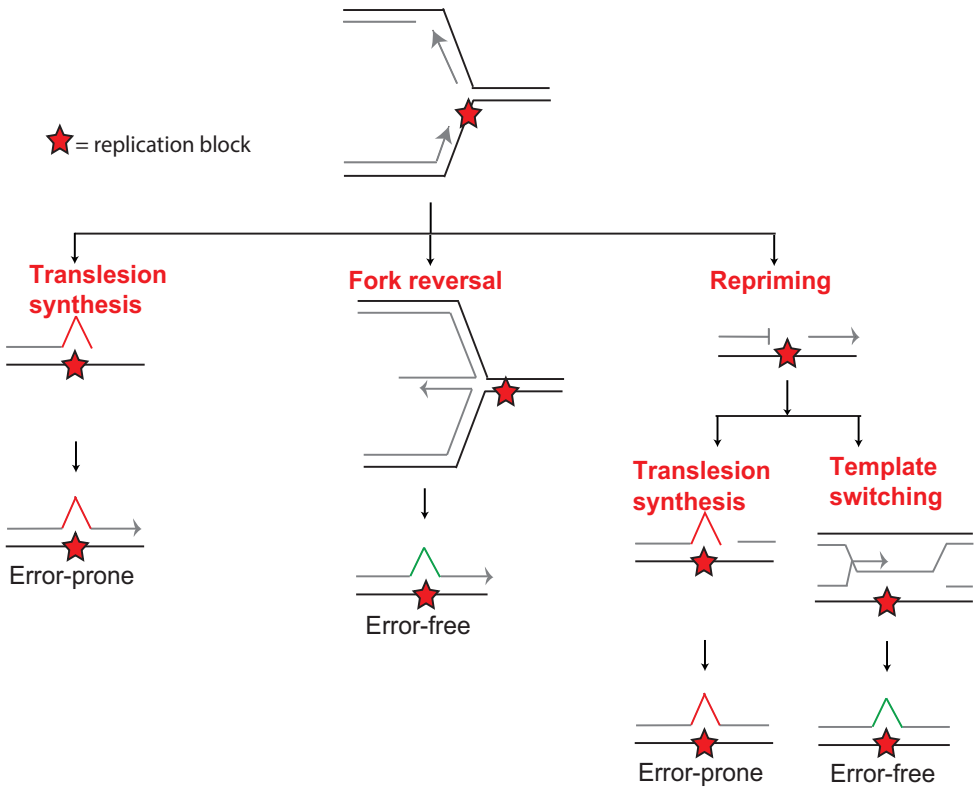

Figure 1. Modes of DNA damage tolerance. Upon stalling of the replication fork, several modes of DDT can be activated. TLS and fork reversal can occur at the stalled fork. Alternatively, TLS or template switching can occur after repriming event. Figure adapted from (23).

nated under DNA damage free conditions and that there is yet another USP responsible in PCNA K164 deubiquitination once TLS is completed. USP1 has been reported to interact with PCNA unloading factor ATPase family AAA domain containing 5 (ATAD5) and this interaction was considered important in the deubiquitination of PCNA-Ub (56,57). The release of TLS polymerase from PCNA-Ub is complex. PCNA-Ub can be modified by ubiquitin-like modification E3 ubiquitin/ISG15 ligase TRIM25/EFP. TRIM25/EFP ISGylates PCNA-Ub on two lysine residues, one of which is K168 (58). ISGylation of the two lysine residues was demonstrated to depend on PCNA K168. ISGylated PCNA (PCNA-ISG) in turn binds USP10, which deubiquitinates PCNA-Ub leading to the release of POLH. PCNA-ISG is then deISGylated by UBP43, enabling the switch back to a replicative polymerase. Apparently, ISGylation signals the release of TLS polymerases from PCNA.

Regulation of TLS by REV1

Next to PCNA-Ub, TLS polymerase REV1 can recruit other TLS polymerases independently of PCNA-Ub, through its C-terminal domain (59–62) (Figure 2). REV1 is ubiquitinated and interacts with Fanconi anemia associated protein 20 (FAAP20), a subunit of the Fanconi core complex, in UV-induced REV1 foci (63). The REV1 FAAP20 complex is also associated with PCNA at the replication fork, though it is not clear whether this process is PCNA K164 ubiquitination dependent. Interestingly, the knockdown of *Polh* in mouse embryonic fibroblasts (MEFs) also leads to a reduction of UV-induced REV1 foci, suggesting that also POLH has a recruiting function (64). The genetic characterization of *Rev1* and *Pcna K164* has shown that REV1 and PCNA K164 are mainly non-epistatic (65,66), indicating that there is a PCNA-K164-dependent DDT pathway and a REV1-dependent DDT pathway. Although REV1 functions independently of PCNA-Ub, it also operates in a PCNA-Ub-dependent manner as seen with increased binding efficiency with PCNA-UB over PCNA (65). Furthermore, the presence of REV1 increases PCNA-Ub levels, likely by stabilizing the RAD18 interaction with PCNA (67).

In summary, the recruitment and exclusion of potentially error-prone TLS polymerases are tightly regulated by a myriad of protein interactions and post-translational modifications. Furthermore, PCNA and REV1 seem to exert mainly non-epistatic functions regarding the regulation of DDT.

HOMOLOGY-DIRECTED DNA DAMAGE TOLERANCE

During homology-directed DDT, the newly synthesized DNA strand of the sister chromatid is used as template in order to bypass the fork stalling entity either at the ongoing replication fork or in a post-replicative gap setting. Two pathways are thought to perform homology-directed DDT, specifically fork reversal and template switching (21,67,68) (Figure 3). In mammals, fork reversal seems the dominant homology-directed pathway, whereas in yeast template switching is more dominant (69). Compared to TLS, both homology-directed DDT pathways are considered rel-

