

# Homologous recombination maintenance of genome integrity during DNA damage tolerance

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**Abbreviations:** BER, base excision repair; DDT, DNA damage tolerance; HR, homologous recombination; HU, hydroxyurea; HJ, Holliday junction; NER, nucleotide excision repair; NHEJ, non-homologous end-joining; MMS, methylmethane sulfonate; PCNA, proliferating cell nuclear antigen; SCJ, sister-chromatid junction; SSBR, single-strand break repair; ssDNA, single-stranded DNA; TLS, translesion synthesis; UV, ultraviolet

The DNA strand exchange protein Rad51 provides a safe mechanism for the repair of DNA breaks using the information of a homologous DNA template. Homologous recombination (HR) also plays a key role in the response to DNA damage that impairs the advance of the replication forks by providing mechanisms to circumvent the lesion and fill in the tracks of single-stranded DNA that are generated during the process of lesion bypass. These activities postpone repair of the blocking lesion to ensure that DNA replication is completed in a timely manner. Experimental evidence generated over the last few years indicates that HR participates in this DNA damage tolerance response together with additional error-free (template switch) and error-prone (translesion synthesis) mechanisms through intricate connections, which are presented here. The choice between repair and tolerance, and the mechanism of tolerance, is critical to avoid increased mutagenesis and/or genome rearrangements, which are both hallmarks of cancer.

## Introduction

Cells have to faithfully replicate their DNA. This task is facilitated by a number of DNA repair mechanisms that “clean” the DNA before replication by specifically dealing with different types of DNA lesions. For example, DNA adducts generated by UV (UV) light are primarily repaired by nucleotide excision repair (NER); alkylated bases and abasic sites are repaired by base

excision repair (BER); and DNA breaks are repaired by single-strand break repair (SSBR), homologous recombination (HR), and non-homologous end-joining (NHEJ).<sup>1</sup> In many cases, however, the replication forks arrive first and have to face DNA lesions that impair their progression and threaten timely completion of DNA replication. When this occurs, an important decision must be taken: the lesion will either be repaired or bypassed with the repair postponed. DNA damage repair ensures fidelity but can excessively extend S-phase duration and induce genetic instability. In contrast, DNA damage tolerance (DDT) might ensure timely replication, but has to be tightly regulated to prevent excessive mutagenesis and genome rearrangements.<sup>2</sup>

The first evidence for DDT mechanisms came from studies of *Escherichia coli* *uvr* mutants that are defective in NER. When irradiated with UV light these mutants stop replication and accumulate single-stranded DNA (ssDNA; detected by separating pulse-labeled DNA in alkaline sucrose gradients) that is later filled in by a RecA-dependent process. The interpretation of these results was that tracks of ssDNA resulting from the encounter between the replication forks and DNA adducts had accumulated and were subsequently repaired by HR.<sup>3</sup> Importantly, although DNA synthesis was restored, the DNA adducts remained unrepaired.<sup>4</sup> These observations, and the finding that filling in the gaps could be mutagenic and was therefore performed by error-prone polymerases,<sup>5</sup> led to the concept of DDT whereby the lesion that causes the replication block is not repaired but is instead tolerated as a means to complete DNA replication in a timely manner. The first models proposed that the forks were able to bypass or circumvent the lesion, leaving ssDNA gaps behind the fork to be post-replicatively repaired.<sup>3</sup> Thus, the term post-replicative repair was coined to define this ssDNA gap filling although this term can be misleading as filling of the gaps might also be coupled to the advance of the fork through the lesion (Fig. 1).

DDT operates in organisms from bacteria to humans. The proteins involved in DDT by HR in the yeast *Saccharomyces cerevisiae* and in humans, and their corresponding functions, are listed in Table 1. In yeast, Rad51, which is essential in the search for homology and in DNA strand exchange during HR,<sup>6</sup> plays a

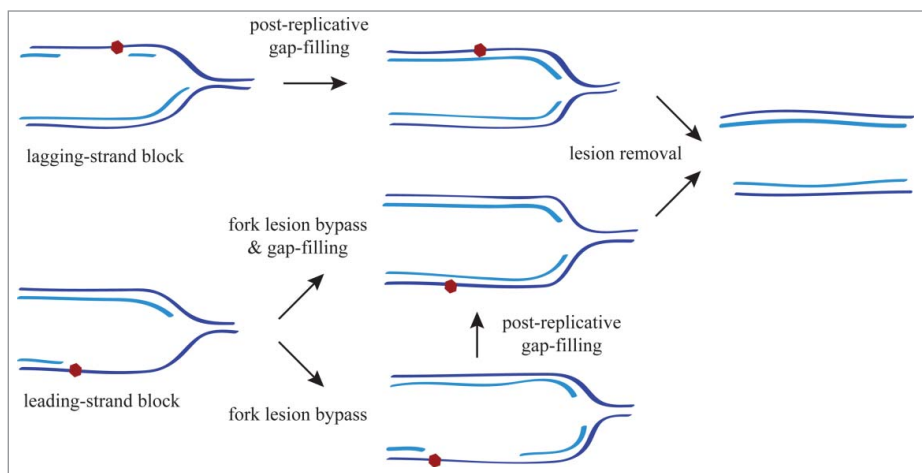
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**Figure 1.** Fork lesion bypass and gap filling after a block of the leading and lagging strand. Blocking the leading strand leads to a transient accumulation of ssDNA through uncoupling of leading and lagging DNA synthesis, whereas blocking the lagging strand is bypassed by priming a new Okazaki fragment. Eukaryotic replication forks can bypass both types of lesions and fill in the ssDNA gaps either during advancement of the fork through the lesion or post-replicatively.

role in DDT appears to be restricted to conditions of high replicative stress. In this article, high and low doses of replicative stress are defined according to whether only the wild-type (low) or both the wild type and the mutants (high) display a discernible biological effect (e.g., cell growth defects).<sup>7,8</sup> In higher eukaryotes, however, HR is probably more important for DDT, as inferred from its essential role during DNA replication even in the absence of DNA damaging agents. In any case, the mechanistic contribution of HR during DDT is unclear and in apparent contradiction with the existence of processes that actively prevent HR during S phase in response to replicative stress. Recent molecular and cellular approaches have started to shed some light on the intricate connections between HR and other mechanisms of DDT during the cell cycle. Here, I will first focus on the mechanisms of gap filling in eukaryotes, regardless of whether they are coupled to the fork or operate post-replicatively, and discuss our current knowledge about how the forks can bypass or circumvent a blocking lesion. Next, I will review the mechanisms that regulate DDT during the cell cycle and their impact on genome integrity and cancer.

