Chapter 16

Roles of RAD18 in DNA Replication and Postreplication Repair

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Chapter Outline

1. INTRODUCTION: THE DDR, DNA DAMAGE–TOLERANCE AND DNA DAMAGE–AVOIDANCE MECHANISMS

DNA damage poses a serious threat to genome stability and the S-phase of the cell cycle is particularly vulnerable to the detrimental effects of bulky replication fork-stalling DNA lesions. Cells have evolved an elaborate signaling network termed the DNA-damage response (DDR) that coordinates DNA replication and DNA repair with cell-cycle progression following genotoxic exposures. DNA damage acquired during S-phase elicits three important protective responses that are mediated at least in large part by the ATR and Chk1 checkpoint kinases [1]: Inhibition of initiation of DNA synthesis at unfired origins of replication and slowing of ongoing replication forks (a mechanism termed the "S-phase checkpoint") [1,2]; Stabilization of stalled replication forks, the crucial function of S-phase checkpoint signaling [2,3]; Inhibition of entry into mitosis in the presence of un-replicated DNA, a mechanism also termed the "replication checkpoint" [3,4]. It has become clear that attenuation of S-phase checkpoint signaling and recovery from DNA damage–induced cell-cycle delays is critically dependent on postreplication repair (PRR) mechanisms that facilitate resolution of stalled DNA replication forks and permit continued S-phase progression on damaged genomic DNA templates [5,6]. PRR of damaged DNA may proceed via trans-lesion synthesis (TLS), a DNA damage–tolerance process that uses error-prone Y-family DNA polymerases to synthesize daughter strand DNA using a damaged template (Fig. 16.1, left). Alternatively, cells may employ an error-free DNA damage–avoidance mechanism termed "template switching" (TS) that depends on the presence of a newly synthesized sister chromatid DNA template (Fig. 16.1, right). Collectively, TLS- and TS-mediated PRR mechanisms allow cells to survive exposure to a variety of genotoxins.

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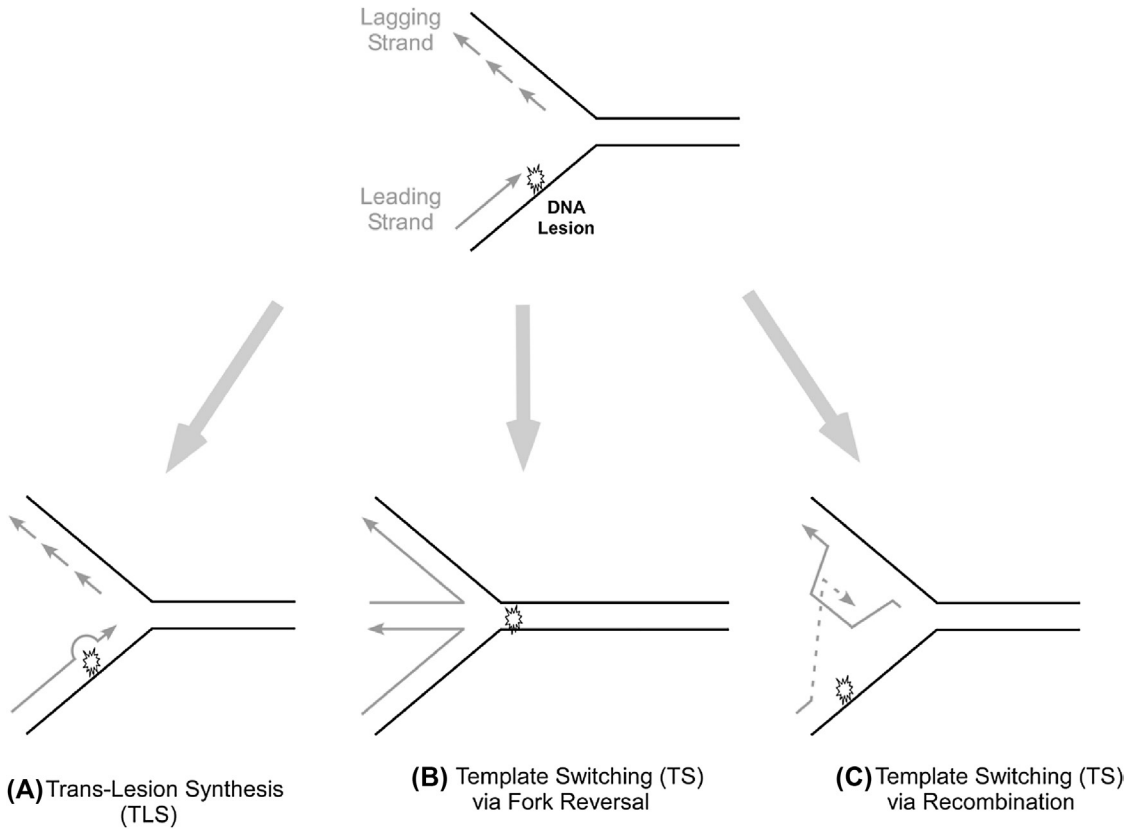

FIGURE 16.1 Potential mechanisms of postreplication repair via TLS and TS. During TLS (A), specialized DNA damage–tolerant Y-family DNA polymerases are recruited to stalled replication forks where they perform error-prone DNA synthesis using damaged templates. TS may proceed via fork reversal (B) or recombination-based (C) mechanisms, both using a newly synthesized undamaged sister chromatid as template for error-free DNA synthesis. See text for details.

TLS and TS are activated by ubiquitination of the DNA polymerase processivity factor Proliferating Cell Nuclear Antigen (PCNA). TLS relies on monoubiquitination of PCNA at Lysine 164 (K164), while TS is promoted by PCNA K164 polyubiquitination. RAD18 is the major PCNA K164-directed E3 ubiquitin ligase in eukaryotic cells. RAD18 exists as a complex with the E2 ubiquitin–conjugating enzyme RAD6 and is activated coincident with the S-phase checkpoint. Therefore, the RAD18–RAD6 complex represents a proximal activator of both TLS and TS pathways. Here we review the activation mechanisms of RAD18, and discuss the roles of its effector TLS and TS pathways in genome maintenance. In particular, we emphasize the basis for coordination of RAD18 with other elements of the DDR. Finally, we consider the potential impact of RAD18-mediated genome maintenance on development and disease.

2. IDENTIFICATION OF RAD18–RAD6 AS A MEDIATOR OF DNA DAMAGE TOLERANCE

The *Saccharomyces cerevisiae RAD18* and *RAD6* genes (encoding E3 ubiquitin ligase and E2 ubiquitin–conjugating enzymes RAD18 and RAD6, respectively) belong to the same epistasis group and were identified based on their roles in conferring tolerance of ultraviolet (UV) light and chemically induced DNA damage [7–9]. *rad18* and *rad6* mutant yeast have PRR defects and accumulate discontinuities in newly replicated DNA following genotoxin exposure [8,10]. Moreover, DNA damage–inducible mutagenesis is attenuated in *rad6* and *rad18* mutants. *S. cerevisiae* RAD6 is a ubiquitin-conjugating (E2) enzyme that can use histones H2A and H2B as substrates [11]. RAD18 associates directly with RAD6, has zinc finger domains that mediate nucleic acid binding [12], binds to ssDNA [13] and has ubiquitin-conjugating and ATP hydrolytic activities [14]. Prakash and colleagues first suggested that DNA-binding and nucleotide-binding activities might enable RAD18 protein to recognize damaged template DNA with high affinity [15]. Furthermore, these workers proposed that ubiquitination of replication factors may be required for activation of postreplicative bypass DNA-repair machinery [13,14].