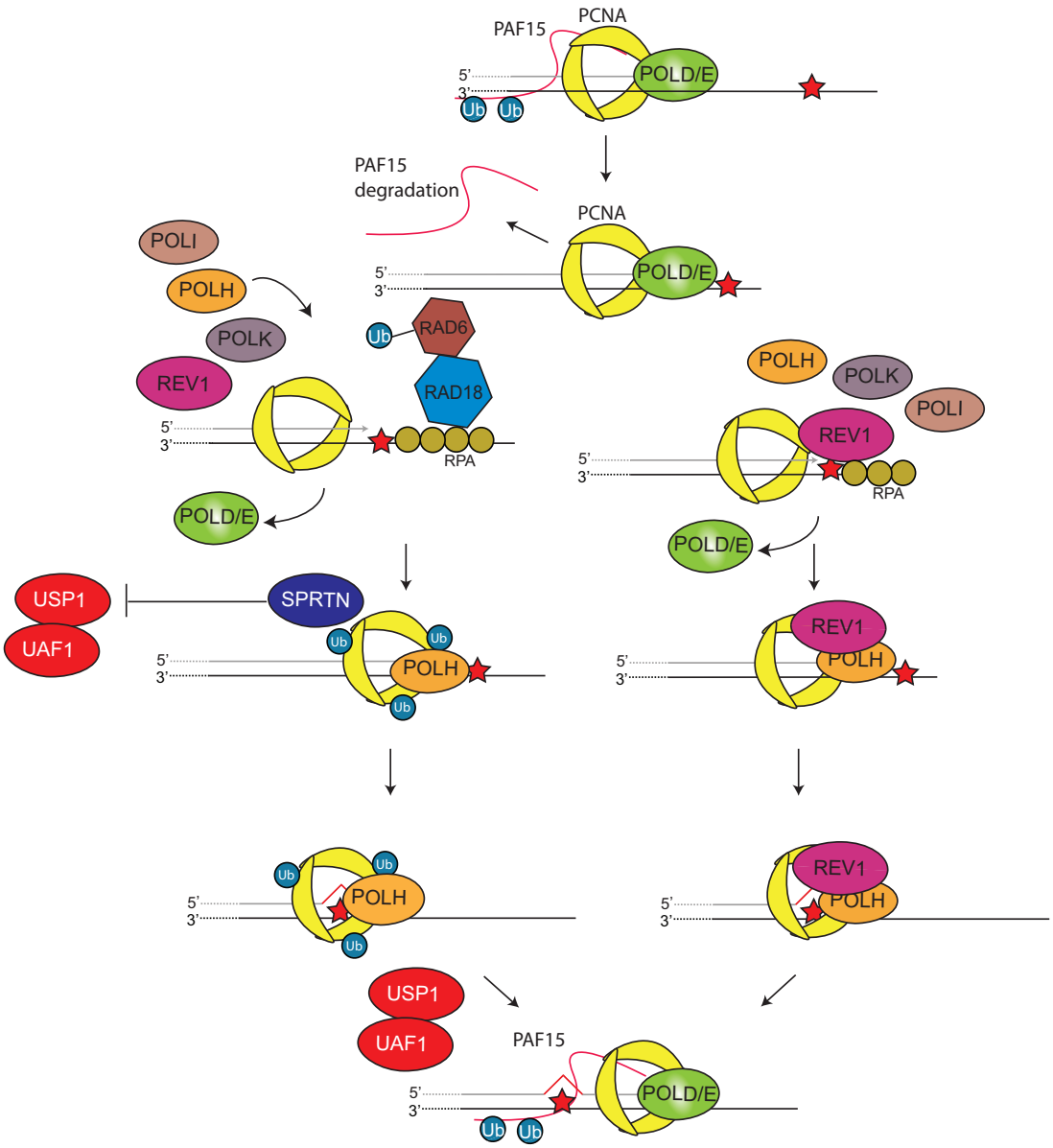

Figure 2. Translesion synthesis. TLS pathways are regulated by PCNA K164 ubiquitination or REV1. PCNA Ub can also interact with REV1 directly, as with other TLS polymerases. PAF15 is degraded upon fork stalling, which allows PCNA to interact with TLS polymerases. RPA recruits RAD6/RAD18 ligase that can ubiquitinate PCNA to recruit TLS polymerases. SPRTN binds PCNA Ub preventing PCNA deubiquitylation. After TLS is completed, the TLS polymerase replaced again by the replicative polymerase. In the REV1-dependent pathway, REV1 can recruit other TLS polymerases. Figure adapted from (23).

atively error-free (70). Both homology-directed DDT pathways depend on the formation of PCNA polyubiquitination (PCNA-Ub^n), where one of the Rad5 homologues SNF2 histone linker PHD RING helicase (SHPRH) or helicase-like transcription factor (HLTF) extends a Ub-K63 linked polyubiquitin moiety to PCNA-Ub. The observation that PCNA-Ub^n is still formed in the absence of both SHPRH and HLTF implies the existence of at least one additional ubiquitin ligase generating PCNA-Ub^n (71). For a more detailed insight into the molecular mechanisms of template switching and fork reversal, see these reviews (21,72). In mouse and human cells, the frequency of PCNA-Ub^n, the main indicator of homology-directed DDT, appears quite rare as suggested by steady state levels of PCNA-Ub versus PCNA-Ub^n (3435,71,73,74). In both homology-directed DDT mechanisms, the fork stalling entity can be avoided by using the newly synthesized template of the other DNA strand.

Fork reversal

During fork reversal, the replication fork regresses from a fork into a chicken foot-like DNA structure (72) (Figure 3). In the chicken foot structure, the replication machinery has the opportunity to use the newly synthesized strand of the intact sister chromatid as alternative to the damaged template. Though it has not been proven directly

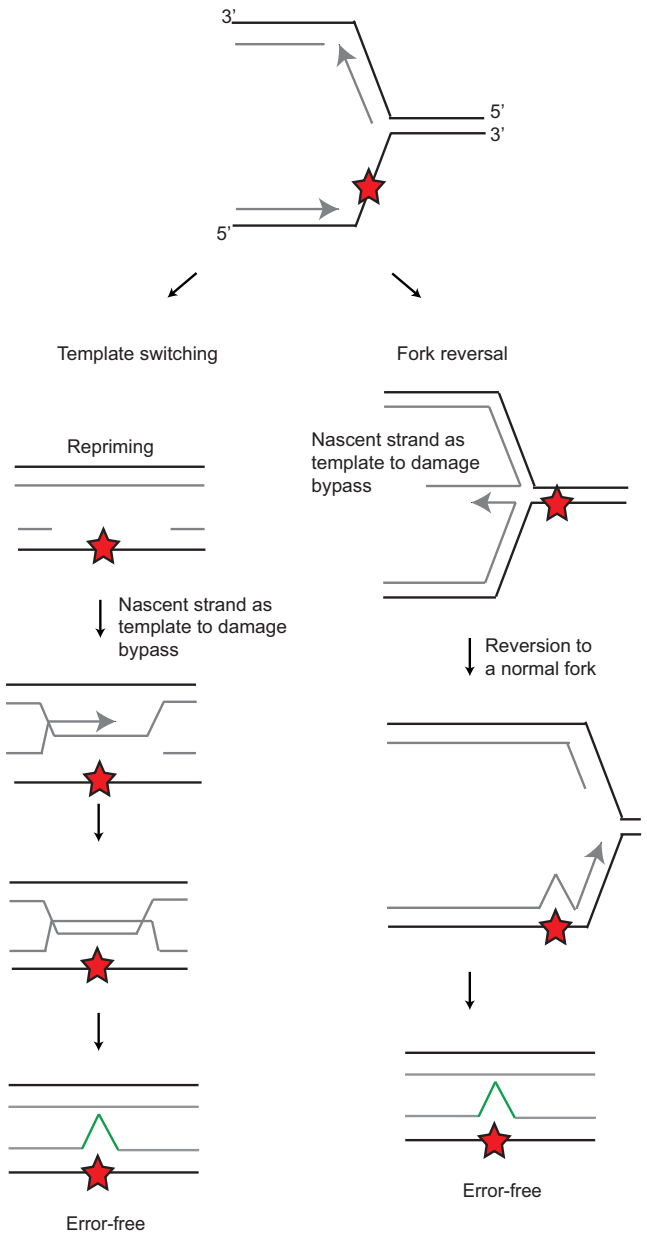

Figure 3. Homology-directed DNA damage tolerance. Homology-directed DDT involves template switching and fork reversal. In order to prevent use of the fork stalling entity as template, the nascent DNA of the sister chromatid is used. Template switching occurs after a priming event in a post replicative gap. After fork stalling, the replication fork can reverse. Fork reversal leads to a chicken foot intermediate structure, which allows access to the nascent DNA of the sister chromatid.

