DOI: 10.1002/cbic.200500139

# The RAD6 Pathway: Control of DNA Damage Bypass and Mutagenesis by Ubiquitin and SUMO

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## 1. Introduction

Our genetic material is constantly exposed to a variety of exogenous as well as endogenous agents that cause DNA lesions of different types. As a consequence, cells have developed a repertoire of repair systems for the maintenance of DNA that can deal with a wide spectrum of lesions and are highly conserved throughout evolution.[1,2] Most repair mechanisms rely on the excision and subsequent resynthesis of the damaged stretch of DNA. Within double-stranded (ds) DNA, the information encoded by the complementary strand is used to correctly restore the original sequence information (Figure 1 A). In contrast, excision repair systems cannot operate on single-stranded (ss) DNA regions as they arise during the duplication of the genome, due to the lack of an instructive template for resynthesis. If left unrepaired, lesions in ssDNA can act as "road blocks" for the machinery involved in the duplication of the genome, because the active sites of replicative DNA polymerases do not accommodate distorted DNA as a template (Figure 1 B).[3] In the absence of a system to resolve a stalled replication fork, the resulting cell-cycle arrest would ultimately lead to cell death. For this reason, DNA-damage-tolerance mechanisms, which allow the bypass of lesions and the completion of replication without the actual removal of the damage, are essential for the survival of a cell in the presence of genotoxic agents, and they are common to all organisms.[4,5]

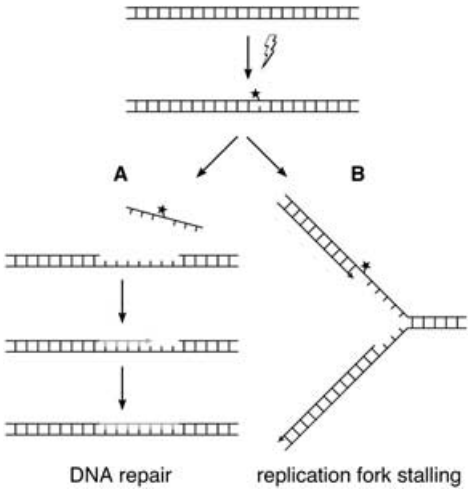


**Figure 1.** Consequences of DNA damage. A) If a DNA lesion (*) occurs in a stretch of double-stranded DNA, it can be removed by an excision repair system, which relies on the removal of the damaged region and its resynthesis based on the coding information of the undamaged complementary strand (grey arrow). B) If the lesion occurs in a stretch of single-stranded DNA or is not removed prior to the onset of DNA replication, it may cause a stalling of the replication fork due to the inability of the replicative DNA polymerases to use damaged DNA as a template.

Although clearly beneficial in terms of cell survival, their action is not always entirely desirable in higher organisms, because the damage-bypass process itself can cause unwanted changes in the genetic information. Genetic instability is a hallmark of cancer and can often be linked to mutations in genes encoding essential regulators of cellular signal transduction pathways, whose inactivation or alteration can lead to uncontrolled cell growth and division. Thus, completion of DNA replication by any means at the expense of accuracy might do more harm than good in a multicellular organism. For eukaryotic cells, it is therefore of crucial importance to keep damage-tolerance mechanisms under tight control and prevent their unrestrained activity in situations in which they are not needed.[6,7]

Control over the activity of eukaryotic DNA-damage-tolerance pathways is exerted by two systems of protein modification unique to eukaryotes: the ubiquitin and the SUMO conjugation systems.[8,9] Ubiquitin and SUMO are small, highly conserved proteins that can be covalently attached to various cellular proteins in a post-translational manner, thereby affecting the stability, localization or activity of the modified targets. In this review, I will summarize our current knowledge about the mechanisms by which ubiquitin and SUMO affect the bypass of DNA lesions. The discussion will focus on baker's yeast, as most of the principles were uncovered in this model organism, but parallels in the mammalian system will be pointed out where appropriate. Finally, possible approaches for intervention with this regulatory system and its consequences for cancer therapy will be mentioned.

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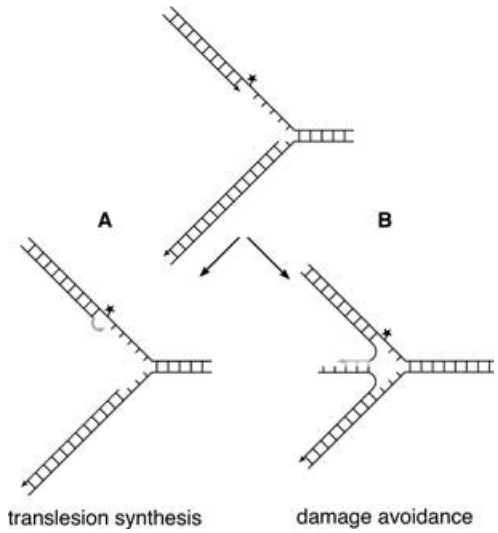


**Figure 2.** Mechanisms of DNA-damage tolerance. A) In a process called translesion synthesis (TLS), specialized, damage-tolerant polymerases are employed to synthesize across the lesion (grey arrow). As most of these polymerases operate with reduced fidelity due to their relaxed active sites, the process is often mutagenic. B) Error-free damage avoidance (DA) relies on the genetic information encoded by the undamaged, newly synthesized sister chromatid. It probably involves a strand switching of the stalled replication machinery and may be associated with a temporary regression of the replication fork (grey arrow). Whether the "chicken-foot" structure depicted here is physiologically relevant, remains to be determined.