Human *RAD18* was identified based on homology to the yeast *RAD18* gene. There are two human RAD6 homologues, RAD6A and RAD6B, both of which interact with RAD18 [16,17]. Human cells expressing hRAD18 protein with

a "really-interesting gene" (RING) finger mutation are compromised for PRR [16]. Similarly, *Rad18*-knockout mouse embryonic stem cells generated by gene targeting are PRR-defective and hypersensitive to multiple DNA-damaging agents [18]. Mutation rates (measured by ouabain resistance) are similar between wild-type and *Rad18*-knockout cells. However, spontaneous sister chromatid exchanges (SCEs), random targeting of exogenous DNA into the genome, and gene targeting at the Oct3/4 locus are increased as a result of Rad18-deficiency, demonstrating that Rad18 represses illegitimate recombination events [18]. Increased SCE rates are also observed in *RAD18*$^{-/-}$ DT40 cells, indicative of a role for RAD18 in suppression of HR-mediated PRR [19]. Therefore, similar to *rad18* mutant yeast, *RAD18*-deficient vertebrate cells exhibit genome maintenance defects, indicating conservation of RAD18 function between species.

3. RAD18-MEDIATED PCNA MONOUBIQUITINATION AND THE TLS POLYMERASE SWITCH

PCNA is the critical target whose modification by RAD18-RAD6 directs PRR pathway activation [20]. Jentsch and colleagues showed that RAD18 recruits RAD6 to chromatin to promote PCNA monoubiquitination at K164. The ubiquitin-conjugating MMS2–UBC13 complex is recruited to chromatin by RAD5 (another RING-finger E3 ligase) leading to further K63-linked multi-ubiquitination of the monoubiquitinated PCNA. Thus, different PCNA modifications target for alternative functions in PRR. Stelter and Ulrich showed that PCNA monoubiquitination activates TLS via DNA polymerases eta and zeta, whereas PCNA polyubiquitination promotes error-free repair [21]. PCNA ubiquitination was also shown to be required for DNA damage–induced mutagenesis. Taken together these important studies demonstrated that PRR activation and the selection of error-prone TLS vs. error-free TS pathways are dependent upon posttranslational modifications of PCNA.

K164 is present in human PCNA, indicating that the mechanism of TLS pathway activation is conserved across species [20]. Lehman and colleagues demonstrated that UV irradiation induces PCNA monoubiquitination in a RAD18-dependent manner in human cells and that DNA polymerase eta (Polη, the mammalian homologue of yeast RAD30) associates preferentially with K164-monoubiquitinated PCNA [22]. Interestingly, RAD18 also has a noncatalytic role in regulating TLS via its interactions with Polη [23] (described in more detail later). RAD18-mediated PCNA monoubiquitination also promotes recruitment of DNA polymerase kappa (Polκ) [24], DNA polymerase iota (Polι) [25], and REV1 [26] to sites of replication fork stalling in genotoxin-treated cells. It is unclear whether Y-family polymerases other than Polη are regulated via direct interactions with RAD18. The presence of specialized ubiquitin-binding zinc finger (UBZ) and ubiquitin-binding motif (UBM) domains in the Y-family DNA polymerases provides the molecular basis for the association of Y-family TLS DNA polymerases with monoubiquitinated PCNA [27].

The extent to which PCNA-monoubiquitination is necessary for recruitment of Y-family polymerases to stalled replication forks has been controversial. In one study, PCNA ubiquitination did not disrupt Polδ–PCNA interactions or enhance the binding affinity of TLS DNA polymerases for PCNA, leading to the suggestion that K164 monoubiquitination displaces putative inhibitors of PCNA–TLS polymerase interactions [28]. A UBZ-deficient Polη mutant retaining the PCNA-interacting peptide ("PIP" domain) was able to complement UV-sensitivity defects of *xeroderma pigmentosum*–variant (XPV) cells which lack endogenous Polη [29], further suggesting that PCNA monoubiquitination is nonessential for TLS polymerase activation. However, in "knock-in" mouse cells harboring K164-mutated ubiquitination-resistant PCNA [30], Polη recruitment to stalled replication forks and TLS-mediated recovery from replication fork stalling are compromised but not completely attenuated. Most probably, therefore, monoubiquitinated PCNA does promote TLS but additional mechanisms (likely involving ubiquitin-independent PIP box interactions) contribute to stable association of Y-family TLS polymerases with PCNA.

4. RAD18 STRUCTURE, ACTIVATION, AND COORDINATION WITH THE DDR

4.1 RAD18 Structure

The domain organization of the 495 amino acid (AA) hRAD18 protein is shown in Fig. 16.2 and illustrates major conserved domains including the RING motif (AAs 25–63), a UBZ4-type zinc finger (AAs 201–225), the SAF-A/B, Acinus and PIAS (SAP) domain (AAs 248–282), the RAD6-binding domain (AAs 340–395), and a Polη-binding motif (AAs 401–445) [31]. A crystal structure for the RAD18–RAD6 complex is not yet available. However, biophysical studies indicate that RAD18 exists as an asymmetric heterotrimer consisting of two RAD18 molecules and a single molecule of RAD6 [32,33]. Multiple contacts between RAD18 and RAD6 are necessary for formation of the $[RAD18]_2$–RAD6 complex. The RAD18 RING domain is necessary for PCNA ubiquitination activity [16]. RING domains generally serve as interaction sites for E2