### Rad51 and Rad6/Rad18 Genetic Interactions During DNA Damage Tolerance

Alkaline sucrose gradient analyses in *Saccharomyces cerevisiae*, in which DDT has been extensively studied, showed early on that eukaryotic cells are also able to fill in ssDNA gaps generated by replicative DNA damage using DDT mechanisms; this led to the elucidation of the first eukaryotic genes involved in gap filling, including the recombination gene *RAD52*.<sup>9-11</sup> Rad52 facilitates loading of Rad51 onto ssDNA filaments, strand invasion, and D-loop formation (Table 1),<sup>12</sup> and is essential for most HR events in yeast.<sup>13</sup> Supporting a role for HR in DDT, other genes encoding recombination proteins, including *RAD51*, have subsequently

been shown to be required for gap filling.<sup>14</sup> However, HR is not central to the replicative DNA damage response in yeast. Indeed, *rad52* and *rad51* mutants are partially defective in gap filling and are resistant to low doses of agents that damage replicative DNA, such as UV light and the alkylating agent methylmethane sulfonate (MMS).<sup>7,8,14</sup>

In yeast, the products of *RAD6* and *RAD18* govern DDT, and accordingly, *rad6* and *rad18* null mutants are extremely sensitive to UV light or MMS and are completely defective in gap filling.<sup>7,8,11</sup> Rad6 and Rad18 form a heterodimer with ubiquitin-conjugating (Rad18) and ubiquitin-ligase (Rad6) activity; additionally, Rad18 has ssDNA binding and ATPase activities.<sup>15,16</sup> In response to replicative stress, the Rad6/Rad18 dimer binds to the ssDNA tracks resulting from the encounter of the fork

with the blocking lesion and ubiquitinates lysine 164 of the replication processivity factor proliferating cell nuclear antigen (PCNA), a modification that is essential for *RAD6*-mediated DDT<sup>16</sup> (Fig. 2A). Further studies have shown that this pathway is highly conserved from yeast to humans.<sup>17</sup>

Intriguingly, yeast double mutants defective in Rad51 and Rad18 activities are more sensitive than single mutants to UV light and MMS,<sup>18</sup> suggesting that HR has *RAD6*-independent roles in response to replicative DNA damage. This additional function of Rad51 is negatively regulated during S phase by a second modification of PCNA: lysines 164 and 127 are sumoylated by the Ubc9/Siz1 complex both during unperturbed S phase and in response to replicative DNA damage.<sup>16</sup> PCNA sumoylation inhibits HR by recruiting the helicase Srs2,<sup>18,19</sup> which disrupts the ssDNA/Rad51 nucleofilament and inhibits DNA repair synthesis.<sup>20-22</sup> Accordingly, *srs2* and PCNA sumoylation-defective mutants (*siz1*, *ubc13*, *pcna-K164R*, *K127R*) suppress the DNA damage sensitivity of *rad6* and *rad18*, and this suppression is strictly dependent on the HR proteins Rad51, Rad52, Rad54, Rad55, and Rad57.<sup>18,19,23</sup> Likewise, in human cells, PCNA is sumoylated at lysine 164, and an Srs2-like protein—PARI—interacts preferentially with sumoylated PCNA to inhibit HR by interfering with the formation of Rad51 nucleofilaments.<sup>24</sup> Therefore, Rad51 and Rad6/Rad18 control DDT via intricate and highly regulated functions during the cell cycle.

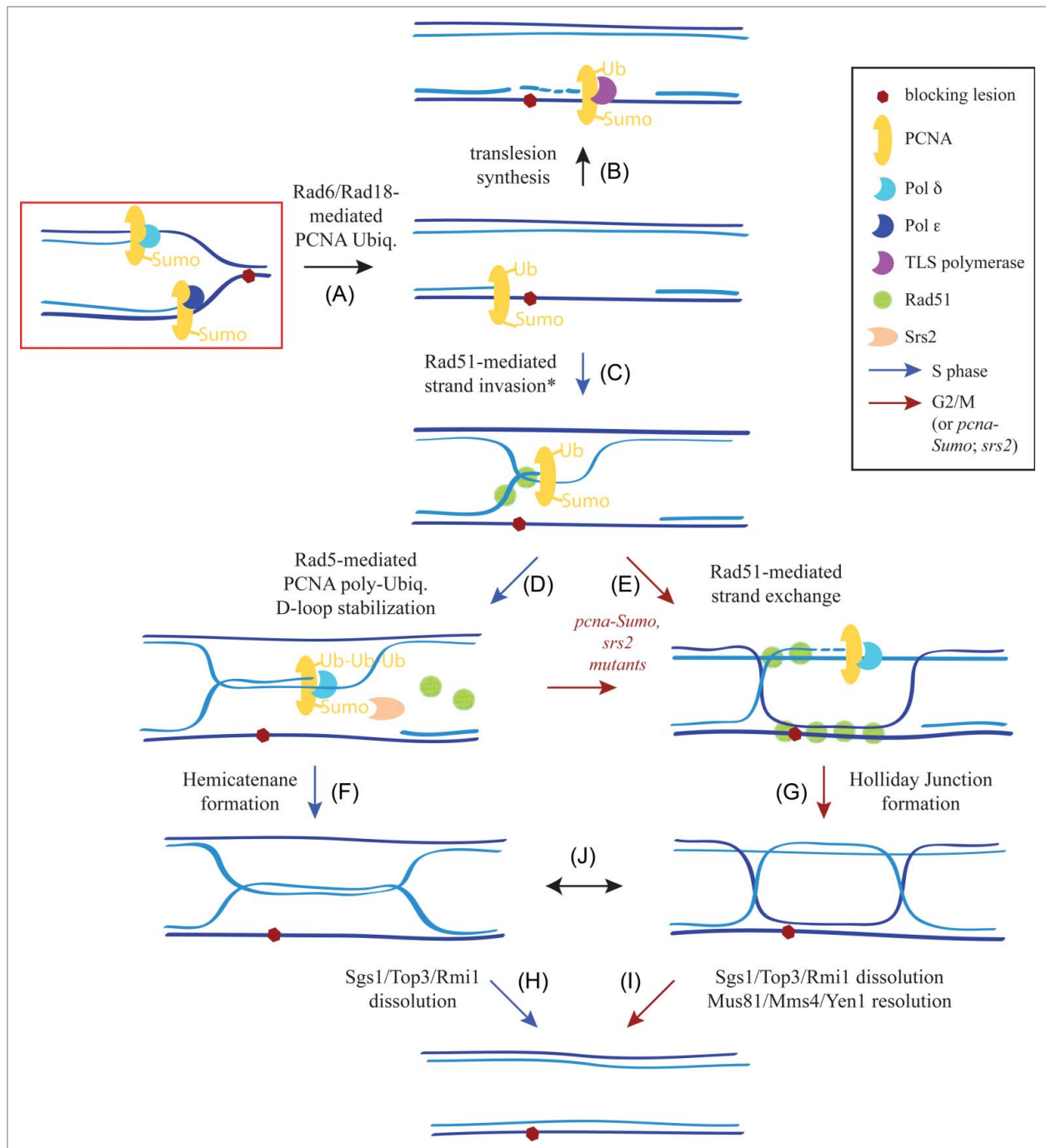
### Mechanisms of ssDNA Gap Filling

As previously mentioned, the accumulation of mutations in response to UV light and alkylating agents suggested the existence of error-prone mechanisms. The search for suppressors of DNA damage-induced mutagenesis, and further biochemical characterization of their corresponding gene products, led to the

