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Regulation of Rad6/Rad18 Activity During DNA Damage Tolerance

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Abstract

Replicative polymerases (pols) cannot accommodate damaged template bases, and these pols stall when such offenses are encountered during S phase. Rather than repairing the damaged base, replication past it may proceed via one of two DNA damage tolerance (DDT) pathways, allowing replicative DNA synthesis to resume. In translesion DNA synthesis (TLS), a specialized TLS pol is recruited to catalyze stable, yet often erroneous, nucleotide incorporation opposite damaged template bases. In template switching, the newly synthesized sister strand is used as a damage-free template to synthesize past the lesion. In eukaryotes, both pathways are regulated by the conjugation of ubiquitin to the PCNA sliding clamp by distinct E2/E3 pairs. Whereas monoubiquitination by Rad6/Rad18 mediates TLS, extension of this ubiquitin to a polyubiquitin chain by Ubc13-Mms2/Rad5 routes DDT to the template switching pathway. In this review, we focus on the monoubiquitination of PCNA by Rad6/Rad18 and summarize the current knowledge of how this process is regulated.

Keywords

DNA damage; PCNA; Rad6/Rad18; ubiquitin; DNA damage tolerance

INTRODUCTION

Replicative polymerases (pols) have very stringent polymerase and proofreading domains and, thus, cannot accommodate damaged template bases. Consequently, progression of replication forks is stalled when such offenses are encountered during S phase. Failure to restart replication often results in double-strand breaks (DSBs) that may lead to gross chromosomal rearrangements, cell-cycle arrest, and cell death. Therefore, it is often more advantageous to circumvent such replicative arrests and to postpone repair of the offending damage in order to complete the cell cycle and maintain cell survival (1, 21, 34, 64–66, 100, 103, 128). Such a process, referred to as DNA damage tolerance (DDT), may be carried out either by translesion DNA synthesis (TLS) or by template switching. In TLS, the replicative pol is replaced with a specialized TLS pol that is able to catalyze stable, yet often erroneous,

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nucleotide incorporation opposite damaged templates, allowing replication to proceed (64, 100, 103, 128). In template switching, enzyme-catalyzed regression of the replication fork creates a chicken-foot structure in which the original template strands are reannealed, and the stalled primer terminus utilizes the newly synthesized sister strand as a damage-free template. Subsequent reversal of the chicken-foot structure reconstitutes the replication fork at a point beyond the damage, bypassing the block in an error-free manner. Both pathways have been observed in organisms ranging from bacteria, such as *Escherichia coli*, to humans, and many components of these pathways are well conserved (64, 100, 103, 122, 128). A major difference between DDT in eukaryotes and prokaryotes, however, is the use of the ubiquitin conjugation system as a regulatory mechanism (34, 122).

Ubiquitin is a small protein that is present exclusively in eukaryotes and that may be covalently attached to a target protein through an enzyme cascade. The E1 ubiquitin-activating enzyme activates ubiquitin and transfers it to an E2 ubiquitin-conjugating enzyme (Ubc), which catalyzes formation of an isopeptide bond between ubiquitin and a lysine residue on a target protein. An E3 ubiquitin ligase can bind an E2 enzyme and a target protein simultaneously, mediating the specificity of this process (29). In response to sublethal doses of DNA-damaging agents that cause replication-blocking lesions, ubiquitin is conjugated to lysine residue 164 (K164) of a PCNA monomer. No such modification occurs in untreated cells during S phase. Furthermore, the number of ubiquitins conjugated to PCNA by distinct E2/E3 pairs orchestrates DDT: Conjugation of a single ubiquitin by the Rad6/Rad18 pair mediates the TLS pathway, whereas extension of this ubiquitin to a polyubiquitin chain by Ubc13-Mms2/Rad5 diverts DDT to the template switching pathway (47). Over the past decade or so, researchers have invested much effort into delineating how the conjugation of ubiquitin to PCNA is regulated and how this posttranslational modification (PTM) orchestrates DDT. In this review, we focus on the monoubiquitination of PCNA by Rad6/Rad18 and the regulation of this process. We begin with a brief overview of the eukaryotic replisome.

THE EUKARYOTIC REPLISOME

Replicative DNA pols alone are distributive and achieve their characteristic high processivity by anchoring to ring-shaped sliding clamps that encircle double-stranded DNA (dsDNA) and slide freely along it. The eukaryotic sliding clamp, proliferating cell nuclear antigen (PCNA) (Figure 1), has two structurally distinct faces and is a trimer of identical subunits. Each subunit consists of two independent domains joined by an interdomain connecting loop (IDCL). The C-terminal face of PCNA contains all C-termini and IDCLs and is a platform for interaction with the eukaryotic replicative pols, pol ϵ and pol δ (45). Specifically, the well-conserved PCNA-interacting peptide (PIP) box within replicative pols makes extensive contact with the IDCLs of PCNA (15). Orchestration of DNA replication also involves many accessory proteins, each of which has a unique role in enhancing the efficiency of this highly complex process. Together, PCNA, the replicative DNA pols, and these accessory proteins (discussed below) compose the replisome, which is responsible for the rapid and accurate replication of genomic DNA (Figure 2).

At each origin of replication, two replicative DNA helicases are loaded onto the DNA, one on each strand, and they utilize ATP to unwind dsDNA. In eukaryotes, the core of the replicative DNA helicase comprises the Mcm2–7 proteins, and it is loaded onto DNA in an inactive form. Subsequent activation occurs by the recruitment of the Cdc45 and GINS accessory proteins, forming the holo-helicase referred to as CMG (Cdc45, Mcm2–7, GINS). In contrast to most helicases, CMG translocates with a 3′–5′ polarity and, hence, tracks along the leading strand of the DNA (12, 80, 115). Upon unwinding of dsDNA by CMG, the single-stranded DNA (ssDNA) templates are bound by replication protein A (RPA), protecting them from cellular nucleases and preventing formation of alternative DNA structures (7, 131). The bifunctional DNA pol α /primase complex then synthesizes short, complementary RNA/DNA hybrid primers on the ssDNA templates for each Okazaki fragment and replication origin (33, 63, 97, 111, 142, 143). These hybrid primers are recognized by replication factor C (RFC), the clamp loader complex in eukaryotes. RFC utilizes ATP binding and hydrolysis to open PCNA rings and place them around the 3′ end of primer/template (P/T) junctions such that the C-terminal face of PCNA is oriented toward the terminal 3′ hydroxyl end of the hybrid primer, where DNA synthesis will initiate. Hence, the C-terminal face of PCNA is often referred to as its “front side” (45). For comparison, the highly conserved K164 residue is located on the opposite face, i.e., the “back side” of PCNA (Figure 1) (32). Replicative pols then bind to the front side of PCNA encircling DNA, completing formation of the replisome. Specifically, the CMG helicase selects and stabilizes DNA pol ϵ on the leading strand while DNA pol δ utilizes the hybrid primers synthesized every 100–250 nucleotides by the DNA pol α /primase complex on the lagging strand (38, 111) (Figure 2).