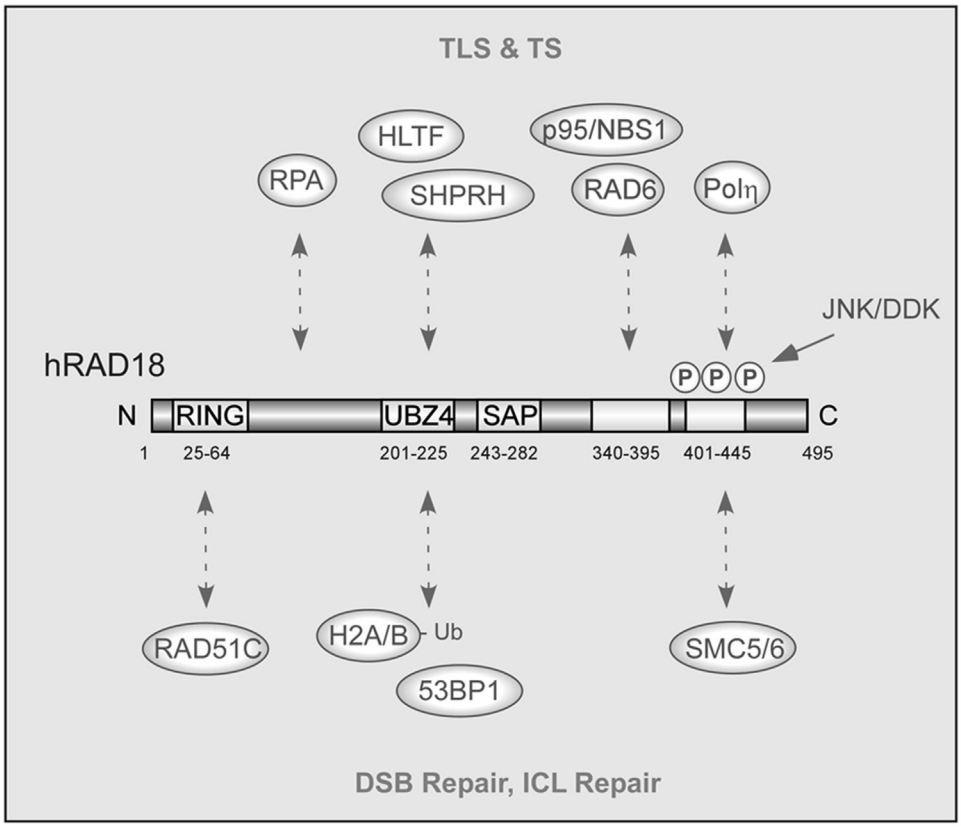

FIGURE 16.2 Domain structure of hRAD18 indicating key domains involved in TLS, TS, and other genome maintenance activities. The diagram shows relative locations of RING, UBZ, and SAP domains in the full-length (495 amino acid, AA) human RAD18 protein. Interaction sites for several key binding partners that mediate TLS and TS (top half of figure) and DSB/ICL repair (bottom half of figure) are indicated. The region spanning AAs 401–445 contains phosphorylation sites for JNK (serine 409) and a cluster of DDK sites (residing in serine residues 432–444). JNK and DDK-mediated phosphorylations are Chk1 dependent and promote associations with Polη (S409, S432–444) and with the SMC5/6 proteins (S432–444) to promote TLS and ICL repair, respectively. See text for details.

enzymes and bring substrates in proximity of the E2 to promote ubiquitination. Similar to other E3 ligases, the N-terminal RING domain of RAD18 contributes to E2 (RAD6) binding [16,31,34]. The RAD18 UBZ domain belongs to the UBZ4 subgroup that is also present in Polκ and WRIP1 [35] The UBZ4 domain is dispensable for RAD18–RAD6 complex formation, catalytic activity, and TLS [33], yet may facilitate DNA binding and may contribute to self-dimerization [16,34]. UBZ-mediated interactions between Rad18 and monoubiquitinated PCNA may also facilitate retention of Rad18 at sites of replication fork stalling, providing a feed-forward mechanism that amplifies the PCNA monoubiquitination response [36].

As discussed later (Section 7), RAD18 participates in DNA double-stranded breaks (DSB) repair independently of its role in TLS and the UBZ motif may facilitate RAD18 recruitment to DSB-flanking ubiquitinated histones [37]. The SAP domain [38] facilitates RAD18 recruitment into Polη-containing nuclear foci, PCNA monoubiquitination, and UV DNA-damage tolerance [39,40], yet is dispensable for the recruitment of RAD18 to DNA DSB [37]. Residues 401–445 of RAD18 interact with Polη and this association is necessary for efficient chaperoning of Polη to sites of replication stalling [23]. The importance of the RAD18–Polη interaction is demonstrated by the observation that Polη interaction–deficient RAD18 mutants that retains E3 ligase activity are compromised for DNA-damage tolerance [23,41]. As discussed later, the Rad18–Polη interaction also integrates TLS with the cell cycle and other genome maintenance pathways.

4.2 RAD18 Activation

DNA damage–induced accumulation of monoubiquitinated PCNA results both from inhibition of PCNA de-ubiquitination [42], and from increased PCNA ubiquitination by RAD18. The RAD18-inducible component of the overall PCNA ubiquitination seems to be a multistep process involving RAD18 recruitment to ssDNA in the vicinity of stalled DNA replication forks, followed by a Polη-mediated "hand-off" to PCNA, as described further on.

DNA damage–induced stalling of replicative DNA polymerases causes uncoupling of leading and lagging strand DNA synthesis and leads to single-stranded DNA (ssDNA) accumulation [43]. In *S. cerevisiae*, UV-induced replication stalling increases the length of replication-associated ssDNA tracts from about 100 to 200 bases [44]. ssDNA is the proximal trigger that activates several branches of the DDR including the ATR/Chk1-mediated S-phase checkpoint [45]. PCNA ubiquitination is selectively induced by genotoxins that generate ssDNA via uncoupling of replicative helicase and polymerase activities [46]. It has long been known that RAD18 has ssDNA-binding activity [13] and ssDNA generated during replication fork stalling is probably the basis for the initial recruitment of RAD18 to the vicinity of damaged DNA. Indeed, RAD18 preferentially recognizes synthetic ssDNAs that resemble replication fork intermediates [40]. ssDNA generated by stalled replication forks is coated by replication protein A (RPA), and RPA-ssDNA is a key mediator of ATR/Chk1 pathway activation. In *S. cerevisiae*, 95% degradation of temperature-sensitive rfa1 (the large subunit of yeast RPA) mutant sustains DNA replication yet abolishes PCNA monoubiquitination, indicative of a role for RPA-ssDNA accumulation in RAD18 activation [46]. Moreover, RAD18–RAD6 complex interacts with RFA1 and RFA2 subunits of yeast RPA, even in the absence of DNA. An N-terminal domain of yeast RAD18 confers RPA-binding activity while the SAP domain (necessary for ssDNA binding) is dispensable for RPA association. Therefore, recruitment of RAD18 to DNA at sites of replication stalling may require independent interactions of RAD18 with RPA and ssDNA, at least in yeast. An RPA-ssDNA-based mechanism of RAD18 activation explains the temporal correlation of PCNA ubiquitination and Chk1 phosphorylation in genotoxin-treated cells and provides a parsimonious mechanism for simultaneous activation of two major elements of the DDR (TLS and the S-phase checkpoint).

Although RPA-coated ssDNA might explain the initial recruitment of Rad18 to the local environment of stalled replication forks, this model does not explain how Rad18 associates with PCNA, its critical substrate in the TLS pathway. RAD18 lacks a PIP box or any known PCNA-interacting motifs. However, the RAD18–Polη interaction may facilitate association of RAD18 with PCNA: Polη interacts with PCNA via a PIP box, thereby providing a potential mechanism for targeting the Polη-bound RAD18 to PCNA. Indeed, Polη promotes association of RAD18 with PCNA *and* enhances PCNA monoubiquitination in vitro and in cultured human cells [47]. A catalytically inactive Polη mutant retains RAD18-binding activity, promotes PCNA monoubiquitination, and stimulates the recruitment of other TLS polymerases to PCNA [47]. Moreover, UV sensitivity of Polη-deficient cells is partially rescued by the expression of catalytically inactive Polη [48]. Therefore, Polη has a noncatalytic scaffolding role in promoting RAD18-mediated PCNA monoubiquitination and DNA-damage tolerance.