## 2. Mechanisms of DNA-damage tolerance

Damage-bypass mechanisms, which can be found in every organism, allow the completion of DNA replication in the presence of lesions. As they are designed to allow replication forks to pass over sites of damage, they do not actually remove the lesion. Although, in effect, they contribute to the resistance of the cell to genotoxic agents, they should therefore not be classified as genuine repair systems. Importantly, these bypass systems differ markedly in the accuracy with which they fill the position opposite a lesion.

### Translesion synthesis is mediated by specialized DNA polymerases with reduced fidelity

The simplest way to continue DNA replication in the presence of a lesion on the template strand is to polymerize across the site of damage (Figure 2 A). Naturally, this strategy, which is called translesion synthesis (TLS), poses several problems for the cell. Replicative DNA polymerases are highly efficient enzymes whose catalytic centers are streamlined to fit an unperturbed template and primer terminus.[10] Any unphysiological change, be it a bulky adduct, a backbone distortion or a small modification that reduces the coding capacity of the affected base, will therefore present an obstacle to the processive activity of the replicative enzyme, causing polymerization to stall. Similarly, abasic sites, which frequently arise by spontaneous hydrolysis, do not serve as templates for replicative DNA polymerases. Thus, most organisms harbor specialized polymerases with more relaxed catalytic centers, which can insert nucleotides opposite a variety of abnormal structures.[11] If the replicative polymerase is transiently exchanged for one of the damage-tolerant enzymes, the lesion can thus be overcome and processive replication can resume. Due to their relaxed active sites, however, damage-tolerant polymerases generally operate with reduced fidelity, in particular if the coding information is obscured by the lesion. Moreover, they are less discriminatory even on undamaged templates. Thus, TLS is generally deemed an error-prone process, and in fact most of the mutations induced by genotoxic agents are believed to result not from the damage per se, but rather from its mutagenic processing by damage-tolerant polymerases.[12] The danger of inducing mutations during TLS might be minimized by employing a large number of polymerases specialized for different types of lesions, but allowing them to incorporate only a few nucleotides before the replicative enzyme takes over again. In fact, most of the polymerases involved in TLS exhibit very low processivity. Nevertheless, keeping a tight control over their activities appears to be essential to prevent them from unscheduled incorporation of nucleotides on undamaged templates.[13]

### Properties of damage-tolerant polymerases

The importance of translesion synthesis is underscored by the diversity of damage-tolerant DNA polymerases encoded by eukaryotic genomes, which have been the subject of many reviews.[4, 10, 11, 14] Most of them belong to a distinct class of enzymes that has been called the Y family.[15] In general, the members of this class of polymerases exhibit low fidelity and low processivity even on undamaged templates. In addition, they are usually quite selective with respect to the types of lesions that can be accommodated in their active sites, and they process different lesions with varying accuracies. An exception among the Y family polymerases is the Rev1p protein in yeast and mammals, which in fact does not exhibit polymerase activity at all, but is limited to the insertion of a single nucleotide (dC) opposite a lesion such as an abasic site.[16] Polymerase (Pol) ζ, on the other hand, is a member of the B family of polymerases and thus more closely related to replicative enzymes. In contrast to most translesion polymerases, it exhibits high processivity and accuracy on undamaged template, and rather than inserting nucleotides opposite a damaged site, it prefers to extend mismatched primer termini.[16] This activity provides the basis for a cooperation between Polζ and other translesion polymerases that leads to the incorporation of damage-induced mutations. The recruitment of most damage-tolerant polymerases to replication forks is mediated by proliferating cell nuclear antigen (PCNA), the sliding clamp for replicative polymerases. PCNA forms a homotrimer that encircles DNA as a ring[17] and functions as a processivity factor for polymerases δ and ε.[18] In addition, PCNA serves as a binding platform for a multitude of other factors involved in different DNA-repair pathways, chromatin assembly, and cell-cycle regulation; this suggests that the clamp acts as a central signal integrator for the coordination of replication, repair, and postreplicational chromatin assembly at the replication fork. Most damage-tolerant polymerases interact directly with PCNA through con-

  

served sequence motifs common to many PCNA-binding proteins.[19] Accordingly, PCNA is generally required for their activity in vivo and in vitro. However, in contrast to its effect on replicative enzymes, PCNA rarely appears to stimulate the processivity of bypass polymerases. It has been hypothesized that the PCNA interaction motif might serve as a tether that allows a loose association of damage-tolerant enzymes with the replication fork even in the absence of damage; this would facilitate the rapid exchange of polymerases when needed.[12] A further contribution to recruitment might come from Rev1p, which in mammals has been demonstrated to interact competitively with many other translesion polymerases.[20] However, the mechanism by which a polymerase switch from the replicative to a damage-tolerant enzyme and back is accomplished is not fully understood yet.