RAD6/RAD18 CONJUGATES UBIQUITIN TO PCNA ENCIRCLING A BLOCKED Primer/Template JUNCTION

During normal DNA replication, the activities of the helicase and the replicative pols are tightly coupled, such that the amount of RPA-coated ssDNA is minimal. When a damaged template base is encountered, leading and lagging strand synthesis and/or helicase and polymerase activities become out of sync, leading to the buildup of RPA-coated ssDNA downstream of the offending damage (17, 19, 89). This structure recruits Rad6/Rad18 to the blocked P/T junction, where the Rad6/Rad18 complex catalyzes the conjugation of a single ubiquitin moiety (a process referred to herein as monoubiquitination) to K164 of the resident PCNA. This response can also be elicited by agents that block progression of replication forks without modifying (damaging) DNA at all, suggesting that RPA-coated ssDNA, rather than the actual DNA lesion, is the signal for Rad6/Rad18-mediated monoubiquitination of PCNA (11, 19, 23, 37, 44, 47, 72, 85, 86, 88, 90, 96, 105, 129). In vitro, Rad6/Rad18 can conjugate ubiquitin to all three monomers of a PCNA ring loaded onto DNA (44, 90). A single report (56) suggests that such conjugation may also occur in vivo, but further experiments are needed to confirm this and to ascertain whether such conjugation is required for DDT within the cell. Although there are roughly 10 E2 enzymes and more than 60 E3 ligases in eukaryotes, Rad6/Rad18 is the predominant E2/E3 pair responsible for the monoubiquitination of PCNA during DDT (29, 51, 118, 140). As the following section

describes, the innate properties of Rad6 and Rad18 are uniquely attuned such that the Rad6/Rad18 complex is specifically targeted to PCNA present at stalled P/T junctions.

RAD6 AND RAD18 ARE A TAILOR-MADE MATCH FOR DNA Damage

Tolerance

Rad18 is a multidomain E3 ubiquitin ligase that functions as a homodimer. Dimerization occurs via an N-terminal RING domain. The Rad6-binding (R6B) domain is located at the C-terminus of Rad18 and interacts with the noncovalent ubiquitin interaction site on Rad6. This site is imperative for ubiquitin chain formation by Rad6, and its association with the R6B domain of Rad18 prevents this activity. The Rad18 RING domain also aids in Rad6 binding, independently of the R6B domain, and together these two domains form a stable interaction with Rad6. Dimerization of Rad18 is asymmetric, however, such that only one of the Rad6 binding sites is open. Thus, the Rad18 homodimer binds only a single Rad6. Whether the R6B and RING domains that bind to Rad6 are from the same or different Rad18 monomers remains unknown (4–6, 46, 50, 77, 90, 117).

Rad18 also contains distinct domains that bind DNA (the SAP domain), PCNA, and RPA (23, 54, 77, 90, 117). The PCNA-binding motif has been mapped to the N-terminal region of Rad18 and acts independently of the other domains in this region. No sequence within this region shows any similarity to the classic PIP box (data not shown), however, and the amino acid residues involved in PCNA binding have yet to be identified (90). Furthermore, the location(s) on PCNA to which Rad18 binds remains unknown. Some reports suggest that Rad18 may not require access to the front side of PCNA for monoubiquitination, as overexpression of proteins that also bind to this side with high affinity, such as p15^{PAF} and p21, does not impair PCNA monoubiquitination (99, 113). Further experiments are needed to identify the Rad18-binding site(s) on PCNA. The SAP domain of Rad18 has a high preference for ssDNA, particularly that present at forked DNA substrates, and is required for assembly of Rad18 at stalled replication forks following DNA damage. Thus, the current model suggests that the SAP and RPA-binding domains together recruit Rad18 to RPA-coated ssDNA that builds up downstream of replication-blocking lesions. Once localized, the PCNA-binding domain of Rad18 directs it to the sliding clamp present at the blocked P/T junction (5, 23, 85, 90, 121). Note that Rad18 also contains ATPase and zinc finger (ZnF) domains. The function of the ATPase domain is unknown, but it has no effect on the DNA binding behavior of Rad18 or the Rad6/Rad18 complex and is dispensable for the interaction of Rad18 with Rad6 (5, 6, 54). The function of the ZnF domain is unclear; conflicting reports suggest that this domain has a role in DNA binding, Rad18 dimerization, ubiquitin binding, and controlling the cellular location of Rad18 (4, 54, 81, 117, 123).

Rad6 is an E2 Ubc that is highly conserved in eukaryotes and that can form independent complexes with alternative E3 ligases (6). Rad6 alone has an intrinsic ability to form polyubiquitin chains, and this activity is inhibited by its association with Rad18, permitting the conjugation of only single ubiquitin moieties to PCNA. Rad6 is also incapable of binding any nucleic acid, so by associating with Rad18, Rad6 is specifically targeted to RPA-coated ssDNA resulting from DNA damage (4). Once localized, the PCNA-binding

domain of Rad18 is surmised to direct the ubiquitin-conjugating activity of Rad6 to PCNA encircling a blocked P/T junction. As discussed in the next section, the expression level, assembly, and cellular location of the Rad6/Rad18 complex are stringently controlled to ensure that the complex is poised for action when needed.