**Table 1.** Proteins involved in DNA damage tolerance through homologous recombination<sup>a</sup>

Yeast	Humans	Functions
Rad51	Rad51	Forms a nucleofilament on ssDNA that is essential for homology search, ssDNA annealing, and strand exchange
Rad52	Rad52; BRCA2	Mediate the assembly of Rad51 on RPA-coated ssDNA; promote ssDNA annealing and D-loop formation
RPA	RPA	ssDNA binding protein; competes with Rad51 for ssDNA binding; modulates DNA damage repair/tolerance and checkpoint activation
Rad55/Rad57	XRCC3/RAD51C/ XRCC2/RAD51B/RAD51D	Rad51 paralogs; counteract Srs2 activity by stabilizing the Rad51 nucleofilament; facilitate Rad51-mediated strand exchange
Csm2/Psy3/ Shu1/Shu2/	RAD51D/SWS1	Like Rad55/Rad57, the yeast Shu complex counteracts Srs2 activities but its function is restricted to replicative non-DSB DNA lesions
Rad54	Rad54	Stabilizes Rad51 nucleofilaments and stimulates Rad51-dependent DNA annealing; dissociates Rad51 from dsDNA via its translocase activity; remodels chromatin
Mre11/Rad50/Xrs2	Mre11/Rad50/Nbs1	Heterotrimeric complex with ATPase and nuclease activities; regulates ssDNA resection; structural role bridging sister chromatids; Nbs1 has an additional role in recruiting Rad18 to UV-induced lesions
Sae2	CtP1; BRCA1	Initiate DNA resection by counteracting the inhibitory activities of the Ku/ DNA-PK complex and Rad9/53BP1; BRCA1 facilitates recruitment of Rad18, HLF, and Pol η to replicative lesions
Exo1	Exo1	Structure-specific nuclease involved in processing stalled forks
Sgs1/ Top3/Rmi1	BLM; WRN; SMARCAL1; BLM/TopoIIIα/ Rmi1/Rmi2	Sgs1, BLM, WRN, and SMARCAL1 are DNA helicases with fork reversal and HJ migration activities; the helicase and topoisomerase activities of Sgs1/ BLM and topoisomerase III are required together with Rmi1/Rmi2 for HJ dissolution
Ubc9/Mms21	Ubc9/Mms21	E2 conjugase (Ubc9) and E3 ligase (Mms21) sumoylation complex that acts in concert with Sgs1/Top3/Rmi1 to resolve HJs, probably by sumoylation of the Smc5-6 complex
Smc5-6	Smc5/Smc6	Multisubunit complex involved in DSB repair and fork restart by HR and in resolution of MMS-induced SCJs
Rad17/Mec3/Ddc1	Rad9/Rad1/Hus1	PCNA-like checkpoint clamp; required for full checkpoint activation, and MMS-induced HR in a checkpoint-independent manner
Pol δ	Pol δ	Lagging strand synthesis DNA polymerase
Pol η	Pol η	TLS polymerase; co-localizes with Rad51 foci after UV irradiation in human cells
PCNA	PCNA	Replication processivity factor; loaded by RFC and interacts with DNA polymerases and DNA repair factors via post-transcriptional modifications that control DNA replication and damage tolerance
Rad6/ Rad18	HHR6A; HHR6B/Rad18	E2 conjugase (Rad6; HHR6A; HHR6B) and E3 ligase (Rad18) ubiquitination complex that monoubiquitinates PCNA to promote TLS; recruited to replicative DNA lesions through the ssDNA binding activity of Rad18
Mms2/Ubc13/ Rad5	Mms2/Ubc13/HLTF; SHPRH	E2 conjugase (Mms2/Ubc13) and E3 ligase (Rad5, HLTF, SHPRH) ubiquitination complex that extends PCNA K164 ubiquitination with a K63-linked ubiquitin chain to promote template switch; Rad5 and HLTF have also dsDNA translocase activity that promotes fork reversal
Mgs1	WRNIP1; ZRAMB3/AH2	DNA helicases that bind to ubiquitinated PCNA; Mgs1 destabilizes the interactions between PCNA and Pol δ; ZRAMB3/AH2 has fork reversal and cleavage activity
Ubc9/Siz1	Ubc9/RFC	E2 conjugase (Ubc9) and E3 ligase (Siz1) sumoylation complex that sumoylates PCNA to recruit Srs2; Ubc9 and RFC are sufficient for human PCNA sumoylation <i>in vitro</i>
Srs2	PARI	Sumoylated PCNA interacting DNA helicase that inhibits the salvage HR pathway during S phase by interfering with the formation of ssDNA/Rad51 nucleofilaments and inhibiting DNA repair synthesis
Elg1	ATAD5	RFC-like clamp loader; interacts with sumoylated PCNA to unload it from chromatin; ATAD5 recruits the ubiquitinating enzyme USP1
Hmo1	HMGB	Chromatin structural DNA bending protein that binds cruciform structures and promotes Rad51-, Rad5-dependent SCJ formation and prevents function of the salvage HR pathway during S phase
Ino80	Ino80	ATP-dependent chromatin remodeling complex; facilitates the recruitment of Rad51 and Rad18 to MMS-stressed forks

<sup>a</sup>Only proteins (and their orthologs or functional counterparts) and functions mentioned in the text are shown. Abbreviations: DSB, double-strand break; HJ, Holliday junction; HR, homologous recombination; MMS, methylmethane sulfonate; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; SCJ, sister-chromatid junction; TLS, translesion synthesis.