that fork reversal promotes damage bypass, it is an attractive model for the function of the fork reversal as fork reversal gives nascent DNA of the stalled strand direct access to the nascent homologues undamaged template. Alternatively, fork reversal may occur to stabilize the stalled fork (22). PCNA-Ub^n formation facilitates fork reversal and E2 ubiquitin ligase UBC13 is needed to generate a K63-linked polyubiquitin moiety at PCNA K164 (69). Translocase zinc finger RANBP2-type containing 3 (ZRANB3) binds PCNA-Ub^n and remodels the replication fork to a reversed fork and is involved in D-loop dissociation (69,74–76). Another translocase, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a like 1 (SMARCAL1) is also involved in promoting fork reversal (75,77). SMARCAL1 can evict RPA from the DNA and reanneal complementary single-stranded DNAs (78–80). In this manner, SMARCAL1 is involved in reversing stalled replication forks. Furthermore, SMARCAL1 phosphorylation by ATR prevents nuclease-mediated fork collapse (81). The phosphorylation of SMARCAL1 by ATR limits fork regression by SMARLCAL1. Other factors that are involved in fork reversal are the homologous recombination proteins BRCA2 and RAD51. These factors protect the fork form Meiotic Recombination 11 Homologue 1 (MRE-11) exonuclease activity (82–84). When forks are reversed, these structures need to be protected from MRE-11 exonuclease activity. Without protection of the reversed fork, MRE-11 degrades the newly synthesized DNA of the leading and lagging strand from 3′ to 5′ and 5′ to 3′, respectively (85). As *in vitro* MRE-11 only has a 3′–5′ resection activity, the 5′ to 3′ degradation of the lagging strand may involve another exonuclease whose activity depends on MRE-11 (86). In addition, RecQ like helicase (RECQL), DNA replication helicase/nuclease 2 (DNA2) and Werner syndrome RecQ like helicase (WRN) helicases are involved in promoting replication fork restart during fork reversal (22). Another report in human cells suggest that RECQL limits DNA2- and WRN-mediated degradation of the reversed fork to create the 3′ overhang in the reversed fork (87). Furthermore, PARP1 is involved in promoting fork reversal, as shown in topoisomerase inhibitor induced fork reversal (88). In addition, helicase FANCM has been implicated in fork reversal in promoting branch migration, as shown using purified FANCM (89,90). This observation has been corroborated independently in fission yeast, where FANCM orthologue Fml1 promotes fork reversal (91).

Template switching

Template switching occurs in post replicative gap filling and serves to close the single-stranded gap containing the lesion in an error-free manner (21) (Figure 3). Template switching involves formation of structures resembling double holiday junctions (92). In *Saccharomyces cerevisiae*, it has been demonstrated that the cohesion/condensing-like Smc5/6 complex stimulates template switching. Furthermore, template switching is needed to replicate through natural pausing sites (93). In the same model system, the helicase Pif1 was described to be key in template switching through enlarging single-stranded DNA in post-replicative gaps (94). The combined action of the E3 ubiquitin ligases Rad18- and Rad5 generates PCNA-Ub^n, which is necessary for template switching. Next to PCNA-Ub^n, PCNA SUMOylation mediated by Mms2 and Ubc13 has been shown to promote template switching in *S. cerevisiae* (95). Furthermore, PCNA SUMOylation prevents the formation of DNA double strand break formation and recombination upon fork stalling (96). Human PCNA is SUMOylated on several residues, including K164. K164 SUMOylation is not site-specific, as K164R mutation does not abrogate PCNA SUMOylation, although the level of SUMOylation was

found to decrease. *Rad18*-deficient cells are used as PCNA-Ub free system, though there are alternative E3 ligases in higher eukaryotes that can ubiquitinate PCNA. Therefore, it remains unclear whether the PCNA K164R mutation leads to SUMOylation related defect in the human system. In line with preventing double strand break formation, PCNA SUMOylation prevents spontaneous gene conversion (97).

RESTARTING DNA SYNTHESIS BY REPRIMING

In order to start DNA synthesis, a primase is required that generates a primer for a DNA polymerase. On the lagging strand this is warranted by continuous priming by POLα. Therefore, a stalled fork does not necessarily cause a stop of replication on the lagging strand (98). On the leading strand, replication is usually continuous and hence repetitive priming by POLα is not required. Therefore, on the leading strand a stalled fork might have more impact on the progression of replication. However, also on the leading strand repriming occurs (99,100). In mammals, this task appears primarily executed by the primase and polymerase PRIMPOL (18,20,101). In contrast to POLα that produces an RNA primer and subsequently extends the primer with DNA, PRIMPOL uses mainly dNTPs for priming (20). PRIMPOL has both primase and TLS polymerase activity, though the primase activity seems to be more important for fork progression and genome stability (102,103). PRIMPOL reprimes after lesions to rapidly restart replication (18,20,101). PRIMPOL binds to single-stranded DNA–RPA filaments. At high concentrations of RPA, RPA inhibits PRIMPOL activation (Figure 4). However, at non-saturating RPA conditions, single-stranded RPA–DNA filaments do activate PRIMPOL (104,105). This model fits with the observation that *Primpol*-defective MEFs do not have a replicative defect upon low dose UV-C exposure, though at a higher dose they were found impaired (106). Furthermore, free RPA becomes rate limiting in high levels of DNA damage or replication fork stalling (107). These data suggest that the concentration of unbound RPA regulates PRIMPOL activation. In chicken DT40 lymphoma cells, loss of PRIMPOL leads to epigenetic instability especially at G4 stacks on the leading strand (108). The epigenetic instability refers to loss of histone marks during replication. Under steady state conditions, histone marks are the same on the DNA before and after replication of a locus as many histones are recycled and distributed over the sister chromatids, but under certain replication stresses the histone marks can be instable possibly due to defective histone recycling (109). Using the same system, also REV1 was shown to exert the same effect on the epigenome (10). In this earlier article, one of the conclusions was that G4s on the lagging strand do not affect epigenetic stability. The authors suggest that this may be due to continuous repriming. Thus, G4-induced epigenetic instability in this setting cannot be used for restriction of activity to the leading strand, due to the absence of an effect on the lagging strand. The only evidence that PRIMPOL selectively targets the leading strand is based on the analyses of the