### Damage avoidance mechanisms operate in an error-free manner

An alternative strategy of damage bypass, which avoids the use of the damaged region as a template for DNA synthesis altogether, takes advantage of the genetic information encoded by the undamaged, newly synthesized sister chromatid to restore the sequence opposite the lesion in an error-free manner.[5,21] Although the mechanistic details of this damage-avoidance (DA) pathway are not yet understood in eukaryotes, it is believed to involve a temporary reversal of the replication fork, which would allow a pairing of the two newly synthesized strands in a so-called "chicken-foot" structure (Figure 2B). In this situation, the stalled primer terminus could be elongated based on the new template strand, and resolution of the structure would result in the bypass of the damaged site. A prerequisite for the strand-switching model is the continuation of replication on one strand upon a stalling on the other strand in order to provide the template for reorientation of the primer. Evidence for this "overshoot" synthesis has indeed been found in mammalian systems.[22]

### DNA damage tolerance is controlled by the *RAD6* pathway

The significance of damage-tolerance mechanisms for the survival of a cell was recognized several decades ago in genetic experiments on collections of yeast mutants sensitive towards ultraviolet irradiation.[23] Three independent groups of DNA repair genes were identified, and each was named after a prominent member:[2] the *RAD3* group, responsible for nucleotide excision repair; the *RAD52* group, whose members are involved in the repair of double-strand breaks (DSBs) through homologous recombination, and the *RAD6* group, whose mutants showed varying sensitivities towards a variety of genotoxic agents and rather heterogeneous phenotypes. It soon became clear that the *RAD6* group of genes is responsible for controlling DNA damage tolerance.[24] One class of mutants was found to contribute little to the overall resistance to damage, but to be unable to accumulate mutations upon treatment with DNA-damaging agents; this is consistent with a defect in error-prone TLS. The other class exhibited defects in a recovery system called postreplication repair, which is important for survival, but has no effect on damage-induced mutagenesis and is therefore deemed error-free. This activity is detected by the cell's ability to convert low-molecular-weight DNA synthesized on damaged templates to the high-molecular-weight form that is normally produced in the absence of damage, and it most likely reflects the action of the error-free damage-avoidance system. Interestingly, some of the members of the *RAD6* group, including the *RAD6* gene itself, are required for both of the activities described above; this suggests that the *RAD6* pathway might act as a control system that regulates the balance between error-prone TLS and error-free damage avoidance.

Genetic relationships between the members of the *RAD6* pathway have been studied extensively in yeast, but insight into the mechanistic aspects of damage bypass has come more recently from a characterization of their enzymatic activities and physical interactions (Figure 3). According to their en-

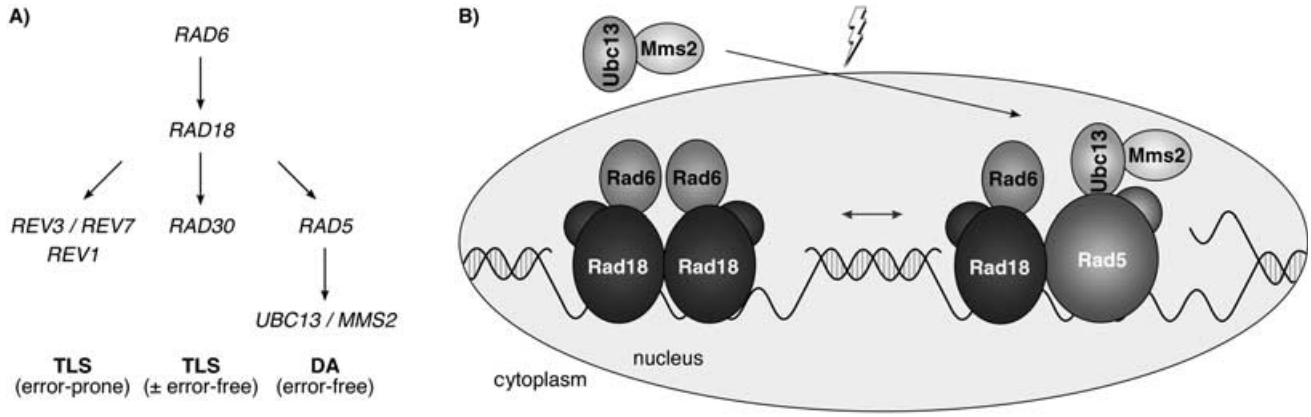


**Figure 3.** The *RAD6* pathway of DNA-damage tolerance in *S. cerevisiae*. A) Genetic relationships between the members of the *RAD6* pathway, as determined from the sensitivities of the respective mutants towards DNA-damaging agents, are indicated by arrows. Two pathways of translesion synthesis (TLS), mediated by different damage-tolerant DNA polymerases, act independently of the error-free damage-avoidance (DA) system. B) The components of the *RAD6* pathway form DNA-associated complexes based on mutual interactions between the RING finger proteins Rad18p and Rad5p. Ubc13p and Mms2p are mostly cytoplasmic proteins in undamaged cells, but partially relocalize to the nucleus upon treatment with DNA-damaging agents. While Rad6p is chromatin-associated by means of its interaction with Rad18p, Ubc13p and Mms2p are recruited to DNA through the interaction between Ubc13p and Rad5p (adapted from ref. [48]).