**Figure 2.** Mechanisms of gap filling. A replication fork block leads to Rad6/Rad18-dependent PCNA monoubiquitination (A), which targets a specialized polymerase able to insert a correct or incorrect nucleotide opposite the lesion (translesion synthesis) (B). Alternatively, Rad51 and Rad5 can promote ssDNA gap filling by a mechanism that employs the intact sister chromatid to circumvent the lesion through the formation and subsequent dissolution of hemicatenanes (C–H). Hemicatenane formation requires the ssDNA binding complex replication protein A (RPA), the recombination proteins Rad51, Rad52, Rad55, Rad57, the Shu complex, Rad53-dependent phosphorylation of Rad55, Rad54, the nuclease Exo1, the polyubiquitin ligase activity of Rad5, the DNA bending activity of the high mobility group protein Hmo1, the cyclin-dependent kinase Cdc28, and Pol  $\delta$ , but neither Pol  $\epsilon$  nor the TLS polymerases.<sup>29,43,44,46,58,84,118</sup> Dissolution of hemicatenanes requires the Sgs1/Top3/Rmi1 complex together with Ubc9/Mms21-dependent sumoylation of the DNA repair Smc5-6 complex.<sup>43,119,120</sup> An alternative, Rad5-independent, Rad17/Mec3/Ddc1-dependent salvage HR pathway, which is inhibited in S phase by sumoPCNA-dependent recruitment of Srs2, might be operating through the formation and further resolution of HJs (C–I). See text for more details. \* It should be noted that Rad51-strand invasion does not require PCNA monoubiquitination.

discovery of specialized polymerases that are able to insert either correct or incorrect bases opposite the lesion.<sup>25</sup> In yeast, translesion synthesis (TLS; Fig. 2B) can operate through 2 distinct pathways: an error-prone pathway that involves Rev1 and the polymerase  $\zeta$  (encoded by *REV3* and *REV7*), and an error-free pathway that involves polymerase  $\eta$  (encoded by *RAD30*); these polymerases are highly conserved from yeast to humans.<sup>2</sup> Importantly, TLS is governed by Rad6/Rad18, which drives filling in of the gaps via monoubiquitination of PCNA.<sup>26</sup> This modification, conserved from yeast to humans, facilitates recruitment of the TLS polymerases.<sup>25</sup> TLS is not a major repair pathway in yeast,<sup>27</sup> and accordingly the sensitivity of a triple *rev1 rev3 rad30 (tls)* mutant to damaging agents is mild.<sup>8</sup> In fact, filling in the UV-induced ssDNA discontinuities is affected only slightly or not at all by the absence of Pol  $\eta$  and Pol  $\zeta$ , respectively,<sup>28</sup> although ssDNA gaps have been detected by different single-molecule analyses in *tls* yeast mutants<sup>29</sup> and in human cells lacking Pol  $\eta$ .<sup>30</sup>

Alkaline sucrose gradient studies in yeast have revealed 2 additional groups of genes, defined by *RAD52* and *RAD5*, which additively contribute to gap filling upon UV irradiation.<sup>14</sup> Consistent with these findings, double mutants affecting both groups are more sensitive to UV and MMS than single mutants.<sup>28</sup> Both pathways require the heterotrimeric complex MRX(N)<sup>14</sup> and operate through error-free mechanisms.<sup>31</sup>

*RAD5* belongs to the same yeast epistasis group of DNA damage sensitivity as *UBC13*, *MMS2*, *RAD6*, and *RAD18*.<sup>31</sup> Yeast Rad5 and its human orthologs helicase-like transcription factor (HLTF) and SNF2 histone linker PHD RING helicase (SHPRH) are proteins with ubiquitin ligase and double-stranded DNA (dsDNA) translocase activities.<sup>16,32-34</sup> They recruit the ubiquitin conjugating complex Ubc13/Mms2 to DNA lesions via physical interactions with PCNA and Rad18 and extend Rad6/Rad18-mediated PCNA monoubiquitination at lysine K164 with a K63-linked polyubiquitin chain.<sup>17</sup> The function of PCNA polyubiquitination has remained unknown for a long time. Recent work has shown that the yeast helicase Mgs1 is targeted to replicative lesions through an ubiquitin-binding zinc finger domain that physically interacts with the polyubiquitin chain of PCNA. This interaction destabilizes the binding of Pol  $\delta$  to PCNA, which might facilitate DNA transitions required during the repair process.<sup>35</sup> Nonetheless, the main function of PCNA polyubiquitination is unlikely to be linked to Mgs1 because *mgs1* null mutants are resistant to DNA damage.<sup>36</sup> In human cells, the helicase ZRAMB3/AH2 is also recruited to DNA lesions through its interaction with polyubiquitinated PCNA.<sup>37-39</sup> ZRAMB3/AH2 has a number of in vitro activities that suggest different modes of action; specifically, it can promote fork reversal, disrupt D-loop structures, and cut replication forks.<sup>37,38</sup>

Extensive genetic and molecular analyses in yeast have demonstrated that Rad5 promotes gap filling by a template switch process. Thus, it governs a TLS-independent, error-free process<sup>40</sup> and causes sister chromatid exchanges.<sup>41</sup> Importantly, Rad5/Mms2/Ubc13 and Rad6/Rad18-dependent sister-chromatid junctions (SCJs) can be molecularly detected in MMS-treated mutants lacking the Sgs1/Top3/Rmi1 complex,<sup>42</sup> which is required for SCJ dissolution.<sup>43</sup> Indeed, Rad5-dependent SCJs

can also be detected in wild-type cells treated with the alkylating agent adozelesin.<sup>44</sup> Recently, it has been shown that both yeast Rad5 and human HLTF can promote strand invasion and D-loop formation in vitro, providing the ability to initiate template switching.<sup>45</sup>

Strikingly, SCJ formation also requires the recombination proteins Rad51, Rad52, Rad55, and Rad57, but not Rad59, which is dispensable for DNA strand exchange.<sup>29,42,43,46</sup> In addition, genetic screens aimed at finding new functions that operate in error-free DDT revealed the Rad17/Mec3/Ddc1 (9-1-1) checkpoint complex and its specific DNA loader Rad24.<sup>47</sup> Interestingly, this complex is required for functionality of the salvage *RAD6*-independent HR pathway.<sup>47</sup> This HR/9-1-1 pathway is also associated with SCJs, as evidenced by their *RAD51*-dependent accumulation in *sgs1 pcna-K164R, K127R*, which is defective in SCJ dissolution and PCNA ubiquitination and sumoylation.<sup>42</sup> Together, these results suggest a mechanism in which the sister chromatid provides the template to bypass the lesion through Rad51- and Rad5-mediated strand invasion, DNA synthesis, and SCJ formation; after resolution of these SCJs the lesion persists in a dsDNA molecule and can be removed by oncoming DNA repair mechanisms (Fig. 2C–H). The salvage HR pathway would also contribute to filling the gaps when either replication is completed or inhibition by PCNA sumoylation is removed<sup>42,47</sup> (Fig. 2C–I).