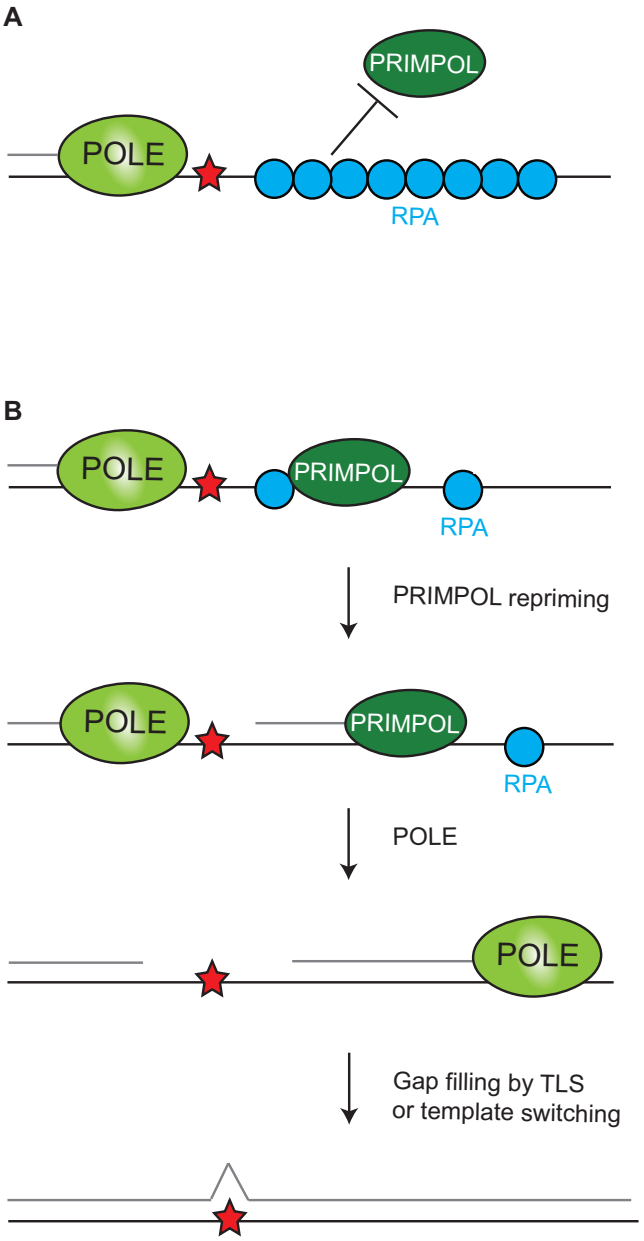

Figure 4. Regulation of PRIMPOL by RPA. (**A**) PRIMPOL is inhibited from access to single-stranded DNA by high concentration of RPA on DNA. (**B**) When the amount of RPA coating single-stranded DNA is low, PRIMPOL can bind single-stranded DNA and form a primer for a replicative polymerase to continue.

different base-to-base substitutions during somatic hypermutation of *Igh* genes in *PrimPol*-deficient B cells, where a strand-biased anti-mutagenic effect of PRIMPOL on the leading strand was observed (106,110). This anti-mutagenic effect likely relates to a preferential PRIMPOL activity on the leading strand, promoting an error-free DDT pathway such as template switching. Additional evidence for this hypothesis awaits further experimentation. At present, it re-

mains unclear how the system targets PRIMPOL preferentially to the leading strand.

TRANSLESION SYNTHESIS DURING INTERSTRAND CROSSLINK REPAIR

Fanconi anemia is a disease characterized by reduced fertility, bone marrow failure induced anemia, and increased risk of acute myeloid leukemia and solid tumours (111). Furthermore, patients show increased chromosomal instability and sensitivity to interstrand crosslink inducing agents such as mitomycin C and cisplatin. This is due to disease-causing mutations found in genes associated with interstrand crosslink repair. The Fanconi anemia pathway is involved in interstrand crosslink repair. Interstrand crosslinks are covalently linked bases on opposite strands of the Watson/Crick double helix, and prohibit the melting of two DNA strands necessary for central biological processes, such as transcription and replication. Consequently, DNA interstrand crosslinks are highly toxic lesions (112). Interstrand crosslink repair appears one of the molecularly most challenging and intricate DNA repair processes. Most relevant in the context of this review, interstrand crosslink repair depends on TLS. During S phase, as demonstrated in Xenopus nuclear egg extracts, interstrand crosslinks require two converged and stalled replisomes for replication-coupled repair (113). Upon stalling of the replication fork, FANCD2 and FANCI are monoubiquitinated by the Fanconi anemia core complex. The monoubiquitination of FANCD2 is necessary for recruitment of nucleases that then make the incisions and unhook the interstrand crosslink (114) (Figure 5A). After unhooking of the crosslink, TLS is required to continue replication opposite the non-instructive, unhooked interstrand crosslink. REV1 and POLζ have been implicated in this step (115). At present, the role of PCNA-Ub is ill defined, as studies in *Xenopus* egg extracts could not adequately deplete PCNA to determine the reliance of interstrand crosslink repair on PCNA K164 (115). Resolving the molecular details of interstrand crosslink repair greatly benefitted from studies in nuclear *Xenopus* egg extracts using cisplatin-induced interstrand crosslinked plasmids.

Of note, many mammalian studies frequently used psoralen, as they are known to induce specifically interstrand crosslinks. However, as shown in *Xenopus* egg extracts, these appeared to be repaired by an alternative interstrand crosslink repair pathway involving the DNA glycosylase NEIL3. NEIL3 has also been shown to repair abasic site induced interstrand crosslinks in *Xenopus*. Both psoralen and abasic site induced interstrand crosslinks do depend on REV1 for TLS (116) (Figure 5B and C). In the case of psoralen-induced interstrand crosslinks, NEIL3 cleavage leaves an abasic site on one strand and an unhooked ICL on the other. Subsequent TLS on both sister chromatids requires REV1. For abasic site induced interstrand crosslinks, however, NEIL3 cleavage leaves one strand intact while the other contains the abasic site. Apparently, the bypass of the abasic site involves REV1 in *Xenopus*.

There is also an alternative model to the converging fork model. However, in this study with mammalian cells, psoralens are used and might be repaired more by the NEIL3 pathway rather than the Fanconi pathway. The authors reveal FANCM is involved in traversing the replication fork over the interstrand crosslink (117). In the model proposed by the authors, the replication fork stalls at the interstrand crosslink and FANCM is needed to restart the replication fork on the opposite side to the interstrand crosslink. Further investigation revealed that the interaction between FANCM and PCNA is necessary for traversing of the replication fork (118). When the replication fork traverses the interstrand crosslink, the replication fork can continue. Therefore, interstrand crosslink traversing can be seen as a mode of DDT.

DNA DAMAGE TOLERANCE DEFECTS AND DISEASES

Defects in genes involved in DDT are associated with several diseases. Similar to DNA damage repair associated diseases, symptoms include cancer susceptibility, neurological disorders, stem cell defects and progeria. The following sections address these genes and their associated genetic defects in an alphabetical order (Table 1).

ATAD5—Ovarian cancer

ATAD5 has been implicated in unloading of the DNA clamp PCNA and is also involved in the deubiquitination of PCNA-Ub through interaction with USP1 (56). In humans, *ATAD5* mutations have been associated as a risk factor for ovarian cancer (119). In line, imice with a heterozygous *Atad5* defect tumours of diverse origins arise (120). As the genome instability observed in *Atad5*-deficient cells is at least partially linked to the unloading of PCNA from chromosomes, it appears that this defect is responsible for the increased genome instability and tumorigenesis (121).

ATR—Cutaneous telangiectasia and cancer syndrome, familial, Seckel syndrome

ATR is a key kinase in the DNA damage response involved in fork stability and the management of replication stress (122). In humans, *ATR* mutations have been associated with familial cutaneous telangiectasia and cancer syndrome and Seckel syndrome (123,124). Seckel syndrome is characterized by short stature, intellectual disability, premature ageing and many more diverse symptoms between patients. One mouse model with an *Atr* mutation also shows Seckel syndrome (125). These homozygous *Atr* impaired mice show also increased dwarfism, increased replication stress and accelerated ageing, but do not show increased tumorigenesis. All these symptoms are in common with the human symptoms of ataxia telangiectasia, with the exception of tumour predisposition. It remains unknown to what extend the fork stabilizing function of ATR contributes in preventing Seckel syndrome and cancer susceptibility.

