Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation

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Protein modification by ubiquitin is emerging as a signal for various biological processes in eukaryotes, including regulated proteolysis, but also for non-degradative functions such as protein localization, DNA repair and regulation of chromatin structure[1–4]. A small ubiquitin-related modifier (SUMO) uses a similar conjugation system that sometimes counteracts the effects of ubiquitination[5]. Ubiquitin and SUMO compete for modification of proliferating cell nuclear antigen (PCNA), an essential processivity factor for DNA replication and repair[6]. Whereas multi-ubiquitination is mediated by components of the *RAD6* pathway and promotes error-free repair, SUMO modification is associated with replication[6–9]. Here we show that *RAD6*-mediated mono-ubiquitination of PCNA activates translesion DNA synthesis by the damage-tolerant polymerases η and ζ in yeast. Moreover, polymerase ζ is differentially affected by mono-ubiquitin and SUMO modification of PCNA. Whereas ubiquitination is required for damage-induced mutagenesis, both SUMO and mono-ubiquitin contribute to spontaneous mutagenesis in the absence of DNA damage. Our findings assign a function to SUMO during S phase and demonstrate how ubiquitin and SUMO, by regulating the accuracy of replication and repair, contribute to overall genomic stability.

RAD6-dependent DNA damage tolerance in *Saccharomyces cerevisiae* encompasses an error-free damage avoidance mechanism believed to involve replication fork regression associated with a template switch to the newly synthesized sister chromatid, as well as two pathways of translesion synthesis by polymerase η (Pol η, encoded by *RAD30*, the yeast homologue of the human xeroderma pigmentosum variant gene *XPV*) and polymerase ζ (Pol ζ, encoded by *REV3* and *REV7*)[4,10]. All three branches require the action of the ubiquitin-conjugating enzyme Rad6p in complex with the DNA-binding RING finger protein Rad18p[11–13]. In the context of error-free bypass the Rad6p–Rad18p complex cooperates with a second ubiquitin-conjugating enzyme, the Ubc13p–Mms2p dimer, and another RING finger protein, Rad5p, in the conjugation of unusual, lysine 63 (K63)-linked multi-ubiquitin chains to K164 of PCNA (*POL30*)[6–9]. Consequently, mutation of this residue causes a sensitivity towards DNA-damaging agents that is genetically linked to the error-free branch of the *RAD6* pathway[6]. In the absence of Rad5p, Ubc13p or Mms2p PCNA is mono-ubiquitinated by Rad6p and Rad18p[6], but the question of whether this modification is merely an intermediate on the way to multi-ubiquitin chains or represents a physiologically relevant state of PCNA has not been addressed. SUMO modification of PCNA by Ubc9p and the SUMO-specific ligase Siz1p[14] occurs primarily at K164 as well, but also at K127 (ref. 6). As SUMO modification is most prominent during S phase, it has been suggested to confer a replicative function to PCNA[6]. An inhibitory function of SUMO on repair was inferred from the notion that *pol30(K127/164R)*, a mutant lacking both lysine residues subject to SUMO modification, is more tolerant to DNA damage than *pol30(K164R)*, which is devoid of ubiquitination but can still be modified by SUMO on K127 (ref. 6). Both the absence of modifications in mutants of the conjugation systems as well as the identities of the acceptor lysines on PCNA were verified as previously described[6] (see Supplementary Fig. 1).

In order to develop a tool for the analysis of ubiquitination of wild-type PCNA in the absence of interfering SUMO modification we examined the effect of a *siz1* deletion on the PCNA lysine mutants. In the *siz1* background both the single- and the double-lysine mutant exhibited virtually identical sensitivities towards ultraviolet irradiation or chemically induced DNA damage, similar to that of *pol30(K127/164R)* in *SIZ1* (Fig. 1a, f), indicating that abolishment of SUMO conjugation has the same effect as the K127R mutation. Thus, the enhanced sensitivity of *pol30(K164R)* compared with *pol30(K127/164R)* in *SIZ1* cells can indeed be attributed to SUMO modification of PCNA at K127. Considering this inhibitory effect of SUMO, we predicted that deletion of *SIZ1*, which by itself has very little influence on repair efficiency[14], would also alleviate the sensitivities of *RAD6* pathway mutants. Figure 1b, f shows that this was indeed the case.