THE SPATIOTEMPORAL REGULATION OF THE RAD6/RAD18 COMPLEX PRIMES IT FOR DNA Damage Tolerance

The expression levels of both Rad6 and Rad18 are cell cycle controlled. Interestingly, Rad18 expression decreases during S phase, whereas Rad6 expression gradually increases during S phase and peaks in late S/G₂ phase (72, 73, 75, 78). Upon exposure to agents that halt progression of replication forks, however, the expression levels of both proteins are upregulated (53, 72, 75, 78, 124). Such a response ensures that the supply of both proteins meets the demand for them, and this response may also both drive formation of the Rad6/Rad18 complex and control its cellular location.

During unperturbed DNA synthesis, Rad6 is present in both the cytoplasm and the nucleus, but very little of it is associated with DNA (73, 78). This is expected, as the DNA-binding activity of Rad6 depends entirely upon Rad18, which is downregulated in S phase. In response to DNA damage, Rad6 is redistributed exclusively to the nucleus, where it binds to chromatin and monoubiquitinates PCNA present at stalled replication forks (72, 73). Rad6 does not contain a nuclear localization signal, however (60), whereas Rad18 contains three (85), suggesting that the cellular location of Rad6 is dictated by Rad18 during DDT as follows. In the absence of DNA damage, low levels of Rad18 ensure that the nuclear concentration of the Rad6/Rad18 complex is low, allowing Rad6 to maintain its relationship with other E3 ligases; then, upon exposure to DNA-damaging agents, upregulation of Rad6 and Rad18 sequesters them into a complex that is directed to the nucleus, where the damaged chromatin resides. Future studies are needed to confirm this pathway.

PHOSPHORYLATION OF THE MAMMALIAN RAD6/RAD18 COMPLEX ENHANCES ITS ACTIVITY

Human Rad6 is phosphorylated at conserved serine residue 120 by cyclin-dependent kinase 9 (CDK9). This PTM increases the activity of Rad6 and is critical for its role in cell cycle progression, suggesting that this modification may also be vital for the role of Rad6/Rad18 in DDT. Indeed, knockdown of CDK9 reduces ubiquitination of PCNA (104, 108, 132). How regulation of this Rad6 PTM correlates with exposure to DNA damaging agents and DDT, however, is not known. Interestingly, the basal level of phosphorylated Rad18 present during unperturbed conditions is greatly enhanced in response to DNA damage by two kinases: Dbf4/Drf1-dependent Cdc7 kinase (DDK) and ATR/Chk1-dependent c-Jun N-terminal kinase (JNK) (9, 25). Identification of the latter kinase agrees with previous independent reports of a reduction in Rad18 foci at stalled replication forks (88) and of PCNA monoubiquitination (11) upon knockdown or inhibition of ATR (ataxia telangiectasia and Rad3-related), a DNA damage checkpoint kinase (14, 19, 23, 31, 86, 135).¹ Recent studies suggest that phosphorylation of Rad18 mediates its interaction with Pol η .

In mammalian cells, Rad18 exists in complexes with Pol η regardless of genotoxic stresses, owing to mutual binding domains within each protein. This interaction is modulated by DNA damage-dependent phosphorylation of the Pol η -binding domain within Rad18. In the absence of DNA damage, a basal level of Rad18 is phosphorylated and associated with Pol η . In response to UV irradiation, both DDK and JNK phosphorylate serine residues within the Pol η -binding domain of Rad18. Each kinase is independent of the other, and both DDK and JNK promote the association of Rad18 with Pol η . Interestingly, both kinases are essential for the cellular tolerance of UV-induced lesions and for the role of Rad18 in TLS (9, 25, 129, 139). Furthermore, Pol η protein expression is induced upon exposure to UV irradiation (26, 69). Together, these distinct responses drive formation of the Rad18-Pol η complex and the subsequent redistribution of cellular Pol η to replication foci. Thus, by associating with Rad18, Pol η may be chaperoned into the vicinity of stalled replication forks upstream of RPA-coated ssDNA. Once the complex is localized, however, Pol η may actually direct Rad6/Rad18 to PCNA encircling stalled P/T junctions. This function is unique to Pol η among TLS pols, distinct from the catalytic activity of Pol η , and dependent upon the ability of Pol η to bind PCNA (via its PIP box and flanking domains). Thus, by bridging Rad18 and PCNA, Pol η stimulates PCNA monoubiquitination following UV irradiation (26). Combining both of these purported roles for the Rad18-Pol η complex in TLS suggests a dynamic “horse-and-carriage” scenario in which (a) a basal level of Rad18 is phosphorylated and associated with Pol η in the absence of DNA damage; (b) phosphorylation of Rad18 is greatly enhanced in response to UV irradiation, promoting its association with Pol η and hitching the “carriage” to the “horse”; (c) functioning as the “horse,” Rad18 pulls the Pol η “carriage” to RPA-coated ssDNA; and (d) once it is localized to the damaged chromatin, Pol η becomes the “horse” and directs the Rad18 “carriage” to PCNA residing at blocked P/T junctions, promoting monoubiquitination of PCNA.

The aforementioned studies primarily utilized UV irradiation as the DNA-damaging agent, suggesting that the horse-and-carriage scenario described above is specific to UV-induced lesions. Indeed, Pol η is responsible for the error-free bypass of cyclobutane pyrimidine dimers (CPDs), the major photoproduct of UV irradiation. Given the prominence of solar UV irradiation and the presence of at least three alternative TLS pols that can bypass CPDs erroneously, such a model may account for the highly efficient and error-free bypass of UV-induced CPDs within mammalian cells (137, 144). Rad6/Rad18, however, is responsible for the monoubiquitination of PCNA in response to all agents that elicit TLS, so this “horse-and-carriage” scenario may apply broadly to any circumstance in which progression of the replication fork is stalled. Several independent studies on benzo[*a*]pyrene diolepoxide (BPDE), suggest this may be the case. BPDE is a genotoxic metabolite of the chemical carcinogen benzo[*a*]pyrene that primarily forms guanine adducts that block the progression of replication forks. Similar to UV irradiation, these studies showed that exposure to BPDE induced JNK-dependent phosphorylation of Rad18 (9). In addition, treatment of DDK-depleted cells with BPDE specifically impaired the association of Pol η with stalled replication forks (25), and in Pol η -depleted cells, PCNA monoubiquitination by Rad6/

¹Numerous studies have observed that Rad6/Rad18-catalyzed monoubiquitination of PCNA did not depend on either ATM or ATR, suggesting that this process is independent of the DNA damage checkpoint. The reason for the discrepancy between these studies and those described above is currently unknown.