BRCA1/FANCS—Familial breast-ovarian cancer susceptibility, Fanconi anemia (complementation group S) and prostate cancer

BRCA1 is involved in genome maintenance through its function in homologous recombination, interstrand

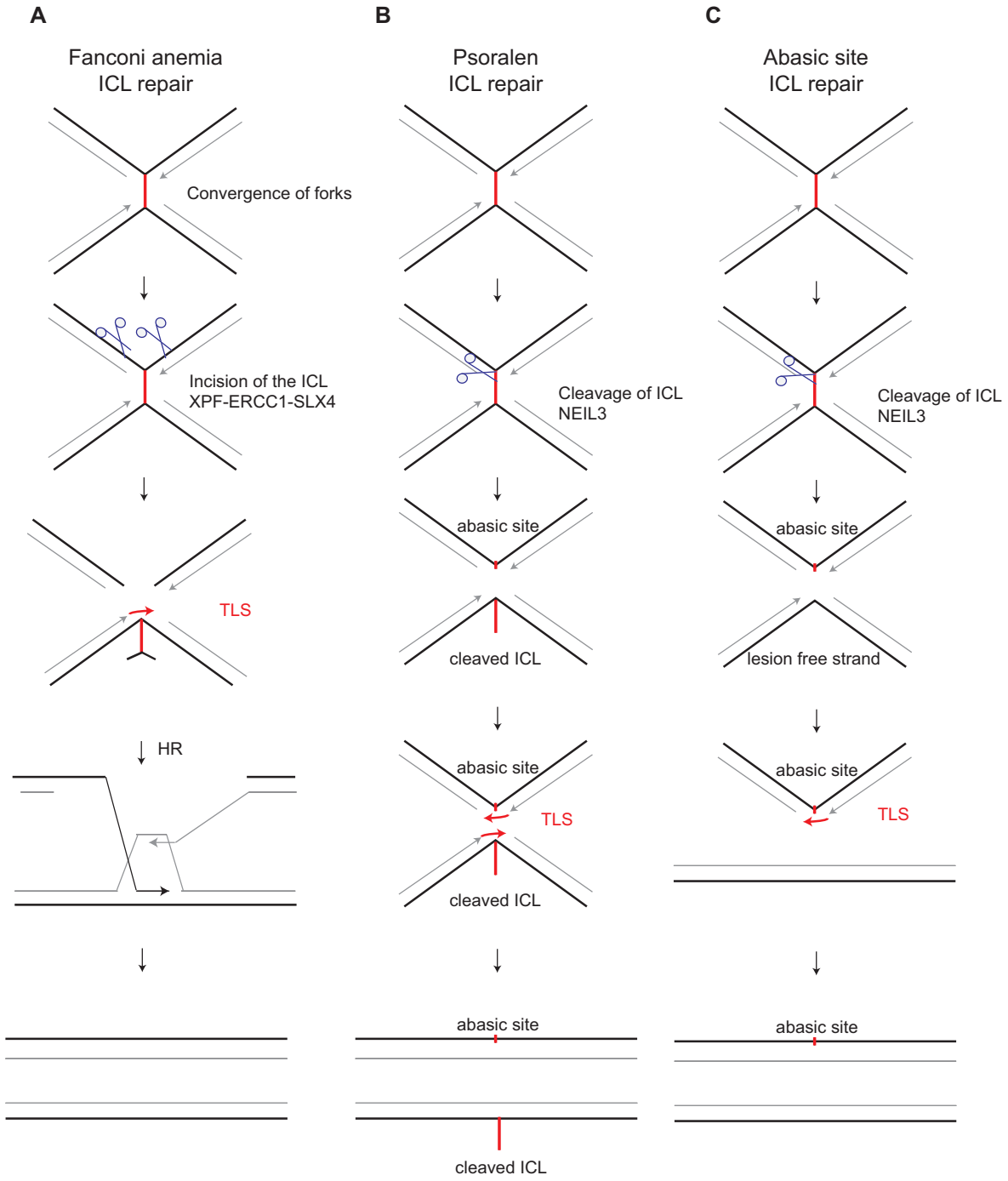

Figure 5. Translesion synthesis during interstrand crosslink repair. Interstrand crosslinks can be induced by cisplatin, mitomycin C, psoralen and abasic sites. Cisplatin and mitomycin C are repaired by the Fanconi anemia pathway (**A**), whereas psoralen and abasic site induced ICL are repaired through NEIL3-dependent pathway (**B** and **C**). Figure adapted from (23).

crosslink repair and fork stability (72,111,126). Mutations in human *BRCA1* contribute to familial breast and ovarian cancer, Fanconi anemia and other cancer types such as prostate cancer (127,128). Mouse models of *Brca1* deficiency are embryonic lethal. But mammary gland specific knockouts and hypo-morphic non-lethal mutations show increased incidence in developing mammary tumours, in line with human phenotypes (129). It remains unknown whether and to what extent the fork stabilization of BRCA1 contributes to cancer formation.

BRCA2/FANCD1—Familial breast-ovarian cancer, Fanconi anemia (complementation group D1), Wilms tumour and other cancers

Similar to BRCA1, BRCA2 is also involved in homologous recombination, interstrand crosslink repair and fork rever-

Table 1. Genetic defects of genes implicated in DNA damage tolerance and their associated diseases

Gene	Disease	Citation
ATAD5	Ovarian cancer	(119)
ATR	Cutaneous telangiectasia and cancer syndrome, familial, Seckel syndrome	(123,124)
BRCA1/FANCS	Familial breast-ovarian cancer susceptibility, Fanconi anemia (complementation Group S) and prostate cancer	(127,128)
BRCA2/FANCD1	Familial breast-ovarian cancer, Fanconi anemia (complementation group D1), Wilms tumour and other cancers	(130,131)
DNA2	Seckel syndrome and progressive external ophthalmoplegia	(134,135)
HUWE1	X-linked syndromic mental retardation turner type and colon cancer	(129–137)
PRIMPOL/CCDC111	Myopia	(142)
RAD51/FANCR	Fanconi anemia, complementation group R, mirror movements and breast cancer	(133–145)
REV3	Möbius syndrome	(149)
REV7/MAD2L2/FANCV	Fanconi anemia	(151)
SMARCAL1	Schimke immunoosseous dysplasia	(154)
SPRTN	Ruijs-Aalf syndrome, hepatocellular carcinoma	(161)
POLH	Xeroderma Pigmentosum Variant	(167)
UBE2A/RAD6A	X-linked syndromic mental retardation, Nascimento type	(176)
UBE2B/RAD6B	Male infertility	(179)
WRN	Werner syndrome	(184,185)

sal (72,111,126). In line, human *BRCA2* deficiency contributes to familial breast and ovarian cancer and Fanconi anemia (130,131). Mice deficient for *Brca2* are also embryonic lethal. Similar to the human pathology, conditional *Brca2* alleles show increased mammary tumour incidence in mice. Also, Fanconi anemia phenotypes are reconstituted in *Brca1* mutant mice, as hematopoietic stem cell defect and increased sensitivity to mitomycin C (132). It remains unknown whether and to what extend the fork stabilization of BRCA2 contributes to cancer formation.

DNA2—Seckel syndrome and progressive external ophthalmoplegia

DNA2 is a helicase and nuclease involved in fork reversal, long-patch base-excision repair and the processing of Okazaki fragments (133). Mutations in human DNA2 is associated with Seckel syndrome and progressive external ophthalmoplegia with mitochondrial DNA deletions (134,135). Progressive external ophthalmoplegia is an increasing weakness of the eye muscles, the cause of droopy eyes. *Dna2* homozygous knockout model is embryonic lethal, though the heterozygous *Dna2* is not lethal. Heterozygous *Dna2* mice show shorter telomeres and show increased tumorigenesis, though no Seckel or ophthalmoplegia symptoms have been reported (136). It remains unclear to what extend the role of DNA2 in fork reversal contributes to Seckel syndrome and progressive external ophthalmoplegia.