  

**Table 1.** Members of the *RAD6* pathway in *S. cerevisiae*.

| Gene | Biochemical activity of the protein | Biological process |
|---|---|---|
| *RAD6* | ubiquitin-conjugating enzyme (E2) | translesion synthesis, damage-induced mutagenesis, error-free damage avoidance |
| *UBC13* | ubiquitin-conjugating enzyme (E2) | error-free damage avoidance |
| *MMS2* | UEV (ubiquitin-conjugating enzyme variant) | error-free damage avoidance |
| *RAD18* | ubiquitin protein ligase (E3) | translesion synthesis, damage-induced mutagenesis, error-free damage avoidance |
| *RAD5* | ubiquitin protein ligase (E3) | error-free damage avoidance |
| *RAD30* | damage-tolerant DNA polymerase η | translesion synthesis, |
| *REV1* | dC-Transferase | translesion synthesis, damage-induced mutagenesis spontaneous mutagenesis |
| *REV3* | damage-tolerant DNA polymerase ζ subunit | translesion synthesis, damage-induced mutagenesis, spontaneous mutagenesis |
| *REV7* | damage-tolerant DNA polymerase ζ subunit | translesion synthesis, damage-induced mutagenesis, spontaneous mutagenesis |
| *POL30* | polymerase processivity clamp (PCNA) | processive DNA synthesis |
| *SRS2* | DNA helicase | facilitation of damage tolerance, inhibition of recombination |

zymatic functions, most of them fall into one of two classes: ubiquitin conjugation factors and damage-tolerant polymerases (Table 1). While the action of the polymerases is restricted to the TLS pathway, many of the ubiquitin conjugation factors contribute to both the mutagenic and the error-free bypass systems.

## 3. Protein Modification by Ubiquitin and SUMO

### Multiubiquitination induces degradation by the 26S proteasome

Ubiquitin is a highly conserved protein of 76 amino acids that is common to eukaryotes and best known for its function in the targeting of short-lived proteins for regulated degradation by the 26S proteasome, a large intracellular protease.[8] Potential substrates are marked for destruction by the attachment of a multimeric chain of ubiquitin molecules in an intricate conjugation reaction that usually requires a cascade of at least three different enzymes (Figure 4A). In an initial ATP-dependent step involving a conserved cysteine in the enzyme's catalytic center, ubiquitin-activating enzyme (E1) undergoes a thioester linkage with the C terminus of ubiquitin. The ubiquitin thioester is then transferred to the active-site cysteine of a ubiquitin-conjugating enzyme (E2), which attaches the ubiquitin moiety to an internal lysine residue of the substrate protein through an isopeptide bond. This reaction is usually aided by a ubiquitin protein ligase (E3). While E3s of the HECT family participate in the thioester cascade, RING finger E3s mediate the contact between the E2 and the substrate without being directly involved in the transfer reaction. Repeated conjugation of ubiquitin, generally to lysine 48 of the previous ubiquitin moiety,[25] results in the formation of multiubiquitin chains that serve as a recognition signal for the 26S proteasome. Selectivity of the reaction is mediated cooperatively by E2 in conjunction with an appropriate E3. The combinatorial nature of the conjugation system given by the existence of multiple E2s and a variety of E3s with differing substrate preferences ensures the discrimination between numerous substrates targeted for modification under different conditions.[26]

### Unconventional functions of the ubiquitin system

In contrast to multiubiquitination, attachment of a single ubiquitin moiety conveys distinct, proteasome-independent signals. For example, monoubiquitination of plasma membrane proteins triggers their selective uptake by endocytosis and subsequent degradation in the lysosome or vacuole. Similarly, intracellular vesicle transport appears to be influenced by the monoubiquitination of a number of membrane-associated proteins.[27] Another prominent function of monoubiquitin is the regulation of chromatin structure and transcriptional activity by the modification of histones, which has been analyzed in detail in yeast, but appears to have similar effects in higher eukaryotes.[28] Finally, the recent identification of the repair-associated Fanconi anaemia protein FANCD2 as a target for monoubiquitination has given evidence for a function of this modification in the DNA damage response in higher organisms.[29]

Not only monoubiquitination, but also multiubiquitin chains can convey signals unrelated to proteasomal degradation. Ubiquitin itself comprises seven lysine residues, and each of them can serve as an attachment site for further ubiquitin moieties. Although the existence of branched chains is still a matter of debate, it is evident that ubiquitin chains linked uniformly through one particular lysine will adopt distinct conformations depending on their topology.[30] Accordingly, they are recognized by the 26S proteasome with varying affinities, and distinct biological functions have been associated with a number of different linkages. While the canonical linkage through K48 usually triggers proteasomal degradation of the modified target, multiubiquitin chains linked uniformly through K63 have been implicated in the inflammatory response,[31] in endocytosis,[32] in ribosome biogenesis[33] and in DNA damage tolerance.[34] Other, less well characterized linkages have also been observed in vivo, albeit with lower abundance.[30]

### Functions of the SUMO conjugation system

In addition to ubiquitin, several ubiquitin-like modifiers have been identified in eukaryotes, each associated with its own specific conjugation system and unique set of targets. Judging by the number of substrate proteins, the small ubiquitin-relat-

  