An important and unresolved question is why the salvage HR pathway is repressed during S phase.<sup>16,18,19,23</sup> One possibility is that both pathways are initiated by Rad51-mediated strand invasion (Fig. 2C) yet differ in their mechanisms of SCJ formation, acting through a currently undefined Rad5 and PCNA polyubiquitination-dependent process (e.g., extended D-loop by DNA annealing and/or synthesis; Fig. 2D) or a Rad51-mediated strand exchange that is prevented by PCNA sumoylation and Srs2 during S phase (Fig. 2E). Whereas Rad5-mediated D-loop stabilization would lead to the formation of a hemicatenane (Fig. 2F), Rad51-mediated strand exchange would lead to the formation of a Holliday junction (HJ) structure (Fig. 2G). An important distinction between these 2 structures is that the hemicatenanes are “dissolved” by the action of Sgs1/Top3/Rmi1, leading to non-crossovers, whereas HJs can be additionally “resolved” by Mus81/Mms4 and Yen1, leading to non-crossovers and crossovers.<sup>48</sup> Crossovers between sister chromatids have no genetic consequences but can be highly deleterious when occurring between DNA sequences located in ectopic or allelic positions (leading to deletions, inversions, translocations, and loss of heterozygosity).<sup>49</sup> For this reason, cells restrict the activity of Mus81/Mms4 and Yen1 to late G2/M to resolve SCJs that escape from the dissolution pathway, a strategy that prevents these resolvases from acting on other branched structures as a result of their limited substrate specificity.<sup>48</sup> Therefore, upregulation of the salvage HR pathway during S phase would result in an excess of unresolved HJs that could interfere with proper chromosome segregation. SCJs with the properties of hemicatenanes and HJs accumulate in *sgs1* $\Delta$  mutants released into S phase in the presence of MMS; such HJ-like SCJs can be removed by expression of heterologous resolvases.<sup>43,50</sup> Interestingly, these resolvases

cannot remove early SCJs.<sup>50</sup> It is possible that hemicatenanes are initially formed via Rad51 and Rad5-dependent activities and dissolved by Sgs1/Top3/Rmi1 (Fig. 2C–H); in the absence of this complex, the hemicatenanes would be interconverted into HJs and become substrates for resolvases (Fig. 2J–I). The salvage HR pathway would thus be operative in G2/M for unresolved SCJs and in mutants defective for PCNA sumoylation or Srs2 inhibition of the Rad51-mediated strand exchange activity.

The finding of a common pathway for Rad51 and Rad5 raises the question of whether the additive defects in gap filling displayed by mutations affecting the *RAD51* and *RAD5* groups of genes<sup>14</sup> reflect partially overlapping functions or additional and alternative gap filling pathways. Studies with chronic low doses of UV light and MMS suggest that at least one Rad6/Rad18/Rad5-dependent, Rad51-independent DDT pathway may exist because only *RAD6*, *RAD18*, and *RAD5* are required to deal with low levels of DNA damage in yeast.<sup>7,8</sup> Interestingly, evidence for a mechanism involving HR and Rad6/Rad18-specific PCNA monoubiquitination has been reported in human cells, which accumulate DNA repair foci containing both Rad51 and Pol  $\eta$  in response to UV light.<sup>51</sup> Indeed, Pol  $\eta$  interacts with Rad51 and can extend DNA synthesis from a 3'-end invading a duplex DNA molecule.<sup>52</sup> However, these events could reflect the repair of broken forks, as Pol  $\eta$  is also required for DSB-induced HR.<sup>53,54</sup>

## Replication Fork Lesion Bypass

A growing amount of evidence suggests that gap filling in eukaryotes can occur coupled to the fork and post-replicatively. Data supporting a post-replicative mode of action include the detection by electron microscopy of ssDNA tracks behind the fork in UV- and MMS-treated *rad52* and *tls* mutants in yeast and *Xenopus* extracts.<sup>29,55</sup> In addition, yeast cells in which expression of Rad18, Rad5, Rad17, or TLS polymerases is genetically restricted to G2/M tolerate MMS and UV-induced damage, suggesting that filling of the ssDNA gaps can occur post-replicatively.<sup>47,56,57</sup> Likewise, mammalian cells accumulate ssDNA gaps after replication in the presence of UV light, especially in Pol  $\eta$ -defective cells.<sup>30</sup> Finally, analysis of Rad52 dynamics in response to MMS in wild-type yeast cells indicates that the recombinational repair of the ssDNA tracks is delayed until G2/M.<sup>58</sup> In fact, as mentioned before, the negative regulation of the salvage HR pathway by PCNA sumoylation suggests that at least this pathway operates post-replicatively.

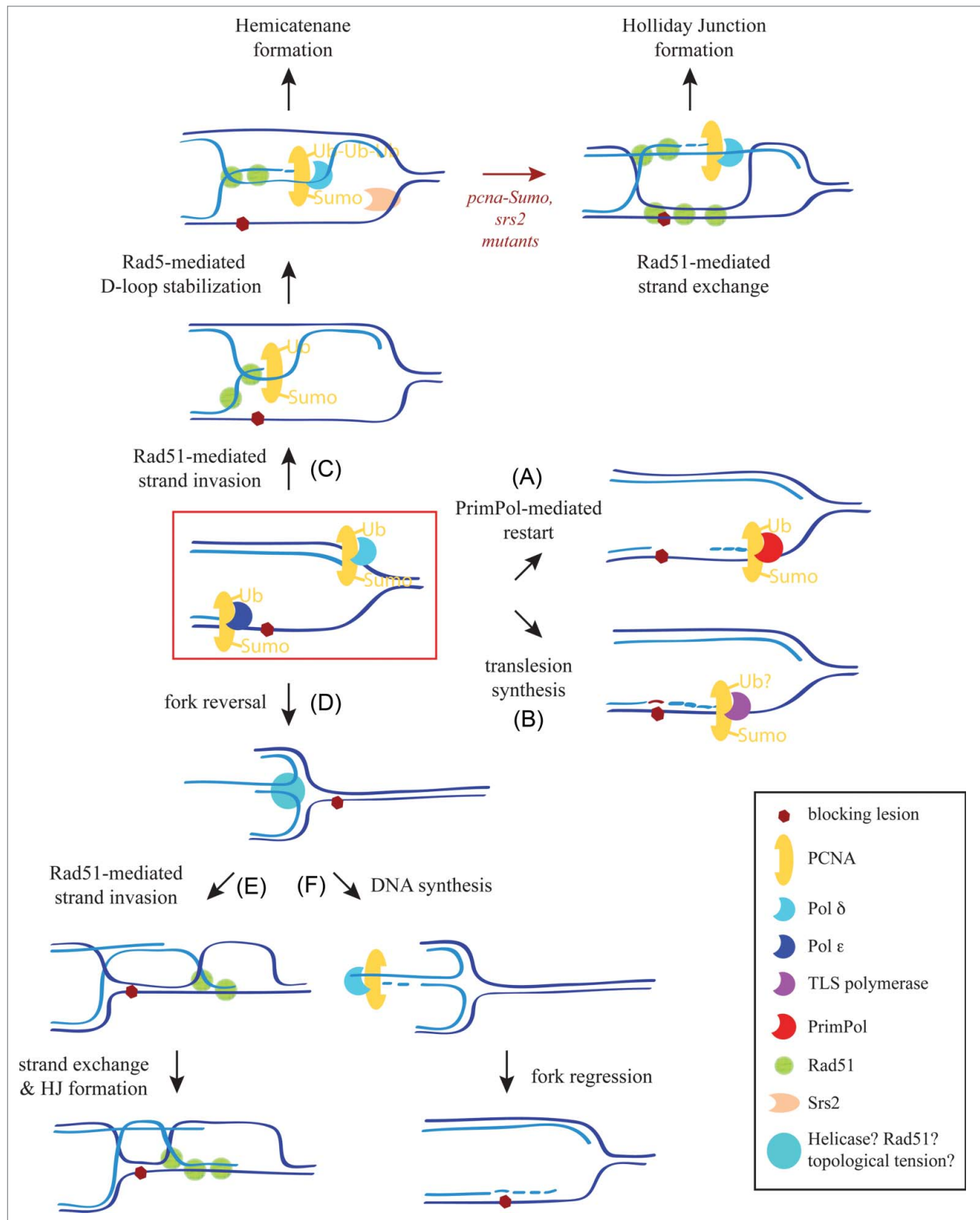
Whereas ssDNA gap filling at the fork directly provides a mechanism for the fork to bypass a blocking lesion, post-replicative repair requires additional mechanisms by which the replication fork either bypasses or circumvents the lesion. This point is intimately related to the position of the blocking lesion relative to the advancing replicative polymerase. Studies in bacteria have shown that a lesion in the leading strand blocks the replication fork, which accumulates ssDNA by transient lagging-strand synthesis, whereas a lesion on the lagging strand inhibits synthesis of