HUWE1—X-linked syndromic mental retardation turner type and colon cancer

The E3-ligase HUWE1 facilitates DDT through its interaction with PCNA, thereby preventing replication stress (43). However, HUWE1 does not affect PCNA ubiquitination. Similar to UBE2A/RAD6, HUWE1 is also associated with X-linked turner syndrome (137,138). *HUWE1* is lost often in colon cancer. Consistent, in *Apc*-deficient mice the loss of *Huwe1* activity increased the tumour incidence (139). Increased tumorigenesis due to *HUWE1* deficiency is likely due to a mixed effect of increased WNT signalling, genetic instability and MYC activity (43,139,140). Mouse models of *Huwe1* are embryonic lethal and neuronal ablation resulted in severe neuronal differentiation defects (141).

PRIMPOL/CCDC111—Myopia

PRIMPOL is associated with leading strand repriming (106). Mutation Y89D in *PRIMPOL* has been associated with myopia (142). The PRIMPOL Y89D mutation leads to both primase and polymerase defects in the DT40 B-cell lymphoma (143). The mutation impairs PRIMPOL function; however, the causal relationship to myopia remains to be determined.

RAD51/FANCR—Fanconi anemia, complementation group R, mirror movements and breast cancer

RAD51 is involved in homologous recombination, fork reversal and interstrand crosslink repair (72,144). In human, *RAD51* gene mutations lead to breast cancer, Fanconi anemia and mirror movements (145–147). Mirror movements are movements of limbs one side that are mirrored by the other. In mice, *Rad51* deficiency leads to embryonic lethality (148). It remains unclear to what extent the role of RAD51 in fork reversal contributes to mirror movements and breast cancer.

REV3—Möbius syndrome

Surprisingly, while *REV7* mutations are linked to Fanconi anemia, mutations in the catalytic subunit of the POLζ complex *REV3* in humans are causal for the Möbius syndrome in an autosomal dominant fashion (149). These observations suggest that REV3 and REV7 may have some differences in activity, in addition to being part of the POLζ complex. Möbius syndrome patients display facial paralysis and eye movement defects (150). In line, in mice heterozygous *Rev3* deficiency closely mimicked phenotypes observed in patients. As the main function of REV3 is TLS, it is likely that the TLS defect in *REV3*-defective cells leads to Möbius syndrome.

REV7/MAD2L2/FANCV—Fanconi anemia

As mentioned above, Fanconi anemia is a disease associated with defects in the interstrand crosslink repair pathway. Patients suffering from Fanconi anemia display bone marrow failure and subsequent anaemia, reduced fertility, congenital defects, an increased cancer incidence, and their cells are highly sensitive to MMC. A biallelic *REV7* mutation has been implicated in the development of Fanconi anemia, and therefore *REV7* was coined *FANCV* (151). As REV7 is a part of the POLζ complex, a REV3/REV7 heterodimer, it is likely functions as extender TLS polymerase activity opposite unhooked interstrand crosslink during the TLS synthesis step. Besides its roles in TLS, REV7 exerts an REV3-independent function by preventing end-resection and thereby promoting non-homologous end joining (152,153). However, since the non-homologous end joining pathway does not play a prominent role in interstrand crosslink repair, the Fanconi anemia phenotype of *REV7* impairment likely relates to defective TLS over the interstrand crosslink.

SMARCAL1—Schimke immunoosseous dysplasia

SMARCAL1 is a translocase involved in reversing stalled replication forks to generate a chicken foot structured replication fork. Mutations in the human *SMARCAL1* gene contribute to Schimke immunoosseous dysplasia (154). This syndrome is an autosomal recessive disease, characterized by T-cell immunodeficiency, dysplasia and renal dysfunction, leading to death at around 8 years (155). In line with observations in humans where the mutation alone is often not enough to cause the disease, knockout mouse models only show phenotypes of Schimke immunoosseous dysplasia upon inhibition of an RNA polymerase II inhibitor (156). These data suggest a *Smarcal1* mutation predisposes to the disease but is not sufficient in causing the disease. Furthermore, differential gene expression was also found in *Smarcal1*-deficient MEFs and speculated that SMARCAL1 regulates gene transcription directly, and that this contributes to disease progression in combination with loss of genomic integrity. On the other hand, *SMARCAL1* mutations found in patients affect SMARCAL1 ability to remodel replication forks and bind to Holliday junctions (157). This suggests that the differential gene expression in *SMARCAL1*-deficient cells is due to replication stress, rather than to direct regulation of transcription.

SPRTN—Ruijs-Aalf syndrome, hepatocellular carcinoma

SPRTN is recruited by PCNA-Ub and once recruited is thought to prevent PCNA deubiquitination through inhibition of USP1 (47–50). SPRTN also plays a role in resolving fork stalling DNA–protein crosslinks (158–160). In human, the *SPRTN* gene is associated with progeroid subtype Ruijs-Aalf syndrome and hepatocellular carcinoma (161). In mice, genetic deletion of *Sprtn* leads to embryonic lethality. A mouse model of *Sprtn* combining a hypo-morphic allele and a knockout allele also show progeroid phenotype and increased tumour incidence, in line with the human disease (162,163). It remains unclear to what extend the interaction with PCNA-Ub and SPRTN prevents Ruijs-Aalf syndrome and cancer.

POLH—Xeroderma pigmentosum variant

The first human disease linked to impaired DDT was Xeroderma Pigmentosum Variant (XP-V). Patients suffering from Xeroderma Pigmentosum (XP) are defective in nucleotide excision repair (NER) and highly sensitive to UV-induced DNA lesions (164–166). Patients suffering from the variant form of XP (XP-V) are nucleotide excision repair competent but defective in the Y-family TLS *POLH* gene (167). XP-V is characterized by an UV-hypersensitivity, a high skin cancer incidence due to higher mutagenicity of UV-induced DNA damage in the absence of *POLH* (168,169). In line with the observation that POLH can perform TLS opposite TT CPDs error-free, *Polh*-deficient mice show increased cancer incidence upon UV treatment (170–172). Furthermore, also heterozygous mice show an increased cancer incidence (173). These data indicate that *POLH* haploinsufficiency in humans might have and increased cancer incidence, due to the loss of the remaining functional allele (173). The increased mutagenesis in *Polh*-deficient mice seems to be related to increased compensatory POLI activity, as *Polh;Poli* double mutation was found to decrease mutagenesis upon UV, compared *Polh* single mutants (174,175). Interestingly, while *Polh;Poli* double mutants showed a lower mutation rate, they suffer from an increased tumour susceptibility following UV irradiation (174). This suggests, in this setting not the UV-induced point mutations, but other types of genomic instability as result of the *Polh;Poli* defect are the main drivers of tumorigenesis. In addition to POLI, POLQ has been reported to be involved in error-prone TLS in UV-induced lesions. Similar to *Polh;Poli* double mutants, *Polh;Polq* double mutates also show decreased point mutagenesis and increased tumorigenesis upon UV, compared to the *Polh* single mutant (30). Collectively, POLH-dependent TLS on UV-induced lesions leads to decreased mutagenesis and thereby contributes prevention of tumorigenesis.

UBE2A/RAD6A—X-linked syndromic mental retardation, Nascimento type

E2 ubiquitin ligase UBE2A/RAD6A is involved in PCNA monoubiquitination (35). In humans, *UBE2A/RAD6A* defect is associated with X-linked syndromic mental retardation (176). X-linked mental retardation syndrome is linked to a lot of different loci on the X chromosome. A mouse model of *Ube2a/Rad6a* shows defects in learning, memory and long-term depression, and female infertility (177,178). In contrast to infertile mouse mutants carrying a non-modifiable $Pcna^{K164R}$ mutation where germ cells are lacking, ovaria and oocytes develop in *Ube2a/Rad6a* mice. The inability of the embryo to survive the two-cell stage causes the infertility in *Ube2a/Rad6a* mice. It is not clear whether the symptoms are due to defective DDT, as ubiquitin ligases have many targets.