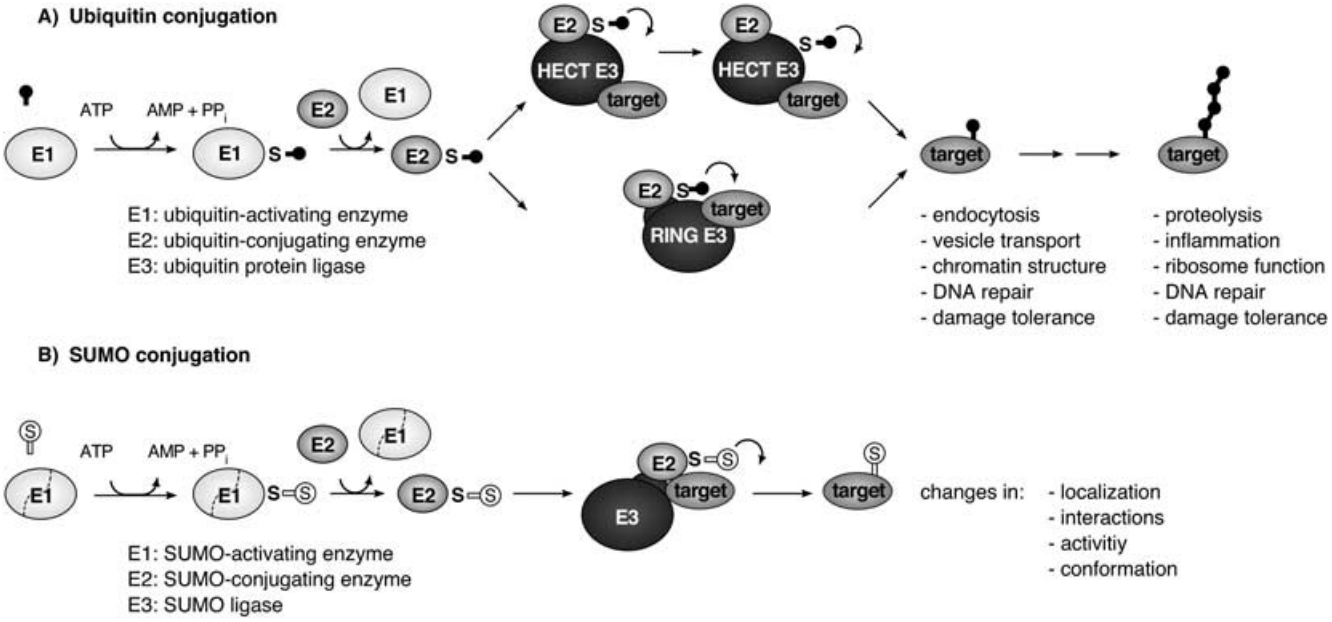


**Figure 4.** The ubiquitin (A) and SUMO (B) conjugation systems. A) Ubiquitin is depicted as a black circle. Its extended carboxy-terminal glycine residue serves for activation and conjugation through a thioester cascade (represented by an S on the E1, E2 and HECT E3 enzymes). Mono- and multiubiquitination result in distinct consequences for the modified target protein. B) SUMO, represented by a white symbol with the letter "S", is activated and conjugated through its carboxy-terminal glycine, like ubiquitin. Its activating enzyme is a heterodimer. In contrast to ubiquitin, SUMO is usually attached to its target proteins as a monomer.

ed modifier SUMO, which in higher eukaryotes comes in three distinct isoforms, appears to be used in the most versatile manner (Figure 4B), but the consequences of SUMO attachment for the modified target are generally less well understood than those of ubiquitination.[35] Sumoylation has been shown to affect the localization of its targets, their shuttling between nucleus and cytoplasm, their protein–protein interactions, and their enzymatic properties. In this manner, the SUMO system exerts a regulatory influence on several signal transduction pathways and transcription factors, and an important, albeit poorly defined role is attributed to SUMO in the maintenance of chromosome stability and genome integrity.[36] There are a few examples of proteins that can be modified by ubiquitin or SUMO, and in at least one instance, the two modifications, targeted to the same lysine residue, were shown to play antagonistic roles.[37] It remains to be seen how general this crosstalk between ubiquitin and SUMO is.

## 4. Ubiquitin and SUMO as Regulators of DNA Damage Tolerance

### The *RAD6* pathway comprises ubiquitin-conjugating factors

A connection between DNA damage tolerance and the ubiquitin system was first established when the *RAD6* gene from *Saccharomyces cerevisiae*, the principal mediator of both TLS and error-free damage avoidance, was discovered to encode a ubiquitin-conjugating enzyme whose catalytic activity as an E2 is required for its function in DNA damage tolerance.[38] Yeast *rad6* mutants are highly sensitive towards any type of DNA-damaging agents, defective in damage-induced mutagenesis, and display elevated rates of spontaneous mutagenesis and recombination.[39,40] In addition, Rad6p affects several other aspects of metabolism unrelated to damage bypass, including the ubiquitination of histone H2B, but also the targeting of several short-lived cellular proteins to the 26S proteasome by multiubiquitination.[41] Two homologues of *RAD6* have been identified in the human genome, the X-linked HHR6A and the autosomal HHR6B, which encode two highly similar proteins with an overall homology of 70% to the yeast E2 and can rescue the DNA-damage sensitivity of yeast *rad6* mutants.[42]

Surprisingly, genetic analysis of ubiquitin mutants in yeast suggested an involvement of multiubiquitin chains of a non-standard topology, linked through K63, in *RAD6*-dependent damage tolerance.[34] The E2 responsible for their synthesis was identified as a heterodimer consisting of Ubc13p, a classical ubiquitin-conjugating enzyme with high homology to other E2 enzymes, and Mms2p, an E2-related protein (dubbed UEV, for Ubiquitin-conjugating Enzyme Variant), whose sequence resembles that of genuine E2s but lacks the conserved active-site cysteine characteristic of catalytically active E2s.[43] The ability of Ubc13p and Mms2p to synthesize multiubiquitin chains correlates with their activity in DNA damage tolerance, and, in fact, the *MMS2* gene had previously been isolated as a member of the *RAD6* pathway based on the complementation of a mutant sensitive to the alkylating agent methyl methane sulfonate (MMS).[44] Like *rad6*, *ubc13* and *mms2* mutants exhibit elevated spontaneous mutation rates, but their sensitivity to UV- or chemically induced DNA damage is only moderate and they show no defect in damage-induced mutagenesis. These observations have placed *UBC13* and *MMS2* in the error-free