The Pol η- and Pol ζ-dependent pathways of translesion synthesis operate independently of the *RAD5*-mediated error-free branch[7,9,15], resulting in additive or synergistic relationships between mutations that inactivate the individual systems. Consequently, the difference in sensitivities between *rad18 siz1* and *rad5 siz1* mutants (Fig. 1b, f) should be due to the two translesion synthesis pathways. Whereas Pol η carries out error-free replication through different types of oxidative or ultraviolet-induced lesions[16] and is known to require direct interaction with PCNA for activity[17], Pol ζ is most efficient in the error-prone extension of primer termini opposite a variety of lesions or mismatches and is responsible for virtually all damage-induced mutagenesis[18]. We asked whether Rad6p and Rad18p control translesion synthesis by means of ubiquitination of PCNA or by modification of a different target protein. We therefore analysed the effect of *rad30* and *rev3* deletions on the DNA repair efficiencies of the PCNA mutants. Deletion of *rad30* had no further effect on the ultraviolet sensitivity of the *pol30(K164R)* mutant (Fig. 1c), implying that the effect of Pol η on repair depends on the presence of K164 of PCNA. The same epistatic relationship was observed in the absence of SUMO modification in *pol30(K127/164R)* and in the *siz1* background (Fig. 1c). Thus, we conclude that it is indeed K164 of PCNA and not some other target protein whose ubiquitination is required for the repair activity of Pol η. From the fact that translesion synthesis is independent of the RAD5-mediated branch[7,9,15], we infer that the modification required for Pol η activity is mono- and not multi-ubiquitination. Analysis of the relationship between PCNA and Pol ζ yielded virtually identical results (Fig. 1d, g). Following the same logic as in the case of Pol η, we conclude that Pol ζ-dependent repair activity also requires mono-ubiquitination of PCNA.

If translesion synthesis by Pol η and Pol ζ fully accounts for the contribution of mono-ubiquitinated PCNA to DNA repair, deletion of both polymerases in combination with a defect in multi-ubiquitination should result in a strain with the same damage sensitivity as a PCNA mutant that is completely devoid of ubiquitination. In order to exclude the effects of SUMO we tested this notion in a *siz1* background. Multi-ubiquitination was prevented by using the *ubi(K63R)* mutant, whose repair defect equals that of a *ubc13* or *mms2* deletion[8,19]. Figure 1e shows that indeed the mutant *ubi(K63R) rad30 rev3 siz1* had the same ultraviolet sensitivity as *pol30(K164R) siz1*, and no further increase was observed in combinations of *pol30(K164R)* with the other mutations. Treatment with DNA-damaging chemicals yielded identical results (not shown). We conclude that the phenotype of the PCNA lysine mutant is caused by a combination of defects that result on one hand from the abrogation of the error-free bypass system, dependent on K63-linked multi-ubiquitin chains, and on the other hand from inhibition of the two translesion polymerases Pol η and Pol ζ by the absence of PCNA mono-ubiquitination. On the basis of our findings, it is likely that these three systems, Pol η, Pol ζ and the multi-ubiquitin-dependent branch, account for most, if not all of the effects of PCNA ubiquitination on DNA repair.

As Pol ζ-mediated translesion synthesis is the major cause of

damage-induced mutagenesis[18], we asked whether mutants deficient in the enzymes involved in ubiquitin or SUMO conjugation or whether mutations affecting the acceptor lysines on PCNA would have an impact on the accumulation of ultraviolet- and methyl methane sulphonate (MMS)-induced mutations (Fig. 2). Whereas *siz1* exhibited no defect in damage-induced mutagenesis either alone or in combination with *ubc13*, mutation of K164 of PCNA completely inhibited induced mutagenesis, similar to the *rev3* mutant itself. On the basis of the notion that *ubc13* and *siz1* are capable of mutation induction, whereas *pol30(K164R)*—similar to *rad6* and *rad18* mutants[20,21]—is not, we conclude that mono-ubiquitination of PCNA is a prerequisite for Pol ζ-dependent damage-induced mutagenesis.