UBE2B/RAD6B—male infertility

E2 ligase UBE2B/RAD6B is involved in PCNA monoubiquitination (35). *UBE2B/RAD6B* mutations lead to male infertility (179). Mouse defective in *Ube2b/Rad6b* show male infertility, due to defects in synaptonemal complex and meiotic recombination in spermatocytes (180,181). It is not clear whether the symptoms relate to defective DDT, as ubiquitin ligases generally have many targets.

WRN—Werner syndrome

The *WRN* gene encodes a DNA helicase and exonuclease and is involved in homologous recombination repair, and fork reversal (182,183). In humans, aberrant mutations in the *WRN* gene lead to Werner syndrome (184,185). Clinical features of Werner syndrome include premature ageing, short stature and cancer predisposition (186). Werner syndrome inheritance is recessive and the average age of death is 54 years. Surprisingly, *Wrn*-defective mice do not recapitulate Werner syndrome symptoms of premature ageing as found in humans, though they are born at a sub-Mendelian frequency (187). However, *Wrn* defective mice kept for 3–5 generations do show increased ageing phenotypes, such as grey hair, osteoporosis, increased cancer susceptibility and other phenotypes of seen during normal ageing. This phenotype could also be accomplished by crossing *Wrn* mice to telomere maintenance factor *Terc*-deficient mice (188). As mice have very long telomeres compared to humans, these data suggest that the Werner syndrome might be mainly caused by defects in telomere maintenance (189). In fact, the *Wrn* defect leads to telomeric loss likely due to G4 stack bypass defect during replication of the G4 stack dense telomeric DNA (190). This suggests that premature ageing in this model relates to defective DDT of G4 stacks, ultimately leading to loss of telomeres.

In summary, DDT plays a key role in preventing disease. Overall, DDT defects are associated with neurological symptoms, accelerated ageing, infertility and cancer. These symptoms are shared with DNA repair defects, highlighting the critical contribution of the DDT system to the DNA damage response network. Mouse models with genetically defined DDT defects will be of value to further study the disease-related DDT defects and develop strategies that alleviate the symptoms or cure the patients.

THE ROLE OF DNA DAMAGE TOLERANCE IN MUTAGENESIS

The role of translesion synthesis in the generation of point mutations

DDT has been divided into error-prone TLS, error-free TLS and error-free homology-directed DDT, the latter includes fork reversal and template switching (191). Error-free definition in this context should be not considered literally but relatively, as error-free DDT can still contribute to mutagenesis, albeit at a lower frequency. Despite the fact that TLS is closely linked to the generation of point mutations, the existence of multiple TLS polymerases implies the existence of specificity per polymerase for the type of DNA lesion. In line with this notion, POLH or POLK knockouts, two closely related Y-family members show selective sensitivity for UV and MMS, respectively (74,192,193). In line, expression of Y-family TLS polymerases POLI and POLK correlated inversely with the mutation load in invasive breast cancers, suggesting that the absence of one TLS polymerase leads to increased point mutagenesis. This mutagenic phenotype likely relates to the fact that the backup TLS polymerases are more error-prone in tolerating a specific lesion. Missing TLS polymerases are likely to be back-upped by other members that further increases the error-rate and consequently mutation-rate (106). In line with this hypothesis, both *Polh* and *Polk* knockout mouse models show increased mutagenesis under specific conditions, suggesting that each TLS polymerase has its preferred lesion(s). *Polh* deficiency leads skin UV-induced mutagenesis in the skin, *Polk* deficiency leads to an increase of spontaneous mutations during ageing in an organ-specific manner, respectively (174,194).

In order to provide a comprehensible overview on the effect of TLS on mutagenesis, we focus here on UV-induced mutagenesis. Exposure of DNA to UV leads to the formation of CPDs and 6-4PP (195). Upon UV-B exposure, these CPDs and 6-4PP are produced in a declining frequency: TT CPD > TC CPD > TC 6-4PP > CT CPD > CC CPD > TT 6-4PP (196). The UV signature is composed of primarily of TC > TT or CC > TT transitions caused by CPDs (197). Daylight UV and more specifically UV-B exposure leads to preferential formation of CPDs at 5-methylcytosine bases, though this preference for 5-methylcytosine bases seems to be absent upon UV-C irradiation (198,199). Replication across the U in CPDs can explain the TC > TT and CC > TT UV mutagenic signature. TLS has been involved in UV mutagenesis, as *POLH*-deficiency leads to increased mutagenesis and XP-V (168,169). As mentioned previously, POLH can synthesize DNA across TT CPDs in an fairly error-free manner (200). The increase of mutagenesis in XP-V seems to be due to POLI activity, as *Polh;Poli* double mutant mice have a lower mutation rate upon UV exposure, compared to *Polh* defective mice (174,175).

In contrast to POLH, POLQ was found to act error-prone on UV-induced lesions even in the presence of POLH, as *Polq*-deficient cells show a level of UV-induced mutagenesis decreased similar to the non-treated control (30). These data are in line with the notion that specific TLS polymerases bypass different types of DNA damage. In order to study selectively CPDs or 6-4PP, CPDs or 6-4PP specific photolyases are often used to repair either CPDs or 6-4PP (201). Whether an impairment in the activity of a given TLS polymerase will be pro- or anti-mutagenic appears to depend on the type of the DNA polymerase and lesion (64). The authors exposed MEFs express (6–4) PP and CPD specific photolyases to UV light and show that knockdown of *Polh* or *Rev1* leads to an increased number of mutations in the 6-4PP lyase setting, but a decrease of mutations when the CPD specific lyase is expressed.

Whereas loss of individual TLS polymerases generally increases mutagenesis, impaired regulation of TLS decreases UV-induced mutagenesis. Regarding UV-damage induced mutagenesis, both REV1 and PCNA K164 were found to stimulate mutagenesis (64,73,202). Thus, if there is inefficient recruitment due to a defective TLS regulator, there are fewer mutations, at least in the case of UV irradia-

tion. Whether the same holds for the plethora of endogenous lesions remains to be determined further. However, the loss of an individual non-regulatory TLS polymerase increases mutagenesis, likely caused by an alternative TLS polymerase more error-prone at a given lesion.

Homology-directed DNA damage tolerance in genome rearrangements

Both template switching and fork reversal prevent point mutations by avoiding the use of the damaged template; however, they are associated with the extension of inverted repeats and other genome rearrangements (70). Homology-directed DDT has been shown to be involved in genome rearrangements induced by template switching during pausing at inverted repeats, as shown in *Schizosaccharo pompe* and *S. cerevisiae* (203–205). The genome rearrangements fuelled by inverted repeats can lead to acentric and dicentric chromosomes. Also in the evolution of the human genome, homology-directed DDT induced genome rearrangements seems to have a large contribution (206).

In conclusion, DDT can have both pro- and anti-mutagenic activities. Both homology-directed modes of DDT prevent point mutations, though they likely come wish the risk of increased genome rearrangements. The error-proneness of TLS depends on the specific lesion and the type of TLS polymerase replicating this lesion. It remains unknown whether there are mechanisms that recruit specific TLS polymerases to specific lesions.