  

damage-avoidance pathway. Homologues with conserved enzymatic function have been identified in mammals.[43,45]

In addition to the E2s described above, two genes encoding RING finger proteins are members of the yeast *RAD6* pathway; this suggests that they function as E3s in cooperation with Rad6p and Ubc13p/Mms2p. Rad18p, a protein with ssDNA-binding activity, exists in a stable complex with Rad6p, and the phenotype of a *rad18* deletion resembles that of *rad6* mutants, although it appears restricted to aspects related to DNA-damage tolerance.[40,46] Homologues of *RAD18* have been identified in higher eukaryotes, and deletion of the gene leads to DNA-damage hypersensitivity and increased levels of recombination and sister-chromatid exchange.[47] Thus, Rad18p exhibits all the features suggestive of an E3 enzyme that targets its cognate E2 Rad6p to DNA and is responsible for mediating damage bypass by TLS as well as error-free damage avoidance. The role of the second RING finger protein, yeast Rad5p, within the *RAD6* group is somewhat more ambiguous. Although, based on its relationship to the other members of the *RAD6* pathway, *RAD5* is now generally viewed as a component of the error-free DA system,[48] additional activities, including a role in TLS and mutagenesis, are likely to emerge.[49] *RAD5* encodes a protein of 134 kDa whose RING domain is embedded in a helicase-like domain that place Rad5p into the SNF2/SWI2 family of DNA and RNA helicases as well as chromatin-remodeling factors.[50] Accordingly, the purified protein was found to exhibit DNA-binding and ssDNA-dependent ATPase activity.[51] Interestingly, the function mediated by the protein's ATPase activity appears to be largely separable from its involvement in ubiquitin conjugation (S. Chen and H. D. Ulrich, unpublished data). Moreover, no convincing mammalian homologue of *RAD5* has been identified so far. Rad5p associates with Ubc13p by means of its RING domain and recruits the Ubc13p/Mms2p heterodimer to chromatin in response to DNA damage.[48] Both Rad18p and Rad5p are capable of self-association, and in addition, a mutual interaction between Rad18p and Rad5p, which most likely competes with homodimerization, yields the assembly of two distinct E2–E3 pairs in a single chromatin-associated complex (Figure 3 B).[48]

### PCNA is a target for ubiquitination by the enzymes of the *RAD6* pathway

Insight into how the chromatin-associated complexes described above regulate damage tolerance came from the identification of PCNA as a target for Rad6p-dependent ubiquitination.[52] Yeast PCNA, encoded by the *POL30* gene, is modified at a single, highly conserved lysine residue, K164, with a K63-linked multiubiquitin chain in response to DNA damage.[52] Modification requires the components of the error-free DA pathway, Rad6p, Rad18p, Rad5p, Ubc13p, and Mms2p (Figure 5). The conjugation reaction is a two-step process in which Rad6p and Rad18p attach the first ubiquitin moiety, which is then extended to a multimeric chain by Ubc13p, Mms2p, and Rad5p, according to the unusual linkage specificity of the dimeric E2. In mammalian cells, monoubiquitination appears to be the predominant modification,[53,54] although genetic evidence[45] suggests that the failure to detect multiubiquitinated forms of PCNA to date may be due to detection problems.

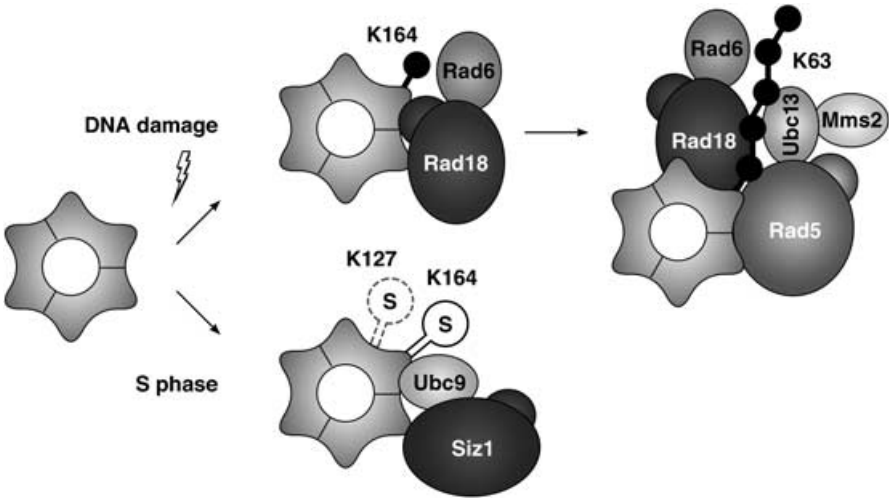


**Figure 5.** Proliferating-cell nuclear antigen (PCNA) is a target of the *RAD6* pathway. Following DNA damage, the homotrimeric PCNA is modified by mono- and multiubiquitination, involving the E2–E3 pairs Rad6p–Rad18p and Ubc13p/Mms2p–Rad5p. Ubiquitin is attached to lysine 164 of PCNA, and the ubiquitin moieties in the multiubiquitin chain are linked via K63 of ubiquitin. Independent of DNA damage, yeast PCNA is modified by SUMO during S phase at K164 and, to a lesser extent, at K127. This reaction involves the SUMO-specific E2 Ubc9p and the SUMO ligase Siz1p (adapted from refs. [52] and [55]).