The RAD18-binding motif of Polη has not been mapped precisely, yet resides in a C-terminal domain (AAs 594–713) that is frequently deleted in XPV patients [49]. Therefore, genome instability in some XPV patients may result from defective Polη scaffold function and altered targeting of RAD18 to PCNA. The extent to which the other Y-family polymerases associate with RAD18 and promote PCNA monoubiquitination is unclear, although in a side-by-side comparison, Polκ fails to promote PCNA monoubiquitination as efficiently as Polη [50]. Interestingly, substitution of the Polκ PIP box with the Polη core PIP sequence plus PIP box-flanking residues confers increased PCNA monoubiquitination activity upon Polκ [47]. Therefore, the high affinity of the Polη PIP box for PCNA may explain why Polη supports RAD18-mediated PCNA ubiquitination preferentially when compared with other Y-family DNA polymerases.

RAD18 can perform sequential monoubiquitinations of multiple units of the PCNA homotrimer and the mono- and multi-monoubiquitinated PCNA trimers might activate distinct modes of DNA-damage tolerance [36]. Interestingly, trimeric PCNA complexes containing one or two K164-monoubiquitinated monomers are ubiquitinated more efficiently by RAD18 when compared with unmodified PCNA trimers [36]. That is, PCNA monoubiquitination appears to stimulate further ubiquitination of the other PCNA subunits. It is possible that the UBZ domain of RAD18 mediates its retention at monoubiquitinated PCNA, establishing a feed-forward mechanism for enhanced monoubiquitination of other PCNA monomers in the same trimer.

In addition to RPA-ssDNA and Polη, several other proteins may influence RAD18-mediated PCNA ubiquitination and TLS at sites of DNA replication stalling. For example, the orphan protein C1orph124 (also designated "Spartan") facilitates RAD18–PCNA association and modestly stimulates PCNA monoubiquitination [51]. Spartan/C1orf124 also interacts with the replicative DNA polymerase POLD3 and PDIP1 in the absence of DNA damage, but preferentially associates with Polη upon UV damage, perhaps indicating additional roles for Spartan in the polymerase switch [52]. Spartan may also promote accumulation of monoubiquitinated PCNA independently of its putative role in RAD18 activation by protecting against de-ubiquitination [53]. It must be noted, however, that the role of Spartan in TLS is not entirely clear since other studies indicate Spartan is not required for PCNA monoubiquitination, but instead interacts with p97 "segregase" to promote removal of Polη from sites of UV-induced DNA damage, thereby reducing mutagenesis [54]. Other reports indicate that Spartan depletion increases rates of mutagenesis [55]. Clearly therefore, the roles of Spartan in regulating TLS are complex and incompletely understood. Han and colleagues in 2014 identified the ARF-directed E3 ligase SIVA1 as another mediator that physically bridges chromatin-bound RAD18 and PCNA [56]. Therefore, SIVA1 may function as substrate receptor for RAD18 ubiquitin ligase that promotes PCNA ubiquitination.

Other proteins with known roles in distinct genome maintenance pathways have also been implicated in RAD18-mediated TLS. p95/NBS1 (mutated in Nijmegen breakage syndrome) interacts directly with the RAD6-binding domain of RAD18 [57] and promotes RAD18 distribution to sites of DNA replication stalling, stimulating PCNA monoubiquitination [57]. The BRCA1 (breast cancer 1) protein, a major component of the HR pathway, also recruits RPA, RAD18, Polη, and REV1 to damaged chromatin to promote TLS and template switching [58]. The participation of major DSB-sensing and repair factors in TLS is indicative of extensive crosstalk and coordination between genome maintenance pathways.

In summary, multiple factors (RPA, ssDNA, Polη, NBS1, BRCA1, SIVA1, and doubtless other proteins) associate with RAD18 and/or create a local environment that is permissive for PCNA monoubiquitination and TLS at stalled replication forks.

4.3 Transcriptional and Posttranslational Regulation of RAD18

Ectopic over-expression of RAD18 in cultured cells induces DNA damage–independent PCNA monoubiquitination, drives TLS polymerases to sites of DNA replication [24], and confers DNA-damage tolerance [59]. Therefore, stringent control of RAD18 expression is important for limiting error-prone DNA synthesis and maintaining genome stability. During the cell cycle, RAD18 protein levels are relatively low in G1, increase during S-phase, and decrease rapidly following mitosis [60]. Interestingly, the *RAD18* promoter is a target of the DNA damage–inducible E2F family member E2F3, which mediates transcriptional induction of RAD18 expression in genotoxin-treated cells [61]. Other mechanisms for transcriptional regulation of RAD18 expression have not been described. However, RAD18 protein levels are regulated via its ubiquitin-dependent proteolysis. RAD18 is polyubiquitinated (via auto-ubiquitination) and the polyubiquitinated species is targeted for proteasomal degradation [34]. A 2015 siRNA screen identified RAD18 as a target of the de-ubiquitinating enzyme USP7 [62]. Thus, USP7-mediated removal of polyubiquitin chains from RAD18 confers stability and represents an important mechanism for maintaining DNA-damage tolerance via TLS.

Integration of TLS with S-phase, checkpoint signaling, and stress kinase pathways is achieved through RAD18 phosphorylation [41,63]. The Polη-binding domain of hRAD18 contains a cluster of DBF4/DRF1-dependent kinase (DDK) phosphorylation sites (including the preferred DDK phosphorylation site at S434) embedded in an acidic region termed the "S-box" [41] and a c-Jun N-terminal kinase (JNK) phosphorylation site at S409 [63]. DDK is a critical protein kinase for the initiation of DNA synthesis [64] and JNK mediates signaling in response to diverse cellular stresses, including many genotoxic agents [65]. The JNK and DDK phosphorylation sites of RAD18 are conserved between species and serve to promote RAD18–Polη complex formation, contributing to DNA-damage tolerance. DBF4, the activating subunit of DDK binds RAD18 and likely directs CDC7 to RAD18 [66]. Interestingly, DBF4 might also promote PCNA monoubiquitination by facilitating RAD18 recruitment to damaged chromatin independently of its role in DDK-mediated RAD18 phosphorylation. RAD18 phosphorylation by JNK and DDK depends on Checkpoint Kinase 1 (CHK1), a key mediator of the S-phase checkpoint [67]. Therefore, RAD18 phosphorylation by DDK and JNK coordinates TLS with DNA replication and stress kinase signaling via the S-phase checkpoint.