Pol ζ is also responsible for 50–75% of all spontaneous mutations[18]. In light of this fact the defect of the *rad6* and *rad18* mutants in many assays of Pol ζ-dependent induced mutagenesis is hard to reconcile with the notion that both display a spontaneous Pol ζ-dependent hypermutator phenotype[22–24]. In order to examine this phenomenon, we determined spontaneous mutation rates in two independent reporter genes (Table 1). As noted before[22,24,25], *rad18* and *ubc13* mutants both exhibited elevated mutation rates, whereas *rev3* had a three- to fivefold reduced level of mutagenesis. Notably, *pol30(K164R)* behaved like the wild type, indicating that in contrast to induced mutagenesis, wild-type levels of spontaneous mutagenesis do not require ubiquitination of PCNA. Interestingly, however, *pol30(K164R)* completely abolished the hypermutator

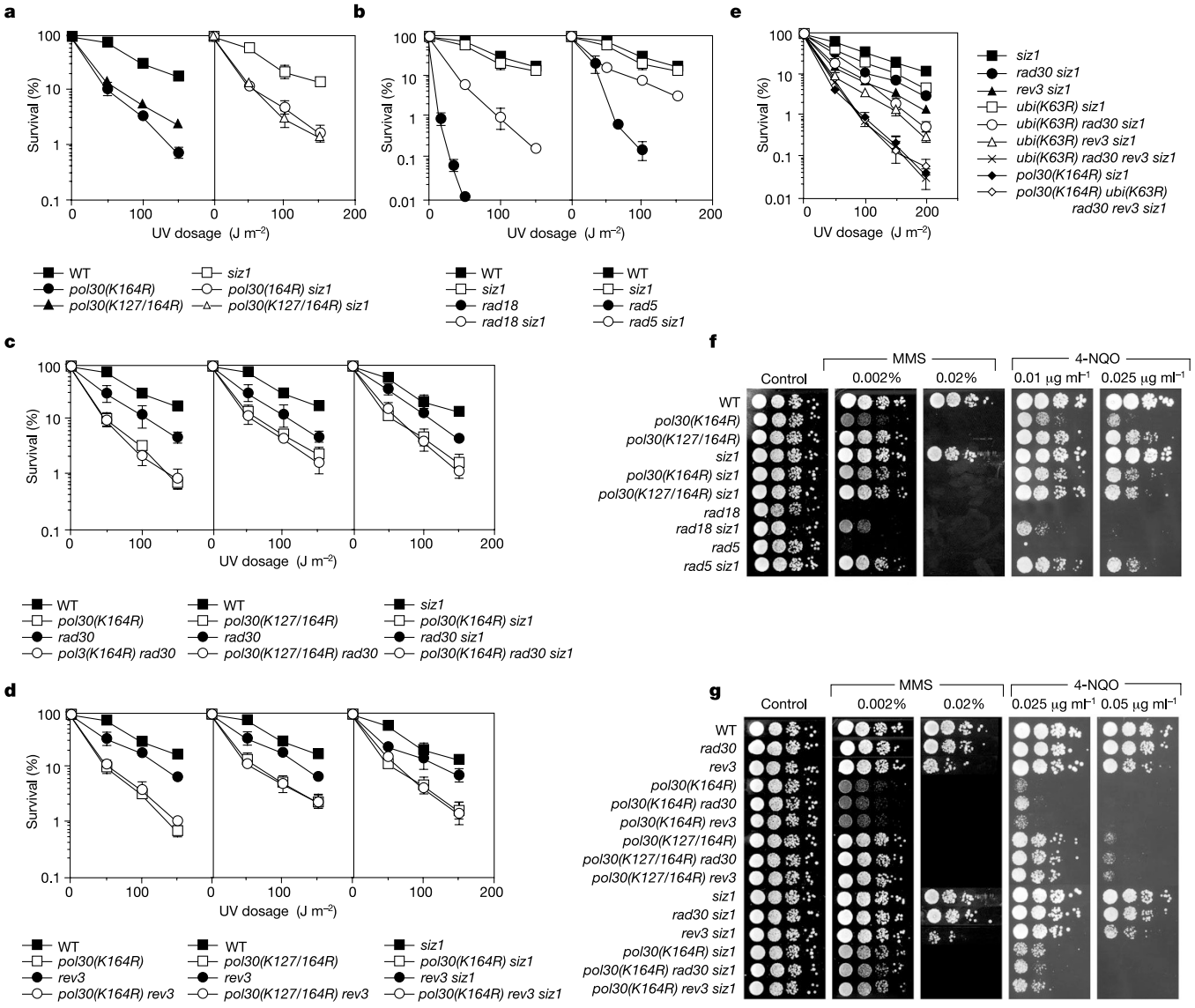

Figure 1 PCNA modifications differentially affect individual branches of the *RAD6* pathway. **a**, Effect of *siz1* on the ultraviolet (UV) sensitivities of PCNA mutants. **b**, Effect of *siz1* on the ultraviolet sensitivities of *RAD6* pathway mutants. **c**, Effect of PCNA mutants on Pol η-dependent repair. **d**, Effect of PCNA mutants on Pol ζ-dependent repair. **e**, Comparison of the ultraviolet sensitivities of strains deficient in individual branches of the *RAD6* pathway and combinations thereof. **f**, Sensitivities to MMS and 4-nitroquinoline-1-oxide (4-NQO) of the same set of strains as in **a** and **b**. **g**, Sensitivities to MMS and 4-NQO of the same set of strains as in **c** and **d**. Mutants, created as described previously[9], were derived from a strain in which a *pol30* deletion was complemented by wild