### Yeast PCNA is subject to SUMO modification during S phase

In addition to ubiquitination, yeast PCNA is also subject to modification by SUMO.[52] Independent of DNA damage, low levels of the modified forms are found in replicating cells during S phase, but not in G1, G2 and mitosis. In addition, extensive modification is observed when cells are treated with lethal amounts of the alkylating agent MMS. SUMO conjugation to PCNA in vivo requires the SUMO-specific E2 Ubc9p and the SUMO ligase Siz1p (Figure 5). PCNA is modified primarily at K164, the same lysine that is also subject to ubiquitination. In addition, modification is observed to a minor extent at K127, which—unlike K164—is part of a consensus motif that was found to serve as a SUMO-attachment site in several other proteins, but is not conserved in the PCNA sequences of other species. Interestingly, sumoylation of PCNA has so far only been observed in *S. cerevisiae*, and it remains to be seen

  

whether this phenomenon is restricted to baker's yeast or will turn out to be more general.

### Consequences of PCNA modifications

Genetic analysis has shown that multiubiquitination of PCNA is the activity that mediates error-free damage avoidance in yeast (Figure 6). Mutation of the acceptor lysine of PCNA to arginine, *pol30(K164R)*, results in a hypersensitivity to DNA-damaging agents that falls into the error-free branch of the *RAD6* group.[52] A distinct function of the monoubiquitinated form has been derived from the effect of PCNA modifications on TLS. In yeast, the contributions of both TLS-specific polymerases, Polζ and Polη, to damage tolerance are dependent on the monoubiquitination of K164 of PCNA.[55] Furthermore, damage-induced mutagenesis, which largely depends on the activity of Polζ, is completely abolished in the *pol30(K164R)* mutant.[55] In the mammalian system, the function of monoubiquitin in the activation of TLS was beautifully confirmed by the observation that the modification directly enhances the affinity of Polη for the clamp, thus resulting in a physical recruitment of the polymerase by monoubiquitinated PCNA.[53,54] Even the mammalian E3 Rad18p, which is responsible for the modification, appears to contribute to the recruitment of Polη to stalled replication forks.[54] Further analysis will be necessary to establish whether other eukaryotic translesion polymerases are recruited in the same manner. Although Polζ was recently shown to cooperate directly with PCNA, it is possible that its activation by ubiquitination of the clamp occurs by a more indirect mechanism.[56]

Although not all aspects of PCNA SUMO modification are fully understood to date, one of its functions during S phase became apparent upon analysis of spontaneous mutation rates in strains deficient in ubiquitin or SUMO conjugation to PCNA. Here—in contrast to damage-induced mutagenesis—the requirement for monoubiquitination of PCNA was not absolute, but could be substituted by SUMO modification; this implies that both monoubiquitin and SUMO are capable of stimulating Polζ activity, with the difference that SUMO modification cannot be induced by DNA damage and thus does not contribute to induced mutagenesis.[55] Activation of Polζ by sumoylated PCNA during S phase in the absence of exogenous DNA damage might serve not only for the bypass of spontaneous lesions, but also to overcome replication fork blocks caused by other refractory sequences such as secondary structures or unedited terminal mismatches.[16]

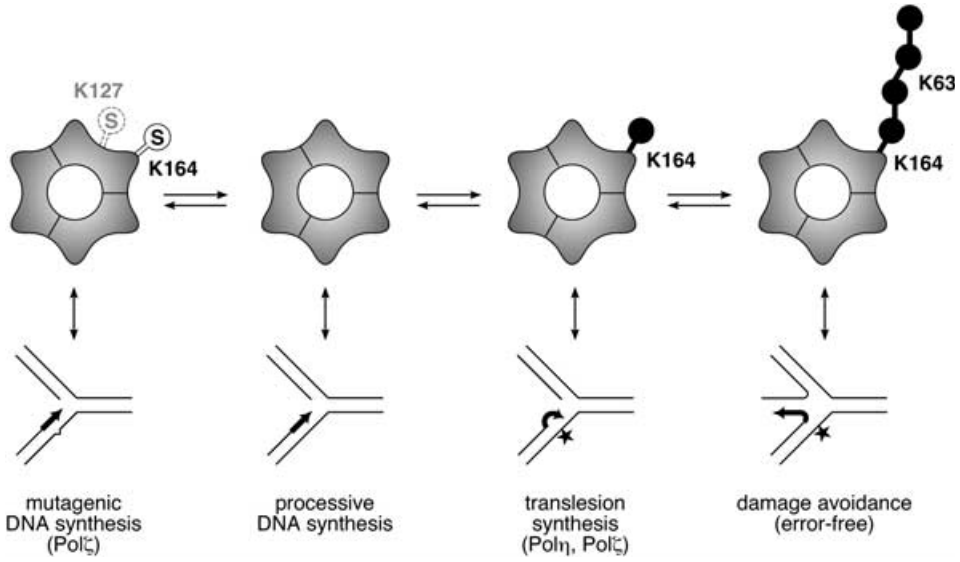


**Figure 6.** Consequences of PCNA modifications. The four different modification states of PCNA elicit distinct cellular responses. SUMO modification during S phase results in damage-independent mutagenic DNA synthesis involving polymerase ζ. Unmodified PCNA acts as a processivity clamp for replicative DNA polymerases. Upon DNA damage, monoubiquitinated PCNA activates the damage-tolerant polymerases ζ and η for translesion synthesis. Multiubiquitinated PCNA is a prerequisite for the error-free damage-avoidance pathway. (Reproduced from ref. [55], with permission. Copyright, Nature Publishing Group, 2003, http://www.nature.com.)