the corresponding Okazaki fragment but the fork can bypass this by priming a new DNA fragment<sup>59–61</sup> (Fig. 1).

Indeed, lesions that impair the advance of the leading strand, such as photoproducts or alkylated bases, and the lack of Pol32, which is required for lagging-strand synthesis, both induce PCNA mono- and polyubiquitination, suggesting that gap filling operates in both strands.<sup>56</sup> Electron microscopy analyses in yeast and *Xenopus* extracts have shown that replicative DNA adducts uncouple DNA synthesis and unwinding—thereby generating ssDNA tracks at the fork—in only one of the 2 sisters, yet lead to the accumulation of ssDNA gaps behind the fork in both sisters.<sup>29,55</sup> Therefore, eukaryotic replication forks are able to bypass a blocking lesion in both the leading and the lagging strands, even though the leading strand is transiently stalled and uncoupled from the lagging strand.

Mammalian cells possess a TLS polymerase, PrimPol, that has also a primase activity able to prime new DNA synthesis with dNTPs downstream of a blocking lesion<sup>62</sup> (Fig. 3A). This primase activity is employed to reinitiate DNA synthesis after UV irradiation and, accordingly, its downregulation reduces fork progression, underscoring the relevance of this mechanism for replication fork lesion bypass in human cells.<sup>63</sup> Yeast lacks a homolog of PrimPol, and it is unknown whether the replicative primase can fulfil this function. On the other hand, *tls* mutations in yeast do not increase UV-induced fork uncoupling<sup>29</sup> or affect fork progression,<sup>64</sup> and the expression of TLS polymerases can be restricted to G2/M without affecting DDT,<sup>56</sup> suggesting that TLS in yeast operates exclusively at ssDNA gaps left behind the fork. Whereas a putative role for Pol  $\eta$  in replication fork lesion bypass after UV irradiation in human cells remains controversial,<sup>30,65</sup> the TLS polymerase Rev1 is required in avian DT40 cells for fork progression through damaged DNA. However, this unexpectedly involves a Rad18- and PCNA monoubiquitination-independent mechanism<sup>66</sup> (Fig. 3B).

A highly conserved and essential mechanism of replication fork lesion bypass relies on HR. Thus, vertebrate cells without Rad51 are not viable as a consequence of defective replication and repair of spontaneous DNA lesions.<sup>67,68</sup> After DNA damage, replication is slowed down by the activity of Rad51 and its paralog XRCC3, indicating that HR modulates fork progression through damaged DNA.<sup>69</sup> In yeast, HR is not essential for unperturbed replication but appears to be the major mechanism by which the fork bypasses lesions at high levels of DNA damage. Different molecular and cellular approaches have clearly demonstrated that replication through alkylated DNA requires Rad52 and Rad51.<sup>44,58,70,71</sup> In fact, Rad52 and Rad51 travel with the fork under unperturbed conditions, and this interaction is not enhanced by replicative DNA damage.<sup>55,58</sup> More importantly, yeast cells in which Rad52 expression is restricted to G2/M are defective in DNA replication and in the repair of MMS-induced DNA lesions, indicating that Rad52-dependent loading of Rad51 during S phase is a prerequisite for their post-replicative functions and, further, that replicative and repair HR activities are mechanistically connected.<sup>58</sup> A separation of replicative and repair functions has also been shown in mammalian cells, which



**Figure 3.** Mechanisms of replication fork lesion bypass. Replication forks can bypass a blocking lesion by PrimPol-mediated DNA synthesis downstream of the lesion (A), a switch of the replicative polymerase for a TLS polymerase (B), Rad5 and Rad51-mediated SCJs (B), and fork reversal followed by either Rad51-mediated strand invasion and HJ formation downstream of the blocking lesion (D–E) or by DNA synthesis and fork regression (D–F). Note that Rad51-mediated strand invasion (step E) would require “chicken foot” processing to generate a 3'-ssDNA overhang. See text for details.

confront replicative stress by distinct replication fork restart and DNA repair Rad51 mechanisms.<sup>72</sup>

How Rad51 facilitates replication past DNA lesions is unclear, but the use of adozelesin, an alkylating agent that causes a detectable block of the forks in yeast, has shown that this block is associated with an accumulation of Rad51- and Rad5-dependent SCJs, and that recovery of this block depends on Rad52 and Rad5.<sup>44</sup> This suggests that the Rad51/Rad5 pathway (Fig. 2C–H) might be operating at the fork to concomitantly promote fork lesion bypass and gap filling (Fig. 3C).

