Conservation of DNA damage tolerance pathways from yeast to humans

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Abstract

Damage tolerance mechanisms, which allow the bypass of DNA lesions during replication, are controlled in eukaryotic cells by mono- and poly-ubiquitination of the DNA polymerase cofactor PCNA (proliferating-cell nuclear antigen). In the present review, I will summarize our current knowledge of the enzymatic machinery for ubiquitination of PCNA and the way in which the modifications affect PCNA function during replication and lesion bypass in different organisms. Using the budding yeast as a reference model, I will highlight some of the species-specific differences, but also point out the common principles that emerge from the genetic and biochemical studies of damage tolerance in a range of experimental systems.

Introduction

The stability of our genetic material is endangered both by DNA-damaging agents from the environment and by endogenous problems during DNA replication arising from spontaneous damage [1]. DNA repair pathways, the cell's first line of defence against many types of lesions, act mostly on duplex DNA and rely on the excision and subsequent resynthesis of the damaged sequence based on the information encoded by the complementary strand. However, during DNA replication, unrepaired lesions can impede the progress of replicative DNA polymerases, highly processive enzymes with active sites that do not accommodate distorted template structures [2]. As stalled replication forks are dangerous structures that can result in either permanent cell cycle arrest or major chromosomal abnormalities, replication blocks need to be overcome to ensure survival. DNA damage tolerance pathways mediate the replicative bypass of replication-blocking lesions without actually removing the damage [3,4].

Importantly, damage bypass systems differ markedly in the accuracy with which they fill the position opposite a lesion. In a process called TLS (translesion synthesis), specialized DNA polymerases that tolerate a variety of abnormal template structures are employed to replicate across a site of damage [5]. Owing to their relaxed active sites, however, damage-tolerant polymerases operate with reduced fidelity on both damaged and undamaged templates. Accordingly, TLS is often error-prone, and in fact most of the mutations induced by genotoxic agents result not from the damage itself, but rather from the activity of the damage-tolerant polymerases [6]. An alternative, error-free pathway of damage bypass avoids the damaged region as a template for DNA synthesis and instead makes use of the genetic information encoded by the undamaged, newly synthesized sister chromatid to restore the sequence opposite the lesion [4,7,8]. Although the mechanistic details of this pathway are not yet understood in eukaryotes, it may involve a transient template switching of the stalled primer terminus [9].

Control over the activity of DNA damage tolerance is important for the maintenance of genome stability, particularly in higher eukaryotes. On the one hand, TLS is a source of genetic instability because of its mutagenic action and should therefore best be avoided. On the other hand, a failure of replicative lesion bypass can induce more severe rearrangements by homologous recombination, which are often associated with malignant transformations [10]. A limitation of damage tolerance mechanisms to situations where they are needed therefore appears to contribute to an appropriate balance between cell survival and genome stability.

Ubiquitination of PCNA (proliferating-cell nuclear antigen) in the budding yeast

Regulation of DNA damage tolerance in eukaryotes is mediated by a group of genes collectively called the *RAD6* pathway (Figure 1). In the budding yeast, *Saccharomyces cerevisiae*, where this system has been studied in detail, the *RAD6* pathway controls both TLS and error-free damage avoidance [11,12]. Mutants in the *RAD6* pathway are sensitive to various DNA-damaging agents, indicating that damage tolerance contributes to cell survival under conditions of genotoxic stress. Its members encode on the one hand the damage-tolerant polymerases that mediate TLS and on the other hand enzymes involved in ubiquitin conjugation: two ubiquitin-conjugating enzymes (E2), Rad6 and the dimeric Ubc13–Mms2, and two ubiquitin protein ligases (E3), Rad18 and Rad5.

These factors form a chromatin-associated complex [13] that operates on the sliding clamp protein PCNA, an essential processivity factor for replicative polymerases and interacting partner for numerous other cellular factors involved in DNA replication and repair. In response to DNA-damaging agents,

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Abbreviations used: PCNA, proliferating-cell nuclear antigen; SUMO, small ubiquitin-related modifier; TLS, translesion synthesis.
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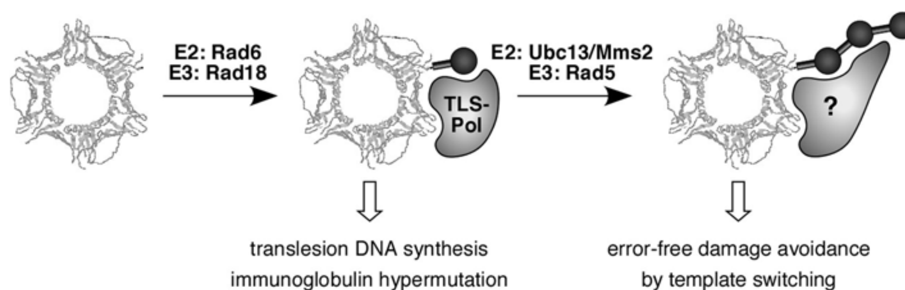
Figure 1 | PCNA is modified by mono- and poly-ubiquitination