### Communication between ubiquitin and SUMO

Despite the apparent cooperation between SUMO and ubiquitin with respect to mutagenesis, in some situations sumoylation of PCNA appears to have a negative effect on the cell's resistance towards DNA damage. It turns out that *pol30(K164R)* mutants, which are no longer able to ubiquitinate PCNA, are less sensitive to DNA damage than *pol30(K127/164R)* double mutants, which in addition have lost the option of SUMO modification.[52] Moreover, deletion of the SUMO-specific ligase gene *SIZ1* alleviates the damage sensitivity of *RAD6*-pathway mutants.[55] Based on these observations, it was postulated that sumoylation antagonizes the nonproteolytic role of PCNA ubiquitination.[52] However, as the detrimental effect of SUMO is only visible in mutants defective in PCNA ubiquitination, a simple competition for the acceptor lysine is unlikely. Instead, the effect of abolishing PCNA sumoylation is reminiscent of the phenotype of a mutant in the helicase gene *SRS2*, a known suppressor of *RAD6*-pathway mutants that has been proposed to facilitate damage tolerance by preventing unscheduled recombination.[57] Indeed, physical interactions between Srs2p and sumoylated PCNA result in a recruitment of Srs2p and an inhibition of homologous recombination at replication forks.[58] Thus, SUMO and ubiquitin appear to cooperate rather than compete in this situation. Finally, crosstalk has been observed even between the respective conjugation systems, as the SUMO-specific E2 Ubc9p was found to physically interact with the ubiquitin-specific E3s Rad18p and Rad5p;[52] however, the consequences of this association have not been analyzed.

  

## 5. Summary and Outlook

The model shown in Figure 6 depicts PCNA as a molecular switchboard that controls the mechanism of replication and damage bypass by means of distinct modification states. While multiubiquitination of PCNA elicits the error-free damage-avoidance response that probably involves a regression of the replication fork, monoubiquitination at the same lysine residue in turn activates the bypass polymerases η and ζ for TLS and damage-induced mutagenesis. Under nondamage conditions, yeast Polζ can also be activated for spontaneous mutagenesis by sumoylation of PCNA. Many of the principles that emphasize the diversity of ubiquitin and SUMO as intracellular signaling molecules are realized in the system of PCNA modifications: three different modifications—monoubiquitin, nonstandard K63-linked multiubiquitin chains and SUMO—trigger distinct cellular reactions when targeted to the same acceptor lysine on PCNA. Intriguingly, both forms of ubiquitination appear to convey unconventional, that is, proteasome-independent signals. Finally, a communication between the ubiquitin and the SUMO system is implicated not only by the identity of the modified residue, but also by physical interactions of the respective conjugation factors. In contrast to previous examples of crosstalk between the two modifiers, however, ubiquitin and SUMO appear to act in a cooperative rather than an antagonistic manner on PCNA.

While it is attractive to invoke PCNA modifications as a means of triggering alternative responses to replication fork stalling, several new questions arise from this scenario. On one hand, upstream signals must exist that determine which of the modifications is appropriate at what time, and these signals should control the activities of the respective conjugation enzymes.[59] On the other hand, the different modifications are expected to elicit distinct cellular responses based on the recognition of these modifications by downstream effectors.

Given its influence on the accuracy of lesion bypass in the eukaryotic cell, the PCNA modification system appears to occupy a key regulatory position in the maintenance of genomic integrity with important implications for the development of strategies to combat cancer. To date most treatments aim at the—more or less selective—killing of malignant cells by radiation or cytotoxic chemicals. As both of these agents target fast-growing cells due to their potential to interfere with DNA replication, either by directly producing lesions or by interfering with the enzymes involved in DNA metabolism, the elimination of DNA-damage-tolerance mechanisms would of course enhance the efficacy of traditional cancer treatments.[60] Moreover, one of the biggest risks of cancer therapy is the development of secondary tumours, caused by the mutagenic action of the anticancer agents themselves. Suppression of the damage-tolerant polymerases responsible for mutagenic lesion bypass would certainly help to minimize these unwanted consequences.[7] It is hoped that an understanding of the regulatory mechanisms controlling DNA damage tolerance will allow us to eventually develop appropriate strategies for their controlled manipulation.

## *Acknowledgements*

*Work in the author's laboratory is supported by Cancer Research UK and grants from the German Ministry for Education and Research (BMBF), the EMBO Young Investigator Programme, and the European Community's Human Potential Programme.*

**Keywords:** DNA damage · mutagenesis · protein modifications · SUMO · ubiquitin

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Received: April 4, 2005
Published online on September 5, 2005

  