Rad18 was decreased after BPDE treatment (26). One study also showed that (a) JNK activity depended upon the ATR kinase recruited to and activated by RPA-coated ssDNA and (b) replication fork arrest induced by either nucleotide depletion [via hydroxyurea (HU)] or UV irradiation promoted formation of Pol η -Rad18 complexes in vivo as well as their association to chromatin (9). Taken together, these findings suggest that the horse-and-carriage scenario is not genotoxin specific; rather, it describes a generic response solicited by RPA-coated ssDNA generated during S phase (139). Thus, of all available TLS pols within mammalian cells, Pol η may be the default choice at any DNA lesion.

The apparent disparity in both accuracy and efficiency among various TLS events within mammalian cells, raises many issues pertaining to how TLS pols are selected for bypass across a given DNA lesion. At one end of the spectrum are lesions induced by chemicals such as BPDE. Pol κ bypasses BPDE-induced lesions in an error-free manner in vitro and is responsible for their faithful replication in vivo (3, 70, 91, 116, 141). Pol η , however, replicates past such lesions in an error-prone manner in vitro and, perhaps owing to the horse-and-carriage model, contributes to the mutagenic bypass of BPDE adducts in vivo (20, 59, 102, 141). At the other end of the spectrum are the two major UV-induced lesions that elicit TLS: the more-prominent CPD and the less-prominent pyrimidine (6-4) pyrimidone photoproducts [(6-4) photoproduct] (10). Pol η accurately replicates across thymine-thymine (TT) CPDs in vitro and is responsible for the highly error-free (>90%) bypass of UV-induced CPDs in vivo (125, 137). In contrast, Pol η has an eightfold preference for inserting an incorrect G residue opposite the 3' T of a (6-4) TT photoproduct, yet TLS opposite these lesions is predominantly error-free (<2% mutation frequency), suggesting that another TLS pol must replicate past (6-4) photoproducts in vivo (52, 136). In the horse-and-carriage model, Pol η binds to the front side of PCNA and is afforded ample opportunity for nucleotide incorporation. Does Pol η simply insert a nucleotide across from a BPDE adduct, but not across from a (6-4) photoproduct? If so, how is this insertion (or lack thereof) ensured? How is the "correct" TLS pol selected for bypass of certain lesions but not others? The linked chaperoning activities of Rad18 and Pol η during TLS raise these and many more intriguing questions.

THE ACTIVITY OF THE RAD6/RAD18 COMPLEX IS MEDIATED BY VARIOUS PROTEINS

Over the last 10 years or so, more and more seemingly unrelated proteins have been implicated in modulation of the activity of the Rad6/Rad18 complex in addition to their primary duties in other cellular pathways. For example, various proteins involved in DSB repair, such as BRCA1, NBS1, PTIP, and Claspin-Timeless, have been shown to interact with Rad18 and/or PCNA and may mediate recruitment of the Rad6/Rad18 complex to blocked replication forks, enhancing monoubiquitination of PCNA during DDT. Similar behavior was also observed for two unrelated E3 proteins involved in initiation of DNA replication (CRL4^{CDT2}) and inhibition of p53-mediated apoptosis (SIVA1) (40, 42, 107, 119, 133, 135). Certain proteins have garnered considerable attention, each of which is summarized below.

ATP-Dependent Chromatin Remodelers

A plausible mechanism for enhancing monoubiquitination of chromatin-bound PCNA would be to provide Rad6/Rad18 with greater access to genomic DNA in response to DNA damage. In the cell, multisubunit ATP-dependent chromatin remodelers utilize the energy from ATP hydrolysis to remodel sections of chromatin, ultimately exposing or shielding the surrounding DNA. Expression of the highly conserved Ino80 complex is upregulated in S phase, during which it is recruited to and moves with active replication forks although the complex is dispensable for fork progression in unperturbed conditions (28, 83, 109, 110). A recent study observed that in *Saccharomyces cerevisiae*, Ino80 and its chromatin-remodeling activity were necessary for recruitment of Rad18 to MMS-stalled replication forks and for PCNA monoubiquitination. MMS is an alkylating agent that primarily forms methylated purine residues that cannot be accommodated by replicative pols, halting progression of replication forks. Ino80 was required for stabilization and efficient restart of MMS-stalled replication forks, a finding which is consistent with the ability of Ino80 to expose DNA neighboring a lesion and promote Rad6/Rad18-mediated ubiquitination of PCNA. It is unclear whether the exposed DNA facilitates recruitment of Rad18 directly or indirectly (through RPA). Interestingly, the role played by Ino80 may be specific to MMS-induced damage, as UV irradiation and HU treatment had marginal, if any, effects on PCNA ubiquitination in the absence of Ino80 (28). Others have observed, however, that Ino80 is required to stabilize HU-blocked replication forks, suggesting that Ino80 may be differentially involved in distinct pathways that deal with specific types of replicative stress (28, 87, 92, 110). What these alternative roles are and how such specificity is attained are unknown.

The RSC (remodels the structure of chromatin) complex is also purported to be associated with replication forks in *S. cerevisiae*, in which two isoforms are present and differ only in whether they contain the Rsc1 or Rsc2 subunit. Only the Rsc2 isoform is required for normal PCNA ubiquitination in response to either UV irradiation or HU treatment. In humans, Rsc1 and Rsc2 are fused together with Rsc4 to form BAF180. When BAF180 is depleted from human cells, ubiquitination of PCNA is reduced in untreated, control cells as well as in cells treated with either UV light, MMS, or HU. Furthermore, in BAF180-depleted cells, the amount of chromatin-bound PCNA, and Rad18, but not RPA, is substantially reduced following UV irradiation and fork progression is less efficient. Together, these observations suggest that the human RSC complex (referred to as PBAF) may enhance Rad6/Rad18-mediated ubiquitination of PCNA following DNA damage by stabilizing the amount of chromatin-bound PCNA as well as by promoting the binding of Rad6/Rad18 to chromatin in a manner independent of RPA deposition (87). How these feats are achieved, however, is unknown.