ATR/CHK1 signaling promotes PCNA monoubiquitination [24,68], although the mechanism of Chk1-induced PCNA monoubiquitination is not known. CHK1-dependent formation of the RAD18–Polη complex (required for targeting RAD18 to PCNA) provides a plausible mechanism for the stimulatory effect of CHK1 on PCNA monoubiquitination [24,68]. The association of RAD18 with Polη also provides a basis for integrating RAD18-mediated PCNA monoubiquitination with p53 signaling. The *POLH* gene (encoding Polη) is a transcriptional target of p53 and Polη protein levels are induced by DNA damage [69]. In cultured cells, RAD18 protein is present in excess of Polη by about 100-fold [47], and consequently Polη levels are limiting for recruitment of RAD18 to PCNA. However, DNA damage–induced p53 activity stimulates Polη expression, increasing the availability of Rad18–Polη complexes that associate efficiently with PCNA.

In summary, we propose an integrated model for initiation of TLS (Fig. 16.3) in which the RAD18–RAD6–Polη complex is first recruited to the vicinity of stalled replication forks via interaction of RAD18 with RPA-coated ssDNA. Subsequent association of the RAD18 complex with PCNA is facilitated by Polη scaffolding activity, leading to K164 monoubiquitination of one PCNA subunit. Additional scaffolding proteins, such as Spartan, p95/NBS, and SIVA1 may facilitate the interaction of RAD18 with PCNA. USP7 and p53 contribute to maintaining RAD18 expression levels. DDK/JNK-mediated RAD18 phosphorylation preserves RAD18–Polη interactions and promotes PCNA monoubiquitination and TLS.

5. DNA REPLICATION–INDEPENDENT RAD18 ACTIVATION AND TLS

There is now considerable evidence that RAD18-mediated lesion bypass occurs postreplicatively and serves to fill ssDNA gaps remaining behind a newly-primed leading strand [70–72]. For example, TLS deficiency does not affect rates of leading

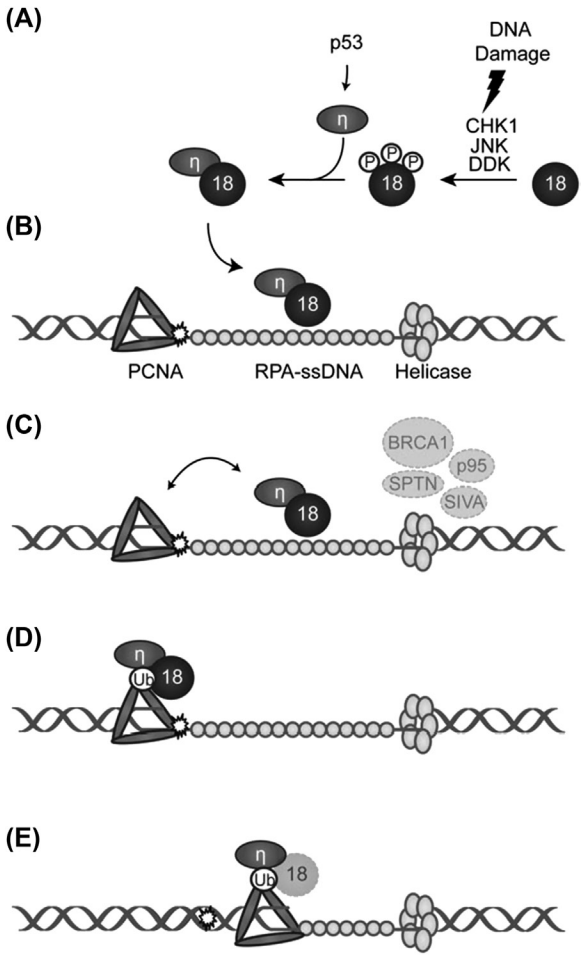

FIGURE 16.3 Mechanisms of RAD18 recruitment to stalled replication forks. (A) DNA damage induces RAD18 phosphorylation (by JNK and DDK), promoting its association with Polη. DNA damage also induces Polη expression via p53-dependent transcription, further contributing to the formation of RAD18–Polη complexes. (B) The RAD18–Polη complex is recruited to the vicinity of stalled replication forks via interactions between RAD18 and RPA-ssDNA. (C) Polη binds PCNA, thereby serving as a scaffold that mediates association of RAD18 with PCNA. Various other factors including BRCA1, p95/NBS1, Spartan, and SIVA may interact with core TLS proteins or create a local environment that facilitates RAD18 interactions with PCNA. (D) RAD18 monoubiquitinates PCNA leading to high affinity binding of Polη and other Y-family TLS polymerases. (E) Association of TLS polymerases with monoubiquitinated PCNA allows replicative bypass of DNA lesions.

strand synthesis on damaged templates, but instead leads to postreplicative gaps [72]. Limiting TLS to G2/M phase efficiently promotes lesion tolerance, fully consistent with the idea that TLS serves to fill ssDNA gaps behind newly re-primed replication forks [70]. In elegant experiments that visualized and quantified PRR tracts, TLS was temporally and spatially separable from global genomic DNA replication [71]. Thus, RAD18-mediated TLS is truly a PRR mechanism that operates distal to active replication forks.

Interestingly, several studies show that RAD18/TLS-mediated patch filling is not necessarily restricted to ssDNA behind replication forks, and also contributes to repair of ssDNA breaks (SSBs) that arise outside S-phase. For example, UV irradiation of quiescent (G0) human fibroblasts induces PCNA monoubiquitination and PCNA association of Polκ [73–75]. Polκ-deficient MEF exhibit reduced repair synthesis activity, particularly in the presence of the ribonucleotide reductase (RNR) inhibitor hydroxyurea (HU), suggesting that TLS polymerases participate in nucleotide excision repair (NER) when dNTP concentrations are limiting [76]. In nonproliferating cells, exonuclease 1 (EXO1) activity converts NER intermediates to long ssDNA gaps that are capable of activating the Chk1 pathway [77,78]. Similarly, it is likely that RPA-coated ssDNA generated at sites of NER could recruit RAD18, thereby initiating TLS independently of DNA replication. Indeed, recruitment of Polκ to monoubiquitinated PCNA is observed in nonreplicating wild-type but not XPA cells [73]. Therefore, DNA intermediates, such as ssDNA generated during the incision phase of NER are likely to initiate TLS outside S-phase.