The conjugation enzymes, modifications and their consequences for DNA damage tolerance are schematically indicated around the PCNA structures. Mono-ubiquitinated PCNA is recognized by damage-tolerant polymerases involved in TLS and immunoglobulin hypermutation (TLS-Pol), whereas the downstream effectors of polyubiquitinated PCNA that initiate error-free damage avoidance are unknown.



the budding yeast PCNA is modified with a polyubiquitin chain at a single lysine (K) residue, Lys^{164} [14]. The conjugation reaction is a two-step process in which Rad6 and Rad18 attach the first ubiquitin moiety, which is then extended to a multimeric chain by Ubc13, Mms2 and Rad5. The ubiquitin monomers within the chain are linked via a non-standard lysine residue of ubiquitin, Lys^{63} [15], and genetic evidence suggests that this modification is a prerequisite for the error-free damage avoidance pathway [14].

Conservation of PCNA ubiquitination among eukaryotes

Although first discovered in *S. cerevisiae*, ubiquitination of PCNA appears to be common to many organisms. The site of ubiquitin attachment itself, Lys^{164}, is absolutely conserved among eukaryotic PCNA sequences, and its modification has been observed in mammals, chicken, frog and fission yeast [14,16–21]. Nevertheless, variations in the type and extent of ubiquitination as well as the conjugation enzymes involved may indicate subtle differences in the way in which cells use the modification.

The fission yeast, *Schizosaccharomyces pombe*, encodes easily recognizable homologues of all the conjugation enzymes for mono- and poly-ubiquitination of PCNA, and both modifications are induced by DNA damage [17]. Unlike *S. cerevisiae*, however, *S. pombe* cells ubiquitinate PCNA during S-phase even in the absence of damage, suggesting either a higher overall activity of the enzymes or a higher incidence of replication-associated problems such as spontaneously stalled replication forks or endogenous lesions. In *Xenopus laevis* egg extracts, mono-ubiquitination of PCNA was found to accompany undisturbed DNA replication, whereas a di-ubiquitinated species was induced by DNA damage [19]. According to a second report, however, both mono- and di-ubiquitination PCNA were limited to conditions of replication fork stalling [22]. The reasons for this discrepancy have not been resolved, but it might indicate that variations can be due to not only the organism, but also the experimental conditions used.

While damage-induced mono-ubiquitination of PCNA is readily observable in mammalian cells [14,18,21], polyubiquitination was not detected until recently and appears to be much less prominent than mono-ubiquitination [23]. Likewise, a mammalian orthologue of the yeast *RAD5* gene, *SHPRH*, has only recently been described, probably because the homology is restricted to the catalytically relevant RING (really interesting new gene) finger and a surrounding helicase-like domain [24,25]. In contrast, mammalian *RAD18* is approx. 20% identical with the yeast gene [26,27], and the genes encoding the E2s are similar enough to complement the respective yeast mutants [28–30].

Correspondence between yeast and vertebrate *RAD6* pathway components is not 100%, however, because there is good evidence for some Rad18-independent ubiquitination of PCNA in the chicken DT40 cell line [16,20]. In addition, removal of ubiquitin from PCNA in mammals and chicken is catalysed by a dedicated isopeptidase, USP1 [20,31]. Although de-ubiquitination of PCNA is clearly detectable in yeast (A.A. Davies and H.D. Ulrich, unpublished work), no specific yeast USP1 homologue has been identified.

Activation of TLS by $PCNA^{Ubi}$

When PCNA modifications were first observed, it was unclear whether mono-ubiquitinated PCNA served a function distinct from that of the polyubiquitinated form, or whether it represented a mere intermediate to polyubiquitination [14]. However, subsequent studies have confirmed that PCNA mono-ubiquitination indeed plays a unique and prominent physiological role in damage bypass.

While the error-free branch of the *RAD6* pathway requires PCNA polyubiquitination, mono-ubiquitination was found to be necessary for the damage-dependent activity of all damage-tolerant polymerases in the budding yeast, polymerases η, ζ and Rev1, indicating that this modification controls TLS and damage-induced mutagenesis [32]. In case of the mammalian damage-tolerant polymerases η and κ, this activation was ascribed to a recruitment of the polymerases to

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stalled replication forks due to a preferential affinity for the mono-ubiquitinated form of PCNA [18,21,33]. In fact, conserved ubiquitin-binding domains have now been discovered in all the damage-tolerant polymerases of the Y family, which include polymerases η, ι, κ and Rev1 [34], and ubiquitination-dependent recruitment of TLS polymerases to PCNA appears to be a common principle of activation following DNA damage [34–36]. In fact, a linear fusion of ubiquitin to PCNA enhances interaction of the clamp with yeast polymerase η and can substitute for physiologically mono-ubiquitinated PCNA in protection against UV irradiation *in vivo* [37]. In an *in vitro* assay with purified components, mono-ubiquitinated PCNA preferentially stimulates lesion bypass by yeast polymerase η and Rev1 [38], and at least in the case of Rev1 this is dependent on its ubiquitin-binding motif [39], although experimental conditions might influence the degree to which a stimulation of the polymerase can be observed [40].