It is important to note, however, that the role of PCNA ubiquitination in replication fork lesion bypass remains controversial. Thus, although Rad18 and Rad5/HLTF are both required for efficient replication through damaged DNA in yeast and humans,<sup>34,70,73</sup> restriction of Rad18 or Rad5 expression to G2/M in yeast does not prevent bulk chromosomal replication in the presence of MMS or UV light.<sup>56,57</sup>

Notably, the dsDNA translocase activity of yeast Rad5 and human HLTF is able to form a “chicken foot” structure in vitro by fork reversal (Fig. 3D).<sup>33,34</sup> This structure, first proposed from mammalian blocked forks,<sup>74</sup> might facilitate replication restart downstream of the blocking lesion by Rad51-mediated strand invasion and HJ formation (Fig. 3E) or, alternatively, lesion bypass by DNA synthesis and fork regression (Fig. 3F). However, the helicase domain of Rad5/HLTF, which is required for its dsDNA translocase activity, appears to be dispensable for DDT in vivo,<sup>75</sup> suggesting that fork reversal either does not occur, is mediated by a different protein, and/or can be bypassed by an alternate mechanism in the absence of Rad5. In fact, fork reversal can be promoted by the helicase activity of RecQ and SMARCAL1,<sup>76</sup> the strand exchange activity of Rad51,<sup>77</sup> and even by topological tension.<sup>78</sup>

Rad51 and the mediator protein BRCA2 are also required to stabilize the stalled forks after dNTP depletion with hydroxyurea (HU) in mammalian cells.<sup>79,80</sup> Interestingly, BRCA2 and Rad51 protect HU-stalled forks from degradation by the nuclease Mre11 through a repair-independent mechanism,<sup>80</sup> further reinforcing the separation of replicative and repair functions of Rad51 in DDT. Likewise, yeast Rad51 is required for the stability of stalled replication forks.<sup>81</sup> Whether this function is associated with the formation of SCJs is unclear because X-shaped structures have not been detected in response to HU,<sup>43</sup> but Rad51-mediated fork stabilization also requires the helicase activity of Sgs1 and Top3.<sup>81</sup> Thus, it is possible that SCJs need to be rapidly processed after fork stalling by HU. In accordance with this, PCNA polyubiquitination, which is required for SCJs formation,<sup>42,44</sup> is also induced by HU.<sup>56</sup> In any case, SCJ dissolution is not required for replication fork advance in the presence of replicative stress.<sup>43,56</sup> In mammalian cells, Rad51-mediated restart after HU treatment also requires BRCA1 and CtIP, which regulate DNA resection.<sup>82</sup> Interestingly, BRCA1 has recently been shown to directly regulate DDT at stalled replication forks by promoting PCNA ubiquitination and physically interacting with Rad18, HLTF, Pol  $\eta$ , and Rev1.<sup>83</sup>

## Regulation of DNA Damage Tolerance

As implicated in the discussion about the mechanisms of DDT, the homotrimeric PCNA sliding clamp plays a decisive role in the choice of mechanism. Whereas the levels of mono- and polyubiquitinated PCNA promote TLS and error-free template switching, respectively,<sup>16,26</sup> PCNA sumoylation controls the salvage pathway of HR during S phase by mechanisms that involve the antirecombinogenic activities of Srs2<sup>16,18,19</sup> as well as the DNA bending activity of Hmo1.<sup>84</sup> The relevance of PCNA modifications is highlighted in mammalian cells, in which a higher level of DDT regulation is in part achieved through multiple factors (e.g., p21, p53, Claspin) acting on PCNA ubiquitination.<sup>85</sup> The levels of PCNA modification are additionally controlled by the clamp loader Elg1, which binds to and unloads sumoylated PCNA from chromatin in yeast<sup>86,87</sup> and targets the PCNA deubiquitinating enzyme USP1 to replicative DNA lesions in humans.<sup>88</sup> Chromatin is also involved in this regulation, and the chromatin remodeler Ino80 is required to recruit Rad18 and Rad51 to promote replication fork progression through alkylated DNA.<sup>89</sup> Mutations in these regulators lead to unscheduled events that are often associated with severe defects in genome integrity and cancer predisposition (see below). Therefore, PCNA acts as an exquisitely regulated molecular switch controlling different DDT pathways, both in unperturbed replication and in response to replicative DNA damage.

DNA damage sensitivity and molecular assays in yeast have shown that the error-free mechanisms are the major DDT pathways both under low and high replicative stress,<sup>7,8,11,27,41</sup> although TLS pathways can also efficiently deal with low doses of DNA damage.<sup>7</sup> Intriguingly, the loss of viability of *rad18* mutants under both DNA damage conditions can be rescued by reactivating the expression of Rad18 in G2/M in the absence of Rad5, but not of Rev3, indicating that *Rad5* requires Rad18 during S phase to tolerate DNA damage.<sup>7,57</sup> This, together with the facts that lack of Mms2 (but not of Rev3) delays G2,<sup>7</sup> that PCNA mono- and polyubiquitination is initially detected in S phase,<sup>56,57</sup> and that the checkpoint is activated during S phase in cells that either lack Rad5 or express it in G2,<sup>56</sup> strongly suggests that the *RAD5* error-free pathway operates preferentially during S phase, regardless of whether its activity is at or behind the fork. In this scenario, TLS would normally be confined to G2 to deal with any lesions that remained unrepaired. Consistent with this model from data in yeast, the levels of Rev1 display an expression peak during G2/M that is approximately 50-fold higher than that in G1 and S phase and is only minimally affected by DNA damage.<sup>90</sup>

As previously mentioned, a number of recent results from different eukaryotic models strongly suggest that the replicative and repair activities of the recombination machinery are separated in the response to replicative stress.<sup>55,58,72,80</sup> In yeast, this separation of functions is regulated by the replicative checkpoint, which inhibits the formation of HR repair centers during S phase despite the fact that Rad51 and Rad52 remain bound to replicative DNA lesions.<sup>58</sup>



The type and amount of DNA lesions are also critical determinants in the choice of the DDT mechanism. Thus, SHPRH and HLTf prevent mutations induced by MMS and UV light, respectively, by interacting with Rad18 and promoting recruitment of the most appropriate TLS polymerases.<sup>91</sup> Likewise, yeast Rad5, but not Ubc13-dependent PCNA polyubiquitination, is required for UV-induced mutagenesis,<sup>92</sup> indicating the existence of additional and conserved intricate connections between the error-free and error-prone pathways.