THE ROLE OF DDT IN AGEING AND STEM CELLS

Stem cells depend on their own genome stability to both maintain an adequate stem cell pool and differentiate to enable long-term tissue homeostasis. The majority of work on genome stability in stem cells and its effect on ageing has been focussed on hematopoietic stem cells (HSC). Long-term HSC (LT-HSC) are mainly quiescent and thought to differentiate into short-term HSC (ST-HSC) (207). ST-HSC further differentiate into multipotent progenitors (MPP) 2, 3 and 4, which give rise to the majority of blood cells to warrant hematopoiesis (207–210).

Increased genome instability leads to a decrease in HSC potential and this progresses with age, suggesting that DNA damage accelerates HSC ageing (211). Similarly, different mouse models have shown that increased replication stress leads to premature ageing of HSCs (125,212–214). DDT impaired $Pcna^{K164R/K164R}$ mice show premature ageing of HSCs and skewing of differentiation toward the myeloid/erythroid lineage and increased DNA damage within the LSKs (213). Both myeloid/erythroid progenitor skewing and increased DNA damage in HSCs are signs of increased ageing. Myeloid/erythroid skewing of HSC differentiation is known to increase upon ageing and is thought to be at the basis of lymphoid decline in aged mice (213,215,216). In line with the role of DDT in HSCs, TLS regulator and Y-family polymerase *Rev1* mutant mice show a reduction of LSK cells in 5-month-old mice. The *Rev1* defect can be increased through introduction of the *Xpc* nucleic excision repair defect (214). As many lesions are not repaired, the *Rev1;Xpc* double defect leads to increased replication stress, anemia and bone marrow failure. Knockout mice lacking regulators of TLS such as *Paf15* a PCNA associated factor suffer from severe HSC defects (217). Another regulator of PCNA, *Sprtn* hypomorphic variant mice displayed a progeroid phenotype. The hematopoietic progenitor compartment was not investigated, though likely to be affected as well. Next to the genes involved in TLS, mutation of genes involved in fork reversal may also have HSC defects. Mouse models of translocase *Smarcal1* also displayed decreased number of HSC upon induction of replication of stem and progenitors (218). Strikingly, *Smarcal1* defective cells are more sensitive to fork stalling by 5-FU, but not double strand break inducing γ-irradiation, indicating that *Smarcal1* deficiency affects DDT but not double strand break repair. In *Brca1/Fancs* hypo-morphic mice, HSCs are marginalized and the mice show reduced bone marrow cellularity (219). In line, a fully defective *Brca1/Fancs* bone marrow specific deletion using a bone marrow specific Cre leads to bone marrow failure and hematologic malignancies (220). Furthermore, *Brca2/Fancd1* contributes to HSC function, as a hypo-morphic homozygous *Brca2/Fancd1* allele also shows an HSC repopulating defect and a decreased number of cells in the bone marrow (132). Next to HSCs, other stem cells also appear sensitive to DDT defects. For example, germ stem cells were completely absent in $Pnca^{K164R/K164R}$ mice, in both male and female mice (221).

In summary, increased DNA damage and replication stress due to DDT defects can accelerate ageing as shown in the hematopoietic system. It will be interesting to assess the relevance of DDT in determining the ageing of other tissues and their stem cells. Although in the liver DNA damage has been shown to contribute to ageing, independent of mutagenesis (222). The role of mutations in HSC and ageing is less clear, many DDT defective mouse models will have both increased DNA damage and mutagenesis, it remains unclear what the contribution of mutations to ageing is.

DDT AS TARGET FOR CANCER THERAPY

Paradoxically, while DNA needs to be kept in pristine condition, both DNA itself and the DNA damage response are prime targets for anti-cancer therapeutics. Many chemotherapeutic agents act through damaging the DNA or impeding replication (1,223). Defective DDT often leads to increased sensitivity to chemotherapeutically induced replication stalling DNA lesions. In line, increased expression levels of *POLH* have been found to correlate with decreased survival in non-small cell lung cancer treated with platinating agents (224). As many cancers have somatic mutations in DDT genes, targeting defects in the DDT system is an attractive way to optimize the treatment of cancers (225). One example already present in the clinic is PARP inhibitors in *BRCA1/2*-deficient cancers, though the mechanism of sensitivity to PARP inhibitors has been hypothesized to be due to defective homologous recombination (226). However, PARP inhibitor resistance mechanisms involve a rescue of replication fork stability, suggesting that one homology-directed DDT mechanism that is defective in *Brca1/2*-deficient cells plays a role in determining the sensitivity to PARP inhibitors (227). Furthermore, PCNA K164 ubiquitination inhibition is also synthetic lethal with *Brca1*,

in combination with UV or cisplatin treatment (228). Another potential candidate for synthetic lethality with DDT defects leading to increased replication stress could be ATM inhibitors, as tumours with mutated DDT genes should show increased replication stress and ATM inhibitors are synthetic lethal with increased replication stress (229). Furthermore, synthetic lethality with *Rad18* or *Polk* can be induced by inhibitor of WEE1- and oncogene-induced stress (230). Individual TLS DNA polymerases may be attractive anti-cancer targets, because they have a bigger active site than replicative polymerases and therefore could be targeted specifically. Screens for TLS polymerase inhibitors for POLK have identified new lead compounds, which await further development (231). As PCNA is a key regulator of DDT, inhibitors of PCNA function are being developed. T2AA is a PCNA ubiquitination inhibitor isolated from fungi (232). Next to T2AA, short peptides inhibiting PCNA interactions revealed efficacy in mouse tumour models (233–235). These PCNA inhibitors affected the AlkB homologue 2 PCNA-interacting motif (APIM), PIP motif or Y211 phosphorylation, and thereby prevent proper response to DNA damage. Furthermore, targeting ubiquitin-binding motif of REV1 that binds PCNA also sensitizes tumours to DNA damaging agents *in vitro* (236). However, mouse models have shown that targeting PCNA K164 for chemo-sensitization strategies is not a good approach, as the side effects of PCNA inhibition combined with platinum treatment become severe, mainly due to bone marrow failure. However, like for high dose chemotherapy, bone marrow failure may be compensated or prohibited by autologous HSC transplantation (213,225). Though, PCNA K164 targeting could be feasible in a setting where there is a tumour-specific mutation in a gene that is synthetic lethal with PCNA K164 inhibition. Phenotypes of mice treated with drugs that target PCNA K164 specifically are likely a lot less sensitive to platinating agents, as compared to a systemic PCNA K164R mutation (213,225).

Inhibition of PCNA deubiquitinase USP1 functioning also leads to cellular sensitization to chemotherapeutics. Targeting USP1 can be accomplished through targeting the USP1 UAF1 complex formation, which is needed for the activity of USP1. The activity of USP1 on FANCD2-Ub and PCNA-Ub has successfully been targeted leading to increased sensitivity to cisplatin *in vitro* (237).

In summary, DDT should be further exploited for synthetic lethal anti-cancer strategies and tumour-specific vulnerabilities to further optimize cancer therapy. In the future, personalized cancer medicine that targets tumour-specific vulnerabilities with adequate drugs will be achieved.

CONCLUSION

DDT is a key component of the DNA damage response network and hence has a central role in genome maintenance, through preventing replication stress and secondary DNA damage. Defects in the DDT system are associated with specific diseases. Disease phenotypes generally include increased ageing, cancer and neurological symptoms. Besides being causal in some cancers, DDT defects can lead to decreased genome stability, which at the same time opens a therapeutic window that renders primarily the tumour vulnerable to targeted therapies. Further research is required to unravel the choice and regulation of specific DDT pathways. These insights will be instrumental in treating DDT-related pathologies.

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