RAD18-mediated PCNA monoubiquitination is also inducible by H_2O_2 (a source of oxidative DNA damage) in nonreplicating cells [74,79]. In contrast with UV-induced DNA damage (which induces PCNA ubiquitination via NER

intermediates), H_2O_2-induced PCNA monoubiquitination depends on the MSH2–MSH6 complex (but not on MLH1) [79]. Thus, oxidative stress-induced clustered lesions evading repair by DNA glycosylases may activate MSH2–MSH6 to load an exonuclease (likely EXO1) that generates the ssDNA tracts needed to activate RAD18. RAD18-mediated PCNA monoubiquitination then facilitates recruitment of Polη, which contributes to repair synthesis. RAD18-mediated TLS is essential for facilitating completion of DNA replication and conferring cell survival after oxidative injury in S-phase [74]. Interestingly, however, the role of RAD18 in preventing H_2O_2-induced DSBs and lethality during G1 is nonessential owing to backup nonhomologous end joining (NHEJ)-mediated DSB repair [74]. Alkylating agents, such as MNNG also induce S-phase-independent PCNA monoubiquitination via noncanonical Mismatch Repair (MMR) [80]. While H_2O_2-induced PCNA monoubiquitination is MLH1 independent, MLH1 is necessary for PCNA monoubiquitination following exposure to MNNG. Therefore, noncanonical MMR in G1 may lead to MUTLα-induced endonucleolytic nicks and loading of EXO1, generating the ssDNA required for RAD18 activation and PCNA monoubiquitination. Extension of ssDNA tracts (by EXO1 and/or other exonucleases) likely represents a general mechanism for replication fork-independent recruitment of RAD18 to sites of NER or SSB repair. RAD18-mediated TLS can also repair ssDNA breaks persisting after replication in G2 and UV-induced PCNA ubiquitination is observed in synchronized metaphase-arrested cells [79]. Therefore, RAD18-mediated PCNA monoubiquitination occurs throughout the cell cycle. Why then would cells use error-prone TLS DNA polymerases in lieu of the error-free polymerases conventionally employed for NER or SSB repair? One possibility is that TLS polymerases may be required for SSB repair when clustered DNA lesions are generated on both strands. In addition, TLS DNA polymerases may be more efficient than high-fidelity DNA-repair polymerases when nucleotide concentrations are low (as is the case in G1 cells). With the realization that TLS is operational outside S-phase, RAD18 and its effector Y-family polymerases represent potential mediators of genome maintenance in diverse nonreplicating cell types including quiescent stem cells, postmitotic and differentiated neurons, and cardiomyocytes that experience high levels of oxidative stress.

6. RAD18 FUNCTIONS IN ERROR-FREE PRR VIA TEMPLATE SWITCHING

The error-free PRR pathway uses a newly synthesized daughter strand of the undamaged complementary sequence as a template for extending stalled leading strands [81]. The molecular basis of TS is not fully understood, but there is evidence for both fork reversal and recombination-mediated template-switching mechanisms (Fig. 16.1, right), as described further on.

In *S. cerevisiae*, error-free PRR involves the *RAD6* epistasis group genes *MMS2*, *UBC13*, and *RAD5* which prevent accumulation of daughter strand discontinuities opposite fork-stalling DNA lesions [82–84]. The ubiquitin-conjugating enzyme UBC13 and a noncanonical UBC variant MMS2 form a heteromeric complex with RAD5 [83]. RAD5 is an SWI/SNF ATPase family member [84] that contains a C3HC4 RING motif [85] and possesses DNA-dependent ATPase activity [84]. RAD5 recruits UBC13-MMS2 to damaged chromatin, to form a complex that cooperates with RAD6–RAD18 to polyubiquitinate PCNA at K164 [86].

There are two known mammalian RAD5 homologues, SHPRH and HLTF. Elegant biochemical studies have shown that purified HLTF and SHPRH cooperate with RAD18-RAD6 to polyubiquitinate PCNA, yet achieve PCNA polyubiquitination via distinct mechanisms. SHPRH polyubiquitinates PCNA via extension of monoubiquitinated K164 [87]. On the other hand, HLTF forms a thiol-linked Ub chain on UBC13 that is transferred to RAD6. RAD18 then transfers the pre-conjugated Ub chain to K164 of unmodified PCNA [20,88].

SHPRH mediates alkylating agent (MMS)-induced PCNA polyubiquitination and confers tolerance to MMS (but not to UV, 4-NQO, and MMC [89]), whereas HLTF mediates PCNA polyubiquitination and confers DNA-damage tolerance in response to bulky DNA lesions [90]. In UV-irradiated mammalian cells, HLTF enhances PCNA monoubiquitination and Polη recruitment, while inhibiting SHPRH function. Conversely, MMS promotes SHPRH–RAD18 interactions, while inducing HLTF degradation. Thus, HLTF and SHPRH promote error-free PRR in a DNA damage–specific manner [91].

It is hypothesized that polyubiquitinated PCNA generated via the concerted actions of RAD18 and RAD5 recruits the mediators of the TS pathway to stalled replication forks. ZRANB3 (Zn finger, RAN-binding domain containing 3, also known as Annealing Helicase two or AH2) is recruited to polyubiquitinated PCNA where it facilitates fork regression, replication fork restart, and DNA-damage tolerance [92–94]. Most likely, additional proteins remain to be identified whose docking at polyubiquitinated PCNA promotes template switching.

Biochemical studies in 2015 suggested a mechanism for HLTF in promoting fork reversal-based template switching [95,96]. Fork reversal occurs when the stalled replication fork is remodeled by pairing of newly synthesized chromatids to form a fourth regressed DNA duplex termed a Holliday junction (HJ). Fork reversal provides an opportunity for error-free DNA synthesis using the undamaged lagging strand as an alternative template (Fig. 16.1).

HLTF and RAD5 possess dsDNA translocase activity with 3′5′ polarity that catalyzes fork reversal and branch migration in an ATP-dependent fashion [97–99]. RAD5 and HLTF share a HIP116/HLTF RAD5 N-terminal (HIRAN) domain that is crucial for fork reversal activity [95,96]. The HIRAN domain is a unique "OB-fold" (a general nucleic acid–binding domain) that recognizes free 3′-ssDNA ends, thereby targeting HLTF and RAD5 to the 3′-end of the leading strand to direct fork remodeling and reversal [95,96]. Replication fork speed is globally increased in HLTF-deficient cells owing to the lack of fork reversal [95]. SHPRH lacks a HIRAN domain, indicating that additional mechanisms exist for recruiting RAD5 homologues to sites of TS.

HLTF can also promote D-loop formation in a Rad51-independent manner [100], possibly indicating dual roles in fork reversal and recombination-mediated modes of TS. Interestingly, ZRANB3 disrupts D-loops formed by strand invasion [92], perhaps suggesting that HLTF and ZRANB3 act in distinct early and late stages of TS, respectively.

Clearly, error-free and error-prone PRR act in opposition, with the RAD5 pathway preventing error-prone (mutagenic) TLS. It is not clear why cells would employ error-prone PRR (TLS) if an error-free (TS) pathway is available. It has been suggested that TS is employed when DNA damage is too severe to be processed via TLS and results in persistence of 3′-ends at stalled DNA replication forks [96]. Nevertheless, selection of error-free TS vs. error-prone TLS could profoundly influence genome stability and mechanisms of carcinogenesis. *HLTF* promoter methylation and loss of HLTF expression are observed in cancer [101] and may contribute to increased TLS and mutagenesis. In summary, RAD18 can direct both TS- and TLS-mediated PRR. The putative mechanisms that dictate the selection of RAD18-dependent TLS and TS remain to be determined.

7. TLS- AND TS-INDEPENDENT ROLES OF RAD18 IN GENOME MAINTENANCE

Although best known for its roles in error-prone TLS and TS, RAD18 participates in additional genome maintenance pathways, including DSB repair and ICL repair. A detailed discussion of noncanonical TLS/TS-independent RAD18 activities is beyond the scope of this review and roles of RAD18 in DSB and ICL repair are summarized very briefly.