type or mutant *POL30* under the control of its own promoter on an integrative plasmid, except for the series of strains used in **e**, which were created in a strain background where endogenous ubiquitin genes were replaced by wild-type ubiquitin or *ubi(K63R)* on a plasmid[19]. Here the *pol30(K164R)* allele was introduced by direct replacement of *POL30*. Details of strain construction are available on request. Survival after ultraviolet irradiation (254 nm) was determined in quadruplicate as previously described[9]. Standard deviations are indicated where the error bars exceed the sizes of the plot symbols. Sensitivities to DNA-damaging chemicals were determined by spotting tenfold serial dilutions of exponential yeast cultures of equal densities onto plates of rich medium containing the indicated concentrations of the relevant agent. Pictures were taken after incubation for 2–3 days.

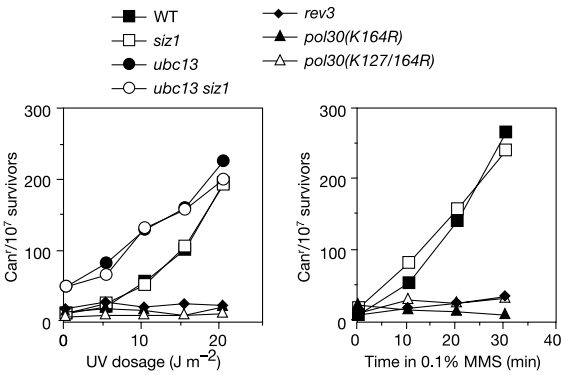

Figure 2 Pol ζ-dependent DNA-damage-induced mutagenesis requires mono-ubiquitination of PCNA. Forward mutation frequencies in the *CAN1* locus[20] were determined in duplicate after irradiation of the indicated strains with increasing ultraviolet dosage at 254 nm (left panel) or treatment with 0.1% MMS in liquid culture (right panel) by relating the number of colonies on minimal medium containing 30 mg l^{-1} canavanine sulphate (Can^r) to the total number of survivors on non-selective medium. The *ubc13* derivatives[9] exhibit a higher frequency of mutants even in the absence of DNA damage due to an elevated spontaneous mutation rate (Table 1). Both PCNA mutants in combination with any other *RAD6* pathway mutant were also completely defective in ultraviolet-induced mutagenesis (not shown).