The nucleosome remodeling deacetylase (NuRD) complex couples ATPase and deacetylase activities to remodel chromatin. Recent evidence suggests that the mammalian NuRD complex may be targeted to UV-damaged chromatin by two transcriptional regulators, ZBTB1 and KAP-1. Upon UV exposure, ZBTB1 clusters into foci that colocalize with both CPDs and PCNA. Such behavior is mandatory for promoting Rad6/Rad18-mediated ubiquitination of PCNA, assembly of Pol η foci, and cell survival following UV-related

damage. Interestingly, recruitment of ZBTB1 to UV-damaged sites requires its ubiquitin-binding domain, suggesting that another unknown ubiquitinated protein recruits ZBTB1 to stalled replication forks. In addition, KAP-1 is strongly phosphorylated by an unknown kinase following UV irradiation and is targeted to chromatin by ZBTB1. Inhibition of either event diminishes both the association of Rad18 with chromatin and PCNA monoubiquitination. Interestingly, increasing chromatin relaxation in ZBTB1-knockdown cells appears to eliminate the need for ZBTB1 in Rad6/Rad18-mediated ubiquitination of PCNA following UV exposure (57). As phosphorylation of KAP-1 is known to initiate chromatin relaxation during DNA DSB repair (41), all of these observations suggest that ZBTB1 enhances PCNA monoubiquitination by targeting pKAP-1-dependent chromatin relaxation to UV-damaged sites. Further experiments, however, have suggested that the contribution of KAP-1 to chromatin remodeling following UV exposure differs from its role in chromatin remodeling during DNA DSB repair. Indeed, phosphorylation of KAP-1 in response to UV exposure is distinct from that observed in DNA DSB repair following treatment with ionizing radiation and is independent of the DNA damage checkpoint kinases ATM and ATR (57). In the realm of transcription, one role of KAP-1 is to recruit the NuRD complex to gene promoters, remodeling the surrounding chromatin (106). In undamaged human cells, a constitutive interaction of ZBTB1 with KAP-1 and a component of the NuRD complex was observed, suggesting that the role of pKAP-1 in chromatin relaxation following UV exposure may be recruitment of the NuRD complex to UV-induced lesions (57). Once localized, the NuRD complex can increase the accessibility of the surrounding DNA, directing Rad6/Rad18 to PCNA residing at a stalled P/T junction and enhancing PCNA monoubiquitination (57). Further experiments are needed to verify this model. As we have already pointed out, many unknowns remain for this pathway, and experiments are assuredly in progress to address each. Nonetheless, these and other studies on ATP-dependent chromatin remodelers clearly show that Rad6/Rad18 activity is modulated by chromatin accessibility.

p21

p21 is a cell-cycle regulator that exerts its control by inhibiting DNA synthesis via two independent domains. Its N-terminal cyclin/CDK-interaction domain binds to both cyclins and CDKs during G₁, particularly those involved in cell-cycle transitions, forming ternary complexes devoid of kinase activity. The activation of certain cyclin/CDK complexes in late G₁ is a key event in the commitment to S phase and is thought to allow the initiation of active replication forks. The C-terminal PIP motif of p21 binds tightly to PCNA, blocking its interaction with DNA pols and inhibiting DNA synthesis (101).

p21 is a target of p53 and is upregulated and transported to the nucleus upon exposure to agents that cause DNA strand breaks. When this occurs before S phase, p21 arrests the cell cycle, primarily via inhibition of CDKs. When DNA damage occurs after the cell has committed to S phase, p21 inhibits ongoing DNA synthesis by binding to PCNA (112). In contrast to the case for DNA strand breaks, modifications to the template bases of DNA may be accommodated during S phase via TLS, allowing DNA replication to resume without first correcting the damage. Under these circumstances, elevated p21 levels are detrimental, as TLS pols must gain access to PCNA. Even the low basal level of p21 that is maintained

throughout S phase is sufficient to impair the interactions of TLS pols with chromatin-bound PCNA (71, 76, 114, 120). Furthermore, elevated p21 levels inhibit ubiquitination of PCNA (71, 113). Indeed, p21 levels decline, rather sharply, specifically in response to agents that halt progression of replication forks during S phase. Interestingly, exposure to these agents still elicits the p53 response, initiating transcription of the p21 promoter. For reasons unknown, however, RNA pol II is less efficient at traversing the p21 gene under these conditions. Thus, p21 mRNA does not accumulate, and no further p21 protein is synthesized. In addition, the basal level of p21 present at the time of damage is degraded by the proteasome (112). Other PCNA-binding proteins may aid in the degradation of p21 that is engaged with the sliding clamp by transiently promoting its detachment in response to DNA damage (71).

Owing to discrepancies in the literature, it is currently unclear how decreased p21 levels promote monoubiquitination of PCNA by Rad6/Rad18. One study observed that dissociation of p21 from PCNA, as well as its subsequent degradation, was necessary for PCNA monoubiquitination in cells, implying that access to the front side of PCNA is imperative for Rad6/Rad18 activity (71). In contrast, another study found that p21 lacking its PIP motif was almost as capable as full-length p21 in inhibiting PCNA ubiquitination, suggesting that the ability of p21 to modulate the ubiquitination of PCNA does not require these proteins to interact. Rather, the CDK-binding capacity of p21 must be negatively regulated to permit efficient PCNA ubiquitination (113). Indeed, p21 mutants lacking only functional CDK-binding domains did not alter monoubiquitination of PCNA in response to UV irradiation (76). Similarly, p15^{PAF}, a small protein much like p21, contains a highly conserved PIP box and interacts strongly with PCNA during S phase, but it has no effect on the ubiquitination of PCNA after UV irradiation (27, 48, 99, 138). Together, these findings suggest that Rad6/Rad18 does not interact with the front side of PCNA and/or that such interaction is not required for monoubiquitination of PCNA. Further experiments are needed to confirm or refute either of these models for the role of p21 in Rad6/Rad18-mediated DDT.

Spartan

Spartan (aka DVC1) is a multidomain protein that comprises a SHP box, a PIP box, and a UBZ4 ubiquitin-binding zinc finger. The SHP box is a known P97-binding motif and is required for association of Spartan with this protein. The latter two domains are highly conserved and mutually exclusive; binding of Spartan to unmodified PCNA is entirely dependent upon the PIP box, whereas only the UBZ domain is required for association with ubiquitin. Interestingly, Spartan also contains a SprT-like domain similar to that found in certain metalloproteases, but no such activity has been observed. It is unclear whether this domain is required for Spartans function(s) in vivo (18, 24, 39, 55, 58, 74, 84).