In DT40 cells and mammalian cancer cells, RAD18 promotes homologous recombination [37,102]. RAD18 mediates HR by binding and chaperoning the RAD51C recombinase to "ionizing radiation induced foci" (ICRF, corresponding to sites of DSB repair) in the nucleus [37]. Association of the RAD18–RAD51C complex with IRIF depends upon RNF8, an E3 ligase which monoubiquitinates Histone H2A (and perhaps other chromatin components) in the vicinity of DSBs, and is mediated via the RAD18 UBZ domain. RAD18-mediated RAD51C chaperone activity does not require the SAP domain or E3 ubiquitin ligase activity. Therefore, the role of RAD18 in RAD51C regulation is fully separable from its PRR activities.

In addition to its role in HR, RAD18 may influence DSB repair via NHEJ.

RAD18 is recruited to X-ray-induced DSB in a 53BP1-dependent manner during G1. Moreover, RAD18 monoubiquitinates and promotes chromatin retention of 53BP1, conferring DNA-damage tolerance [103]. The RAD18 UBZ domain (which is dispensable for RAD18-mediated PCNA modification) is required for formation of 53BP1 IRIF. Therefore, mechanisms of RAD18-mediated PCNA and 53BP1 monoubiquitination are separable. 53BP1 plays important roles in the choice of DSB-repair mechanism, promoting NHEJ and inhibiting homology-directed repair (HDR) [104]. Therefore, RAD18–53BP1 signaling might promote DSB repair via NHEJ, although a direct role of RAD18 in NHEJ has not been formally demonstrated.

RAD18 is also implicated as a potential upstream activator of the Fanconi Anemia (FA) pathway. FA is an autosomal-recessive chromosomal instability syndrome characterized by developmental defects, bone marrow failure, and cancer propensity [105]. FA cells are hypersensitive to interstrand cross-link (ICL)-inducing agents including cisplatin and mitomycin C (MMC). There are at least 18 complementation groups of FA and the protein products of the *FANC* genes mutated in FA patients (termed "FANCA" through FANCT) function in a common ICL-repair pathway. When DNA replication forks encounter ICL, an FA "core complex" comprising "FANCs A, B, C, E, F, G, L, and M" functions as a multi-subunit E3 ubiquitin ligase to monoubiquitinate FANCD2 and FANCI. Monoubiquitinated FANCD2-FANCI is the presumed effector of the FA pathway and directs ICL repair, most likely promoting endolytic processing of cross-linked DNA [106].

RAD18 promotes FA pathway activation and FANCD2-dependent DNA-damage tolerance [107–111], although the mechanisms of RAD18-dependent FANCD2 ubiquitination are lesion specific. For bulky benzo[a]pyrene and cisplatin adducts and UV-induced DNA lesions, FA pathway activation requires PCNA monoubiquitination and Polη activation [107,110]. However, FA pathway activation in response to the Topoisomerase inhibitor camptothecin (CPT, which induces replication-dependent DSB) is RAD18 mediated but TLS independent [108]. Precisely how RAD18 facilitates FA pathway activation in response to DSB is unclear. However, catalytically inactive (C28>F-mutated) RAD18 does not support CPT-induced FANCD2 monoubiquitination, possibly indicating that an unidentified RAD18 substrate must be ubiquitinated to mediate FA pathway activation following Topoisomerase I inhibition. In addition to its proximal role(s) in FA pathway

activation, RAD18 contributes to ICL repair by facilitating association of Structural Maintenance of Chromosome 5 and 6 (SMC5/6) to ubiquitinated histones in the vicinity of damaged chromatin [112]. RAD18 scaffold function in SMC5/6 recruitment and ICL repair is RAD6 independent and does not require E3 ubiquitin ligase activity. Similar to RAD18 function in RAD51C chaperoning, the recruitment of RAD18 to sites of ICL requires UBZ-mediated interactions with ubiquitinated chromatin. Interestingly, although the scaffolding role of RAD18 in ICL repair is TLS independent, the same DDK-mediated phosphorylations that promote RAD18–Polη [41] mediate SMC5/6 complex formation [112]. Therefore, DDK-dependent phosphorylation of RAD18 promotes both TLS and ICL repair, providing a common mechanism for S-phase-specific activation of two important genome maintenance pathways.

8. PHYSIOLOGICAL ROLES OF RAD18

Although numerous studies suggest roles for RAD18 in multiple genome maintenance pathways, physiological functions of RAD18 in vivo are poorly defined. Genome maintenance pathways often have enormous impact on development and tumorigenesis. The few known developmental roles of RAD18 and the potential impact of RAD18 on genome stability and tumorigenesis are considered briefly here.

8.1 Developmental Roles of RAD18

Rad18 (but not Polη) is expressed at high levels in mouse testes and localizes to undifferentiated spermatogonia and the XY body (a region containing transcriptionally silent unpaired XY chromosomes) [113], and to a subset of Spo11-induced meiotic DSB [114]. *Rad18*$^{-/-}$ mice are viable yet have decreased testes size and fertility defects upon aging. For example, while young (2-month old) *Rad18*$^{-/-}$ mice have normal spermatogenesis, 25% of the seminiferous tubules in aged animals (>12 month) lack germ cells, due to depletion of spermatogonial stem cells. Thus, Rad18 is important for long-term maintenance of spermatogenesis [115]. It is likely therefore that stem cells tolerate endogenous forms of DNA damage via Rad18-mediated DNA repair. However, the Rad18 effector pathways (TLS, FA, HR) required for maintenance of spermatogonial stem cells are not known. In stable *Rad18* knock-down (KD) mice, H3K4me2 is increased on the XY body (and elsewhere in the nucleus) and there is increased frequency of XY asynapsis when compared with WT mice [114]. Therefore, the roles of Rad18 in spermatogenesis and meiosis are probably TLS independent and involve DSB processing. Since FA patients and *Fanc*-deficient mice have fertility defects, it is possible that meiotic roles of Rad18 also involve the FA pathway. Indeed, the Spo11-induced redistribution of Fancd2 to the XY body is compromised in *Rad18*$^{-/-}$ mice [115a], consistent with a role for the Rad18-FA signaling axis in normal germ cell function. However, Rad18 mutant mice do not recapitulate baseline hematopoietic defects of FA patients and Fanc mutant mice [115a]. Therefore RAD18 is not an obligate component of the FA pathway in hematopoietic cells.

8.2 RAD18 Roles in Tumorigenesis

From cell culture studies, RAD18 clearly impacts many genome maintenance pathways: RAD18 has the potential to promote both error-free and mutagenic DNA-damage tolerance (via TS and TLS, respectively). RAD18 deficiency can generate DSB owing to defects in recovery from replication fork stalling. Moreover, RAD18 can promote DSB repair via error-free HR or perhaps stimulate indiscriminate genome-destabilizing NHEJ via 53BP1. Therefore, RAD18 could influence the fidelity or DNA replication/repair in ways that preserve genome stability (TS, HR) and suppress tumorigenesis or that cause mutations (via error-prone TLS or NHEJ) and drive tumorigenesis. Effects of *Rad18* on tumorigenesis in vivo have not been addressed experimentally. Nevertheless, the potential impact of Rad18 on mechanisms of genomic instability and carcinogenesis are considered further on.