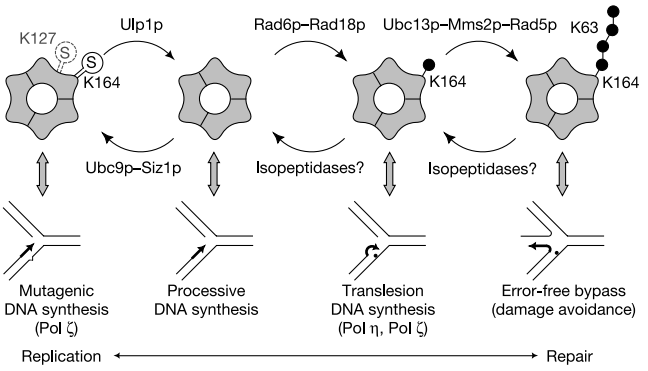

Figure 3 Model of the interplay between the SUMO and ubiquitin conjugation systems and their consequences for DNA replication and repair. PCNA is modified alternatively by ubiquitin or SUMO as depicted by black and white lollipop symbols, respectively. Lysine residues on PCNA and ubiquitin used for conjugation are indicated. Whereas K63-linked multi-ubiquitin chains synthesized by Ubc13p, Mms2p and Rad5p promote error-free bypass by a damage avoidance mechanism possibly involving a template switch to the newly synthesized sister chromatid (fourth column), mono-ubiquitination of PCNA by Rad6p and Rad18p is essential for Pol η- and Pol ζ-dependent translesion DNA synthesis, resulting in the accumulation of damage-induced mutations (third column, see also Figs 1 and 2). During normal DNA replication Pol ζ is stimulated by SUMO modification of PCNA, generating spontaneous mutations in the absence of DNA damage (Table 1, first column); in this mode Pol ζ activity is not damage-inducible. In the absence of exogenous damage, mono-ubiquitin can partially substitute for SUMO in the activation of Pol ζ (Table 1). We postulate a reversible distribution between the various modification states of PCNA, which would require the action of the relevant isopeptidases in addition to the conjugation machinery (see Supplementary Fig. 2).

phenotype of both *rad18* and *ubc13*, reducing it down to the level of the wild type or even below that. Thus, modification of this residue apparently is responsible for the elevated mutation rates observed in *rad18* and *ubc13* mutants. Moreover, the double-lysine mutant *pol30(K127/164R)* consistently yielded even lower mutation rates than the wild type either in isolation or in combination with *rad18* or *ubc13*. This implies that SUMO modification of PCNA contributes to spontaneous mutagenesis. We predicted that in this case deletion of *SIZ1* should negatively affect spontaneous mutation rates. In fact, although the *siz1* deletion in isolation did not affect mutagenesis, it reduced the mutation rate of *rad18* cells down to the level of *pol30(K127/164R)*. In *ubc13* mutants the *siz1* deletion reduced the elevated spontaneous mutation rate only partially. We conclude that Pol ζ-dependent spontaneous mutagenesis can be stimulated by either mono-ubiquitination or SUMO modification of PCNA.

Reduced mutation rates are seen only when neither SUMO nor ubiquitin can be attached to PCNA (as in *pol30(K127/164R)* and in *rad18 siz1*). Modification by either ubiquitin at K164 or SUMO at K164 or K127 is sufficient to afford wild-type levels of mutagenesis (as in *pol30(K164R)* and in *siz1*), indicating that SUMO and ubiquitin can partially substitute for each other. Residual Pol ζ activity in the presence of unmodified PCNA may explain the difference remaining between *rev3* and *pol30(K127/164R)*. Whereas SUMO, mono-ubiquitin and multi-ubiquitin chains normally compete for modification of PCNA, elevated spontaneous mutation rates seem to result from an imbalance in this distribution: in *rad18*, SUMO modification predominates in the absence of ubiquitination; consequently, the elevated mutation rate is completely abolished by abrogation of SUMO modification on deletion of *SIZ1*. In contrast, the hypermutator phenotype of *ubc13* is of twofold origin: as this mutant is incapable of multi-ubiquitination, mono-ubiquitin and SUMO are the only possible PCNA modifications, both contributing to mutagenesis; here only the SUMO-dependent portion is abolished in the *siz1* background. Consistent

Table 1 **Ubiquitination and SUMO modification of PCNA contribute to spontaneous mutagenesis**