Spartan expression is cell cycle regulated such that it peaks in S phase, persists through G₂ into early M phase, and is rapidly downregulated upon exiting mitosis. Spartan condenses into nuclear foci that colocalize with PCNA, and the proportion of cells with such foci peaks during S phase, suggesting that Spartan may have a role in unperturbed DNA replication (24, 74, 84). Upon exposure to agents such as UV irradiation, Spartan foci are dramatically enhanced. These foci are enriched in chromatin and colocalize with sites of DNA synthesis

and DNA damage, ubiquitinated PCNA, Rad18, and RPA (18, 24, 39, 55, 74). Such behavior is entirely dependent upon both the PIP box and the UBZ domains of Spartan and is specific to agents that halt the progression of replication forks and induce monoubiquitination of PCNA (18, 24, 39, 55, 74, 84). Furthermore, Spartan levels gradually decline following UV-induced PCNA ubiquitination, suggesting that Spartans function is transient and dependent on DNA damage (18). Although Spartan preferentially binds monoubiquitinated PCNA in vitro, one cannot assert that Spartan is directly recruited to monoubiquitinated PCNA in vivo, owing to conflicting reports in the literature (18, 24, 55, 74, 84). Interestingly, a fraction of cellular Spartan is ubiquitinated. This modification greatly reduces the ability of Spartan to bind ubiquitin and eliminates its recruitment to UV-induced nuclear foci, suggesting that ubiquitination of Spartan may mediate its role in DDT. However, the mechanism by which ubiquitination of Spartan is regulated in vivo is unknown (84).

Knockdown of Spartan sensitizes cells to killing by agents that cause replication forks to stall and increases the extent of UV-induced mutagenesis (18, 24, 39, 55, 74, 84). Furthermore, although relocalization of Spartan to sites of replication stress does not require its SHP domain (or P97 binding), only wild-type Spartan is able to rescue, suggesting that binding of Spartan to PCNA, ubiquitin, and P97 is required for its role(s) in DDT (18, 24, 39, 55, 84). Two opposing descriptions of Spartan's mode of action have emerged, and these descriptions are based on conflicting results. In one description, depletion of wild-type Spartan or mutation of its UBZ domain led to a pronounced persistence of UV-induced Pol η foci, yet neither depletion nor mutation had any effect on either PCNA monoubiquitination or the association of Rad18 to chromatin following UV exposure (24, 84). However, overexpression of wild-type Spartan did suppress the interaction between Pol η and monoubiquitinated PCNA, enhance recruitment of P97 into UV-induced foci, and lead to the removal of Pol η from such foci and chromatin. Furthermore, an ATPase-deficient P97 targeted to UV-induced foci by Spartan failed to efficiently trigger removal of Pol η . P97 (also known as valosin-containing protein, VCP) remodels ubiquitinated proteins via its ATPase activity. Thus, once targeted to a stalled replication fork by its UBZ domain, Spartan may recruit P97 via its SHP domain to actively displace Pol η from chromatin (24, 84).

In the second description, knockdown of Spartan decreased PCNA monoubiquitination and significantly reduced Pol η recruitment to DNA damage sites following exposure to UV light, and only full-length Spartan rescued these behaviors (18, 39, 55). Similarly, transient overexpression of Spartan dramatically increased formation of Pol η foci and eliminated the requirement for UV irradiation. This effect was impaired by mutation in either the UBZ domain or the PIP box of Spartan (55). Thus, this opposing description purports that Spartan binds to chromatin-bound PCNA through its PIP box and UBZ domain and suppresses the reduction of PCNA ubiquitination, perhaps via transient inhibition of USP1 (ubiquitin-specific protease 1), which is responsible for cleaving ubiquitin from PCNA following DDT (55, 127). Knockdown of Spartan in cells lacking USP1 still led to a reduction in PCNA ubiquitination, however, suggesting that Spartan may suppress the reduction of PCNA ubiquitination by other means (18, 55). Indeed, others studies observed that Spartan binds directly to Rad18 and to P97, inhibiting the activity of P97 and preventing extraction of Rad18 from chromatin in the latter case (18, 39, 55). Thus, Spartan may promote the formation of ubiquitinated PCNA in addition to inhibiting its destruction. Collectively, these

activities serve to transiently enhance recruitment of TLS pols to stalled replication forks. Although there is no doubt that Spartan plays an imperative role in DDT, future experiments are needed to discern which, if either, of these models is correct.

RAD6/RAD18-MEDIATED DNA DAMAGE TOLERANCE IS SELECTED FOR WHEN A REPLICATION-BLOCKING LESION IS ENCOUNTERED

At least three distinct mechanisms may rescue a stalled replication fork during S phase. The two Rad6/Rad18-dependent pathways are primarily elicited when a small DNA adduct is encountered. The third possibility, homologous recombination (HR), is a very complex process that utilizes the undamaged sister chromatid as a homologous template to resume DNA synthesis. HR is functional throughout the S and G₂ phases of the cell cycle and is primarily summoned for the repair of more drastic genomic offenses that cannot be replicated past, such as DNA strand breaks. HR can also rescue replication forks arrested at small DNA adducts, but it may be more harmful than good in such cases, as the process can trigger cell cycle arrest, and failed attempts can lead to gross chromosomal rearrangements. Indeed, eukaryotic cells have established a temporal hierarchy to avoid such disasters: The Rad6/Rad18-mediated pathways are elicited first at replication-blocking lesions, whereas HR serves as a so-called salvage pathway and is summoned only when the other pathways are malfunctioning (61, 79). In *S. cerevisiae*, conjugation of SUMO (small ubiquitin-like modifier) to PCNA proctors this hierarchy.