The importance of the load of DNA lesions has already been anticipated by the differential genetic requirements displayed by yeast in response to an acute high dose and a chronic low-dose treatment. Chronic low doses of DNA damage do not activate the DNA damage checkpoints; indeed, checkpoints are not required for viability under these conditions and become activated and arrest cells in G2/M only in the absence of Rad18 as a consequence of an accumulation of ssDNA that is left unrepaired.<sup>7,8</sup> In contrast, the checkpoints are activated even in wild-type cells after an acute high-dose treatment.<sup>78</sup> Thus, checkpoint activation by unrepaired ssDNA gaps provides the time required for repair and tolerance mechanisms to deal with the lesions before the cell progresses into mitosis; accordingly, checkpoint defective cells are sensitive to replicative DNA lesions.<sup>93</sup>

Notably, checkpoint null mutants in yeast are proficient in TLS but partially defective in gap filling,<sup>93,94</sup> yet it is unclear whether this effect is related to the roles of the replicative checkpoint in maintaining the stability of stressed replication forks or in the regulation of DDT factors.<sup>78</sup> For example, phosphorylation of Rad51 on Ser192 by the sensor checkpoint kinase Mec1 is essential for growth in the presence of MMS.<sup>95</sup> In any case, and separate from coordinating repair with cell cycle progression, the checkpoint machinery appears to modulate DDT in response to the load of DNA damage, as suggested by the observation that a partial reduction in the activity of the yeast checkpoint effector Rad53 increases tolerance to MMS by augmenting the amount of Rev1 bound to chromatin and TLS activity.<sup>96</sup> This finding is somehow unexpected because, rather than supporting TLS as a backup mechanism for situations of high stress, it suggests that TLS might be permitted only under conditions of low DNA damage. As proposed for bacteria,<sup>97</sup> the logic behind this strategy could be modulation of TLS to ensure a certain level of mutagenesis for genetic diversity while preventing a deleterious level of mutations when the number of DNA lesions is excessive.

## DNA Damage Tolerance Versus Repair

In yeast, DNA damage repair and DDT mechanisms are both essential when the load of DNA lesions is high.<sup>1</sup> However, NER-defective cells can efficiently replicate plasmids carrying a photoproduct.<sup>41</sup> In addition, NER and BER repair pathways are dispensable when cells are exposed to chronic low doses of UV light or MMS, whereas Rad6, Rad18, and Rad5 are essential for viability.<sup>7,8</sup> Importantly, this loss of viability is not due to a defect in the removal of the blocking lesions, as would be expected for a DDT mechanism.<sup>8</sup> Therefore, when yeast forks face DNA

blocking lesions, the decision is biased toward tolerating the damage and completing replication. Similar experiments in human cells are lacking, but the high sensitivity to sunlight (chronic low doses) displayed by cells from NER-defective patients<sup>98</sup> and the fact that NER is active in the S phase of human, but not yeast, cells<sup>99</sup> highlight the importance of DNA damage repair during replication in mammalian cells.

## DNA Damage Tolerance, Homologous Recombination, and Cancer

Recent experimental findings suggest that oncogenes induce replicative stress in precancerous cells, which thereby activate replicative checkpoints and DNA repair/tolerance mechanisms that act as a barrier to prevent the proliferation of cells with high levels of genetic instability. Mutations that impair these mechanisms promote cell proliferation and increase genomic instability and, consequently, are selected during tumorigenesis.<sup>100</sup> This scenario highlights the importance of HR and DDT in cancer.

The isolation of Pol  $\eta$  as the protein responsible for xeroderma pigmentosum variant (XPV), an inherited genetic disorder that is associated with increased incidence of skin cancers, provided the first evidence for a direct connection between TLS, mutagenesis, and cancer.<sup>101</sup> Interestingly, the tumor suppressor genes p53 and p21 negatively control TLS, thus reducing excessive mutagenesis.<sup>102</sup> Likewise, the Rad5-mediated pathway appears to play an important role in preventing cancer, as suggested by the high incidence of HLTf gene silencing in human colon cancer<sup>103</sup> and the formation of intestinal cancers in *Httf*-deficient mice.<sup>104</sup> In fact, a role for Rad5 in tumor suppression is supported by the observation that yeast Rad5 and human HLTf and SHPRH prevent chromosomal rearrangements and mutagenesis.<sup>32,105,106</sup> The importance of maintaining strict control of the state of PCNA modifications is also exemplified by the genomic instability and high incidence of tumors in mice that are haploinsufficient for Atad5 (the mouse Elg1 homolog).<sup>107</sup>

However, HR displays the most critical connections with cancer development. HR plays an active role in preventing tumorigenesis, as can be inferred from its essential functions in replication and repair during replicative stress and as evidenced by the number of tumor suppressor genes that are directly involved in HR.<sup>108</sup> Importantly, HR can also be a source of genetic instability, which fuels tumor progression. Thus, HR is highly mutagenic compared to normal replication (1,000-fold more mutagenic)<sup>109,110</sup> and can lead to loss of heterozygosity and genome rearrangements when it uses allelic and ectopic DNA sequences to repair the lesions.<sup>13</sup> In addition, the accumulation of strand exchange intermediates during DDT can in turn have deleterious effects on genome integrity if they are not tightly controlled. Accordingly, HR intermediates are inhibited during S phase by different mechanisms, such as PCNA sumoylation-dependent recruitment of the anti-recombinogenic helicase Srs2,<sup>18,19</sup> checkpoint-dependent regulation of the fork reversal activity of the helicase SMARCAL1 that prevents fork cleavage and unscheduled recombinational repair,<sup>111</sup> and cell

cycle-regulated recruitment of proteins involved in SCJ dissolution that prevent crossovers and genetic instability.<sup>112</sup> Interestingly, the tumor suppressor protein p53, considered to be the guardian of the genome, inhibits replication-associated HR.<sup>113</sup> p53 inhibits human Rad51/ssDNA formation, strand exchange, HJ branch migration, and Rad51-mediated fork reversal, probably by direct contact with Rad51.<sup>77</sup> Therefore, cells have to tightly control HR to allow its essential functions in DNA replication while simultaneously preventing the deleterious effects of unscheduled recombination events. In yeast, activation of the replicative checkpoints inhibits the formation of recombination centers during S phase.<sup>58,71,114-116</sup> I have recently proposed that this inhibition might provide a mechanism to coordinate replicative and repair activities and to prevent genetic instability, since the assembly of repair factories at stressed replication forks might interfere with proper DNA replication and promote fork restart by unscheduled and mutagenic break-induced replication and fork stalling and template switching events using the homology provided by ectopic DNA sequences.<sup>117</sup>

We have only just started to glimpse the intricate mechanistic connections underlying DDT in eukaryotes, and in particular the critical roles of Rad51 and other recombination proteins such as the tumor suppressor genes BRCA1 and BRCA2. A better understanding of the different DDT pathways will require us to differentially address parameters such as the load of DNA

damage, its control by the checkpoints, and DNA lesion specificity. A particular challenge will be deciphering the mechanisms that regulate the replicative and repair activities of HR during the cell cycle, a separation of functions that might also be shared by other DDT mechanisms and which seems to be critical for protection of the genome and prevention of cancer. In addition to the basic and evolutionarily conserved mechanisms ruling DDT addressed in this review, we need to gain a deeper insight into the factors and mechanisms that specifically control the complex DNA damage response in human cells and the genetic consequences of their misregulation.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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