	CAN1 forward assay		*his1-1* reversion assay	
Strain	Mutation rate × 10^7	Relative rate	Mutation rate × 10^9	Relative rate
Wild type	3.1 ± 0.8	1.0	1.4 ± 0.5	1.0
rad18	14.4 ± 4.3	4.6	3.9 ± 1.2	2.8
ubc13	12.9 ± 3.8	4.1	4.5 ± 1.3	3.2
rev3	0.9 ± 0.2	0.3	0.3 ± 0.2*	0.2
pol30(K164R)	3.4 ± 0.9	1.1	1.2 ± 0.4*	0.9
pol30(K164R) rad18	3.3 ± 0.8	1.1	0.6 ± 0.2*	0.4
pol30(K164R) ubc13	2.9 ± 0.7	0.9	0.8 ± 0.3*	0.6
pol30(K127/164R)	1.9 ± 0.5	0.6	0.7 ± 0.2*	0.5
pol30(K127/164R) rad18	2.0 ± 0.5	0.6	0.3 ± 0.1*	0.2
pol30(K127/164R) ubc13	2.0 ± 0.5	0.6	0.7 ± 0.3*	0.5
siz1	3.1 ± 0.8	1.0	1.4 ± 0.5	1.0
rad18 siz1	2.3 ± 0.6	0.7	0.7 ± 0.2*	0.5
ubc13 siz1	5.2 ± 1.4	1.7	2.3 ± 0.7	1.6

Spontaneous mutation rates per generation were determined in the *CAN1* and *his1-1* locus by a modified fluctuation analysis[29]. The *can1* mutants were detected as described in the legend to Fig. 2. Revertants of *his1-1* were detected on histidine-free medium. For this purpose, the *his1-1* ochre allele, which is not subject to suppression by extragenic mutations[30], was introduced by replacement of *HIS1*. Absolute rates were calculated from a total of 11 (*CAN1*) or 22 (*his1-1*) cultures per strain by the method of the median except where indicated. Relative rates are given with respect to the wild type.
*Absolute rates were calculated based on the fraction of cultures without mutants[29].

with the stimulating effect of SUMO on spontaneous mutagenesis, we found a slightly increased rate (1.3-fold) in the *ulp1^{ts}* isopeptidase mutant[26,27], which displays a partial defect in SUMO deconjugation of PCNA at the permissive temperature (see Supplementary Fig. 2).

Our findings lead us to propose a model for the interplay between ubiquitin and SUMO conjugation during DNA replication and repair (Fig. 3): according to ref. 6, PCNA acts as a molecular switch that in its SUMO-modified form may promote replication, whereas multi-ubiquitination stimulates error-free repair. We have now shown that mono-ubiquitination of PCNA is essential for translesion DNA synthesis by Pol η and Pol ζ, resulting in the accumulation of damage-induced mutations. In addition, Pol ζ is also involved in spontaneous mutagenesis during DNA replication. For this purpose Pol ζ can be stimulated by SUMO modification of PCNA; however, in this replicative mode its activity is not inducible by DNA damage, thus explaining the puzzling mutagenesis phenotypes of *rad6* and *rad18* strains. Our model involving differential activation of Pol ζ by ubiquitinated and SUMO-modified PCNA for mutagenesis is consistent with a twofold origin of mutations: according to current beliefs, spontaneous mutations arise not only from replication across unrepaired lesions[22], but also from Pol ζ-dependent extension of terminal mismatches, hairpins or other structural features of template DNA difficult to overcome by purely replicative polymerases[18]. Thus, we postulate that one function of SUMO during normal S phase is to harness Pol ζ to overcome replication fork blocks not caused by damage but by other refractory DNA structures. Activation of the repair polymerases by PCNA could be explained by a preferential interaction with the modified forms of PCNA. However, as unmodified, recombinant PCNA interacts productively with Pol η *in vitro*[17] and we find that even the PCNA lysine mutants are not impaired in their interaction with Pol η (not shown), we consider it more likely that ubiquitin and SUMO may be responsible for the dissociation of other PCNA-binding proteins, such as replicative polymerases[28], to allow access of the translesion polymerases to the primer terminus. Alternatively, the modifications might not affect binding of the polymerases at all, but could exert a more subtle, modulating effect on their activity or processivity. Future studies will have to address the molecular mechanisms that regulate the balance between SUMO modification, mono-ubiquitination and multi-ubiquitination of PCNA and thereby control the accuracy of DNA replication and repair. □

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Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan

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In diverse organisms, calorie restriction slows the pace of ageing and increases maximum lifespan. In the budding yeast *Saccharomyces cerevisiae*, calorie restriction extends lifespan by increasing the activity of Sir2 (ref. 1), a member of the conserved sirtuin family of NAD$^+$-dependent protein deacetylases[2–6]. Included in this family are SIR-2.1, a *Caenorhabditis elegans* enzyme that regulates lifespan[7], and SIRT1, a human deacetylase that promotes cell survival by negatively regulating the p53 tumour