SUMO is a small posttranslational modifier in eukaryotes that is reversibly conjugated to a plethora of proteins involved in a broad range of cellular processes (30). During normal DNA replication within *S. cerevisiae*, SUMO is conjugated to K127 and/or K164 within PCNA, a process referred to herein as SUMOylation. K164 is highly conserved and is the predominant attachment site, whereas K127, which is less conserved, is a secondary site (47, 93). The interaction of DNA pols with PCNA is not affected by SUMOylation of either site (16). Similar to ubiquitination, only PCNA encircling DNA is SUMOylated in vivo (94). In complete contrast to ubiquitination, however, SUMOylation of PCNA (*a*) is upregulated in and limited to unperturbed S phase, (*b*) is not induced by sublethal levels of agents that elicit TLS, and (*c*) has a role in maintaining the integrity of the genome during normal DNA replication (47, 93, 94, 98, 130). This role is manifested through recruitment of the Srs2 helicase, which contains distinct domains that bind PCNA, SUMO, and Rad51 independently (2, 16, 93, 98). Rad51 is an imperative HR protein responsible for locating a homologous template within the sister chromatid and annealing it to the invading strand primer (61). Because of its tandem SUMO- and PCNA-binding domains, Srs2 preferentially interacts with SUMO-PCNA (2, 93). This behavior directs a portion of cellular Srs2 to SUMO-PCNA that is encircling genomic DNA (engaged in DNA replication), where Srs2 prohibits the initiation of HR during unperturbed S phase either by dissolving inappropriately placed Rad51 nucleoprotein filaments or by preventing their formation (16, 43, 62, 67, 93, 98, 126).

During normal DNA replication within *S. cerevisiae*, a significant portion of cellular PCNA is SUMOylated, predominantly at K164. Conjugation of SUMO to a given PCNA trimer is

not refractory toward its subsequent ubiquitination, however, so removal of SUMO is not a prerequisite for ubiquitination (93, 94, 130). In fact, genetic studies in *S. cerevisiae* revealed that polyubiquitination-dependent template switching actually requires previous SUMOylation of PCNA (13), possibly to keep the HR machinery at bay in order to permit conjugation of ubiquitin to PCNA. Furthermore, the activity of Rad6/Rad18 toward a given PCNA trimer is directly enhanced by the presence of SUMO on the same clamp. The N-terminal half of Rad18 from *S. cerevisiae* contains both a PCNA-binding domain and a SUMO interaction motif (SIM) that mediates its noncovalent interaction with SUMO. This pairing accounts for preferential binding of SUMO-PCNA by Rad18 and dramatically improves the efficiency of ubiquitin conjugation to SUMO-PCNA compared to native PCNA. In vivo, this SIM-SUMO interaction enhances the recognition of PCNA engaged in DNA replication and contributes to the full activity of Rad6/Rad18 required for efficient ubiquitin-dependent DDT (95). Taken together, these findings suggest that SUMOylation of PCNA during normal DNA replication functions as a traffic cop in *S. cerevisiae*. SUMOylation of PCNA directs the recovery process toward Rad6/Rad18-mediated bypass in the event that a replication block is encountered by recruiting Srs2 to inhibit HR and/or by recruiting Rad6/Rad18 to promote ubiquitination of PCNA that is engaged in DNA replication (93, 95, 98).

SUMOylation of PCNA has only recently been observed in human cells and occurs at very low levels compared with those observed in *S. cerevisiae* (36, 82). Hence, studies analogous to those cited above are in their infancy. Nonetheless, some insight has been provided. The human Rad18 sequence lacks an obvious SIM, and the presence of SUMO on PCNA has no effect on the ability of the human Rad6/Rad18 complex to conjugate ubiquitin to PCNA in vitro (95). Thus, SUMO does not seem to promote ubiquitination of PCNA by recruiting the Rad6/Rad18 complex. However, a mechanism similar to that described above for Srs2 may exist in humans. The human protein PARI (PCNA-associated recombination inhibitor) contains a UvrD helicase-related domain, a PIP-box, a SIM, and a Rad51-binding domain. In cells, PARI is found at very low levels, and a recent study suggests that SUMO-PCNA concentrates PARI to replication forks, where it suppresses HR by disrupting Rad51 filaments. Interestingly, PARI is not an active helicase and, in contrast to Srs2, which can move along DNA and remove multiple Rad51 molecules within a single binding encounter with DNA (82), PARI must be present in amounts stoichiometric to Rad51 in order to efficiently inhibit HR. As mentioned above, however, SUMO-PCNA levels are very low in human cells, so this method of HR suppression may not be the predominant one. Indeed, other human helicases may suppress HR by inhibiting various stages of initiation independently of SUMO-PCNA (8, 35, 49).

CONCLUDING REMARKS

It was first observed in 2002 that Rad6/Rad18 conjugated ubiquitin to eukaryotic PCNA in response to DNA-damaging agents that cause replication-blocking lesions. In the ensuing years, substantial efforts have begun to unveil the complexity and intricacies of this process. Because it involves multiple levels of regulation for a wide variety of circumstances, this process has been optimized to operate mostly during S phase and only on PCNA trimers encircling damaged DNA. In the pursuit of answers, however, more questions have been

raised, particularly those pertaining to the spatiotemporal control of this process. For instance, where in relation to the replication fork does Rad6/Rad18-mediated DDT occur? There are currently two models that address this question (please see the excellent review, Reference 134). In one, replicative DNA synthesis on the afflicted strand and progression of the replication fork do not resume until the offending damage is bypassed. Hence, Rad6/Rad18 must conjugate ubiquitin to PCNA rings present at or near a replication fork, where the replisome may or may not remain intact. In the contrasting model, the replisome progresses ahead and replicative DNA synthesis resumes downstream of the lesion, leaving behind a gap opposite the offending damage. In this case, Rad6/Rad18 and the ensuing DDT function behind the progressing replication fork (and the replisome) to fill in the gap. Given the ample experimental evidence for each, the two models may not be mutually exclusive. However, each model has many persisting questions. For instance, if DDT occurs at or near the replication fork, what is the composition of the blocked P/T junction? Do the replicative polymerases or other PCNA-binding proteins remain engaged? If so, how does Rad6/Rad18 gain access to the resident PCNA clamp? In contrast, if DDT occurs behind the replication fork, does the original PCNA ring stay behind with the gap? If so, how is it retained at the stalled P/T junction? Another pressing issue is the temporal correlation between the ubiquitination events (monoubiquitination versus polyubiquitination) and the DDT pathways (TLS versus template switching). The E2/E3 pairs involved in DDT act sequentially, suggesting that the DDT pathways also do so. Indeed, UV-induced lesions are bypassed predominantly by TLS in *S. cerevisiae*; template switching functions only as a backup (22, 96). Furthermore, a recent in vivo report suggests that the two Rad5-related proteins in human cells, HLF and SHPRH, actually bind to Rad18 in a DNA damage-specific manner to enhance monoubiquitination of PCNA and recruitment of the appropriate TLS pol (68). Although instances in which template switching is solicited first cannot yet be ruled out, these reports suggest that the simpler yet potentially mutagenic TLS pathway is preferred over the faithful, more complex template switching pathway. But what dissuades Ubc13-Mms2/Rad5 from extending the single ubiquitin moieties on PCNA into polyubiquitin chains? Perhaps one of the many known ubiquitin-binding proteins involved in TLS? Is it a timing issue, i.e., does TLS occur prior to the arrival of Ubc13-Mms2/Rad5 to the stalled P/T junction? Furthermore, what ultimately signals the extension of the monoubiquitin and solicitation of the template switching pathway, and when does this occur? A failed TLS attempt? These questions, in addition to those raised throughout the text, underscore the need for additional studies. Because the answers will require scrutiny of more quantitative data, in vitro biochemical experiments will play a vital role in answering these questions.