Because Rad18 promotes Polη activity, *Rad18*$^{-/-}$ mice might recapitulate the UV-sensitivity and UV-induced skin cancer-propensity phenotypes of Polη-deficient mice [116,117]. Alternatively, *Rad18* deficiency and Polη deficiency could result in distinct phenotypes: UV-induced mutations in Polη-deficient cells result from error-prone compensatory lesion bypass by other Y-family DNA polymerases [118] whose activities are also RAD18 dependent. Therefore, it is possible that overall mutagenic bypass will be reduced when *Rad18* is absent—potentially leading to reduced carcinogenesis. On the other hand, because *Rad18*-deficiency in carcinogen-treated cells leads to of DSB [24,74], *Rad18*-deficienct cells could show reduced rates of point mutations (owing to reduced TLS), and increased translocations due to NHEJ-mediated DSB repair.

In addition to its potential roles in determining the balance between mutagenesis and gross chromosomal rearrangements, RAD18 might affect tumorigenesis by influencing tolerance of oncogenic stress. Oncogene expression in primary

cells elicits "DNA replication stress" via diverse mechanisms including generation of genotoxic reactive oxygen species (ROS) [119–121], depletion of dNTP pools [122], and re-replication (repeated "firing" of replication origins every S-phase [123]). RAD18 is activated by many stresses commonly incited by oncogenes including ROS [74,79], dNTP depletion [124], and origin re-firing in geminin-depleted cells [125]. Importantly, RAD18 facilitates ongoing DNA synthesis in the face of excess ROS, dNTP shortage, and origin re-firing. Therefore, RAD18-mediated genome maintenance might enable proliferation and survival of neoplastic cells, thereby contributing to tumorigenesis. By analogy, the ATR-mediated S-phase checkpoint pathway (which is activated coincident with TLS) may in some instances promote survival of neoplastic cells and contribute to tumorigenesis [126]. Experiments with genetically engineered mice are required to elucidate the roles of Rad18 in tumorigenesis in response to different oncogenic drivers.

Cancer cells typically express very high levels of RAD18 and TLS polymerases when compared with primary untransformed cells—an observation that is potentially consistent with a selective advantage for TLS-proficient cells in oncogenic stress tolerance. Unfortunately, RAD18/TLS polymerase activity in cancer cells is likely to confer resistance to genotoxic therapeutic agents. Cisplatin is an important therapeutic agent for many cancers [127]. However, the success of cisplatin therapy is limited due to several mechanisms that confer cisplatin resistance including increased DNA-damage tolerance [128,129]. Polη allows replication of cisplatin-damaged DNA templates [130–137] and is a reliable marker of cisplatin resistance and poor outcome in patients with non-small cell lung cancer (NSLC) [138,139]. In cell culture studies, cancer cells lacking Polη [136,140,141] or RAD18 [19,142] fail to replicate cisplatin-damaged genomes and instead accumulate unfilled postreplicative gaps, collapsed replication forks, and lethal DNA DSBs. Therefore, RAD18-mediated TLS represents an appealing therapeutic target pathway whose inhibition may sensitize cells to cisplatin [143,144]. Cisplatin therapy also leads to serious side effects including ototoxicity, nephrotoxicity, and neurotoxicity [145–148]. Therefore, inhibition of RAD18-mediated TLS could lower the therapeutic dose of cisplatin and help minimize toxic side effects. Because RAD18 also participates in DSB repair [37,108], suppression of RAD18 function might also be a promising approach for sensitizing cancer cells to camptothecin or radiotherapy.

9. CONCLUSIONS AND PERSPECTIVES

The E3 ubiquitin ligase RAD18 is a major apical component of the DDR with important roles in both TLS and TS pathways of PRR, namely. RAD18 also has TLS/TS-independent roles in DSB repair and ICL repair. RAD18 functions in genome maintenance are integrated with the cell cycle, DNA replication, and checkpoint signaling via transcriptional and posttranslational mechanisms. RAD18 functions in genome maintenance have been identified mainly based on studies with cultured cell lines. However, *Rad18* is a nonessential gene (at least in mice) and *Rad18* deficiency does not result in any overt developmental defects or cancer propensity. Further work is necessary to define the physiological roles of Rad18 and to identify putative genes and pathways that may explain why *Rad18* is nonessential. We speculate that redundant genome maintenance mechanisms must be eliminated to reveal important roles of Rad18. Since RAD18 deficiency in cultured cells leads to DSBs, it is possible that back-up DSB-repair pathways compensate for Rad18 deficiency in vivo. In this regard, perhaps 2014 studies with *Caenorhabditis elegans* DNA-repair mutants are instructive: In *C. elegans* strains lacking Y-family TLS polymerases, DSBs are repaired via the A-family polymerase theta (PolQ, which mediates alternative NHEJ) [149]. It is possible that interesting genome maintenance defects will be revealed in mice harboring combined deficiencies in Rad18 and NHEJ or other DSB-repair genes. RAD18 deficiency sensitizes human cancer cells to therapeutic genotoxic agents. Therefore, understanding RAD18 signaling mechanisms in cancer cells may facilitate identification of synthetic lethalities and development of small molecule inhibitors that augment the anti-neoplastic effects of existing genotoxic therapies.

GLOSSARY

D-Loop A DNA structure formed during HR in which two strands of a double-stranded DNA molecule are separated for a stretch and held apart by a third invading strand of DNA.

Synthetic lethality Death resulting from combined mutations in two or more genes whose individual mutations do not compromise viability.

Template switch An error-free "DNA damage–avoidance" mechanism that allows continued DNA replication of damaged genomes by using a newly synthesized undamaged sister chromatid as a template.

LIST OF ABBREVIATIONS

CPT Camptothecin

DDR DNA-damage response

D-loop Displacement loop
DSB Double-stranded DNA break
FA Fanconi anemia
HDR Homology-directed repair
HJ Holliday junction
HR Homologous recombination
HU Hydroxyurea
ICL Interstrand crosslinker
IRIF Ionizing radiation-induced foci
MEF Mouse embryonic fibroblast
MMC Mitomycin C
MMR Mismatch repair
MNNG Methylnitronitrosoguanidine
NER Nucleotide excision repair
NHEJ Nonhomologous end joining
NSCLC Nonsmall cell lung cancer
PIP PCNA-interacting peptide
PRR Postreplication repair
RING Really interesting gene
RNR Ribonucleotide reductase
SCE Sister chromatid exchange
ssDNA Single-stranded DNA
TLS Trans-lesion synthesis
TS Template switching
UBM Ubiquitin-binding motif
UBZ Ubiquitin-binding zinc finger
UV Ultraviolet radiation
XPA *Xeroderma pigmentosum* complementation group A
XPV *Xeroderma pigmentosum* complementation group V

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