In contrast with the Y family polymerases, the damage-tolerant polymerase ζ, a B family enzyme, lacks obvious ubiquitin- or PCNA-binding domains; yet it is effectively stimulated by the replicative clamp [41]. Although *in vitro* its activity in lesion bypass is not further enhanced by mono-ubiquitination of PCNA [38], the modification is required *in vivo* for polymerase ζ-dependent damage-induced mutagenesis [32]. This may well be explained by an indirect effect mediated through physical interaction of polymerase ζ with the Rev1 protein [42]. However, recent observations indicate that polymerase ζ may also operate independently of the ubiquitin-mediated damage tolerance pathway both in the generation of spontaneous mutations [32,43] and in mutagenic events induced by γ irradiation [44]. Finally, polymerase ζ has been found to interact with the PCNA-like 9-1-1 complex, involved in checkpoint activation, and although this complex does not stimulate damage bypass *in vitro* [41], it appears to be partially required for damage-induced mutagenesis in yeast [45].

The ‘error-free’ pathway of damage tolerance

Compared with the function of mono-ubiquitinated PCNA in TLS, much less is known about the mechanism of error-free damage avoidance and the involvement of PCNA polyubiquitination in this process. Elegant genetic experiments with lesion-bearing plasmids have demonstrated that the pathway mediates a gene conversion event that copies information from the undamaged sister chromatid to the position opposite the lesion and that this occurs in a Rad52-independent manner, i.e. not via classical homologous recombination [9]. In practice, yeast *ubc13* and *mms2* mutants, defective in PCNA polyubiquitination, display only mild damage-sensitivity, and their most prominent phenotype is an elevated polymerase ζ-dependent spontaneous mutation rate, suggesting that lesions normally processed by the error-free pathway can alternatively be bypassed by TLS [46,47]. Down-regulation of human Mms2 by antisense RNA or overexpression of a dominant-negative ubiquitin mutant defective in Lys^{63}-linked polyubiquitination has given similar results in mammalian cells [23,48], and the recent identification of SHPRH as a vertebrate Rad5 orthologue [24,25] strongly suggests that not only mono-ubiquitin-dependent TLS, but also polyubiquitin-dependent damage avoidance are conserved among eukaryotes.

PCNA SUMOylation

Independent of DNA damage, the budding yeast PCNA is also subject to modification by the SUMO (small ubiquitin-like modifier) during S-phase [14]. PCNA is SUMOylated primarily at Lys^{164}, the same lysine that is also subject to ubiquitination, and to a minor extent at Lys^{127}, which (unlike Lys^{164}) 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. PCNA SUMOylation at replication forks has been shown to recruit a helicase, Srs2, that prevents unscheduled homologous recombination events [49,50]. Details of this pathway have been reviewed elsewhere [51,52]. Preferential interaction of Srs2 with SUMOylated PCNA is mediated by a C-terminal SUMO-interaction motif in the helicase that appears to be unique to the *S. cerevisiae* protein and is not found in homologues such as the *S. pombe* protein, suggesting that Srs2 activity is controlled in a different manner in fission yeast. Accordingly, PCNA SUMOylation has not been observed in this organism [17]. Surprisingly, however, PCNA in chicken DT40 cells as well as *X. laevis* egg extracts is SUMOylated at Lys^{164}, despite the apparent absence of a convincing SUMO-interacting Srs2 homologue [16,19]. The functional relevance of these modifications still awaits clarification.

Conclusion

Owing to their multicellular nature, higher eukaryotes have evolved a need for much greater complexity in the regulation of signal transduction and cell differentiation than fungi. This might explain why the *RAD6* pathway appears to have acquired additional functions in vertebrates for which there is no equivalent in yeast. One such role, which can be ascribed directly to the mono-ubiquitination of PCNA, is the generation of antibody diversity by somatic hypermutation [16,53]. Most likely, ubiquitinated PCNA contributes to mutagenesis by recruitment of damage-tolerant polymerases following deamination of cytidines in immunoglobulin variable regions. But even processes completely unrelated to genome stability can be influenced by components of the *RAD6* pathway, such as the signal transduction pathway, leading to activation of the nuclear transcription factor NF-κB (nuclear factor κB) in the regulation of inflammation and apoptosis [54]. In this context, degradation of the inhibitor protein, IκBα (inhibitory κB), requires Lys^{63}-linked polyubiquitin chains synthesized by Ubc13 and an Mms2-related protein, Uev1A [55].

Despite some apparent variations, however, the *RAD6* pathway of PCNA ubiquitination is an important mediator of genome stability that is highly conserved in most of its

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details throughout eukaryotic organisms, both in terms of the conjugation factors responsible for the modification and with respect to the cellular processes that are initiated by ubiquitinated PCNA. A deeper understanding of damage tolerance will now require the elucidation of the signals that trigger the modifications and differentiate between mono- and poly-ubiquitination.

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