SUMMARY POINTS

1. Translesion DNA synthesis (TLS) is one of two DNA damage tolerance (DDT) pathways that can replicate past DNA lesions that halt the progression of replication forks. In this pathway, the replicative polymerase is replaced with a specialized TLS polymerase that is able to catalyze stable, yet often erroneous, nucleotide incorporation opposite damaged templates so that replicative DNA synthesis can resume.

2. DDT in eukaryotes is regulated by the E2/E3-catalyzed attachment of ubiquitin to lysine residue 164 of the the sliding clamp, PCNA. An E2 ubiquitin-conjugating enzyme catalyzes the attachment of ubiquitin to the target protein while an E3 ubiquitin ligase binds to an E2 enzyme and the target protein simultaneously, mediating the specificity of the conjugation.
3. Conjugation of a single ubiquitin by the E2/R3 pair of Rad6/Rad18 mediates the TLS pathway of DDT, whereas extension of this ubiquitin to a polyubiquitin chain by the Ubc13-Mms2/Rad5 E2/E3 pair routes DDT to the other DDT pathway, template switching.
4. The buildup of RPA-coated ssDNA downstream of a replication blocking lesion recruits Rad6/Rad18 to the blocked P/T junction where the Rad6/Rad18 complex attaches a single ubiquitin to K164 of the resident PCNA. The innate properties of Rad6 and Rad18 are uniquely attuned such that the Rad6/Rad18 complex is specifically targeted to PCNA present at stalled P/T junctions.
5. Rad6 has an intrinsic ability to form polyubiquitin chains and does not contain a nuclear localization signal (NLS) or any domain that interacts with PCNA, RPA or DNA whereas Rad18 contains multiple NLS as well as distinct domains that bind DNA, PCNA, and RPA. Thus, by pairing with Rad18, Rad6 is specifically recruited to PCNA residing at a blocked P/T junction within the nucleus where Rad18 permits the conjugation of only single ubiquitin moieties by Rad6.
6. The expression level, assembly, and cellular location of the Rad6/Rad18 complex are stringently controlled by the cell cycle such that the nuclear concentration of the complex is low during unperurbed S-phase. Upon exposure to agents that halt the progression of replication forks, upregulation of Rad6 and Rad18 sequesters them into a complex that is directed to the nucleus where the damaged chromatin resides.
7. The activity of the Rad6/Rad18 complex is regulated by posttranslational modifications to each protein, phosphorylation in particular, as well as by a multitude of accessory proteins that modulate the recruitment of Rad6/Rad18 to RPA-coated ssDNA by various mechanisms.
8. Eukaryotic cells have established a temporal hierarchy of DNA repair pathways when dealing with replication-blocking lesions; Rad6/Rad18-mediated TLS is elicited first, followed by Ubc13-Mms2/Rad5-mediated template switching, and homologous recombination serves as a salvage pathway that is summoned only when the other pathways are malfunctioning. In essence, this is achieved by promoting the monoubiquitination of PCNA by Rad6/Rad18 while simultaneously inhibiting the initiation of homologous recombination.

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Figure 1.

Structure of human PCNA. Model of PCNA generated using Pymol (PDB ID:1AXC) and shown in cartoon form. (*a*) The PCNA monomer consists of two independent domains, shown in gray (N-terminal) and green (C-terminal), joined by an interdomain connecting loop (IDCL), shown in magenta. Lysine residue 164 (K164) is shown as red spheres. Panels *b* and *c* show the front and side views of the PCNA trimer, respectively. The three PCNA monomers are arranged in a head-to-tail manner, resulting in structurally distinct faces. The face from which the C-termini protrude is referred to as the “C-terminal face” or “front side,” and is highlighted in panel *b*. The side view of the PCNA trimer shown in panel *c* highlights the distinct differences between the two faces of PCNA. The “C-terminal face” or “front side” of PCNA shown on the left contains the IDCLs and interacts with replicative polymerases during normal DNA replication. K164, which is monoubiquitinated by Rad6/Rad18 in response to replication-blocking lesions, is on the opposite face; this face is referred to as the “back side” of PCNA.

Figure 2.

Model of the eukaryotic replisome shown in cartoon form. The leading and lagging strand replicative DNA polymerases (ϵ and δ , respectively), sliding clamp (PCNA), clamp loader complex (RFC), holo-helicase complex (CMG helicase), primase complex (DNA polymerase α /primase), and single stranded DNA-binding protein (replication protein A; RPA) are involved in the formation of the eukaryotic replisome at each replication fork. The primase complex primes the lagging strand DNA generated via unwinding of the duplex DNA by CMG helicase. The RNA/DNA hybrid primers (*red*) are recognized by the RFC clamp loader, which subsequently loads PCNA onto the 3' end of the primer. The replicative polymerases anchor to PCNA at the 3' hydroxyl termini of the primer strands and extend them in the 5' to 3' direction. The single-stranded DNA generated during CMG helicase unwinding is coated with RPA.