

Mechanistic analysis of PCNA poly-ubiquitylation by the ubiquitin protein ligases Rad18 and Rad5

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Poly-ubiquitylation is a common post-translational modification that can impart various functions to a target protein. Several distinct mechanisms have been reported for the assembly of poly-ubiquitin chains, involving either stepwise transfer of ubiquitin monomers or attachment of a preformed poly-ubiquitin chain and requiring either a single pair of ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3), or alternatively combinations of different E2s and E3s. We have analysed the mechanism of poly-ubiquitylation of the replication clamp PCNA by two cooperating E2–E3 pairs, Rad6–Rad18 and Ubc13–Mms2–Rad5. We find that the two complexes act sequentially and independently in chain initiation and stepwise elongation, respectively. While loading of PCNA onto DNA is essential for recognition by Rad6–Rad18, chain extension by Ubc13–Mms2–Rad5 is only slightly enhanced by loading. Moreover, in contrast to initiation, chain extension is tolerant to variations in the attachment site of the proximal ubiquitin moiety. Our results provide information about a unique conjugation mechanism that appears to be specialised for a regulatable pattern of dual modification.

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Introduction

Like other post-translational modifiers, the small, highly conserved protein ubiquitin mediates its biological functions by reversibly altering the properties of its targets (Ciechanover *et al*, 2000; Hochstrasser, 2000). However, unlike simple modifications such as methylation or acetylation, ubiquitylation has the potential for a widely expanded range of signalling by means of its ability to form polymeric chains (Pickart and Fushman, 2004; Ikeda and Dikic, 2008). Despite the significance of poly-ubiquitylation for cellular regulation and the

abundance of poly-ubiquitylated substrates, surprisingly little is known about the mechanism of ubiquitin chain assembly (Hochstrasser, 2006). Both mono- and poly-ubiquitylation are mediated by a cascade of enzymes, involving activation of ubiquitin as a high-energy thioester intermediate by an activating enzyme (E1), transfer of the ubiquitin thioester to a conjugating enzyme (E2) and attachment of ubiquitin's carboxyl (C)-terminus to a lysine residue in the target protein with the help of a ubiquitin protein ligase (E3) (Kerscher *et al*, 2006). Whereas in the case of mono-ubiquitylation, E3 is generally responsible for selecting one or more lysines directly on the substrate for ubiquitin conjugation, poly-ubiquitylation requires formation of at least two distinct types of linkage, that between the substrate and the proximal ubiquitin and those between the individual ubiquitin moieties within the chain. Mechanistic studies of selected conjugation factors have shown that this task can be accomplished in several distinct ways. As exemplified by the action of the ubiquitin ligase SCF^{Cdc4} with the E2 Cdc34 on the cyclin inhibitor Sic1 (Petroski and Deshaies, 2005) or the human anaphase-promoting complex (APC) with the E2 UbcH10 on securin or cyclin-B1 (Jin *et al*, 2008), a single E2–E3 pair is able to mediate both initiation and elongation of the ubiquitin chain. In contrast, budding-yeast APC or the virally encoded ligase K3 sequentially cooperate with two distinct E2s for mono-ubiquitylation and chain extension (Duncan *et al*, 2006; Rodrigo-Brenni and Morgan, 2007). Whereas in these examples the poly-ubiquitin chain is presumably assembled in a stepwise manner, some E2s, such as the human Ube2g2 or yeast Ubc7, are capable of preforming poly-ubiquitin chains on their active-site cysteine (Li *et al*, 2007; Ravid and Hochstrasser, 2007). Ube2g2 then transfers these en bloc onto a substrate protein. In a variation of this mechanism, the conjugating enzyme E2-25K is able to use unanchored poly-ubiquitin chains activated by E1 (Piotrowski *et al*, 1997).

Modification of the eukaryotic replication clamp PCNA in the context of DNA damage tolerance is as of now the only reported case where separate E2–E3 pairs appear to be responsible for mono- and poly-ubiquitylation of a common substrate (Moldovan *et al*, 2007; Ulrich, 2009). In response to DNA-damaging agents, PCNA is mono-ubiquitylated at a single, conserved lysine, K164, by the E2 Rad6 in complex with the RING-finger E3 Rad18, whereas poly-ubiquitylation at the same site additionally requires the heterodimeric E2 Ubc13–Mms2 and a second RING-finger E3, Rad5 (Hoegge *et al*, 2002). As a consequence, *rad5*, *ubc13* and *mms2* mutants can mono-, but not poly-ubiquitylate, PCNA, whereas in *rad6* and *rad18* mutants, ubiquitylation is completely abolished. The two modifications label PCNA for alternative functions: mono-ubiquitylation activates translesion synthesis through damage-tolerant DNA polymerases

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(Stelter and Ulrich, 2003; Kannouche *et al.*, 2004; Watanabe *et al.*, 2004), and poly-ubiquitylation is required for an error-free pathway of damage avoidance possibly involving a template switch (Hoeye *et al.*, 2002; Zhang and Lawrence, 2005).

Poly-ubiquitin chain assembly by Ubc13–Mms2 has been studied in detail (Hofmann and Pickart, 1999, 2001; McKenna *et al.*, 2001, 2003; Moraes *et al.*, 2001; VanDemark *et al.*, 2001; Eddins *et al.*, 2006; Yin *et al.*, 2009). The Ubc13–Mms2 complex is unusual among E2 enzymes in that it polymerises ubiquitin exclusively through lysine 63 (Hofmann and Pickart, 1999), its specificity dictated by a ubiquitin-binding site within the Mms2 subunit (Moraes *et al.*, 2001; VanDemark *et al.*, 2001; Eddins *et al.*, 2006). Moreover, it is particularly active at catalysing the synthesis of free, unanchored chains (Hofmann and Pickart, 1999, 2001). In contrast, the mechanism of cooperation between the two E2–E3 pairs with PCNA as a substrate has not been addressed. In addition to interacting with their cognate E2s, both Rad18 and Rad5 interact with PCNA, with each other and with themselves (Bailey *et al.*, 1994; Ulrich and Jentsch, 2000; Hoeye *et al.*, 2002). These interrelations suggest several alternative models of how the enzymes may cooperate in PCNA poly-ubiquitylation. The models, shown schematically in Figure 1, differ with respect to the questions of whether the two E2–E3 pairs act sequentially (A, B) or in concert (C, D), and whether the ubiquitin moieties are added in a stepwise manner (A, C) or transferred en bloc to PCNA (B, D). We have now reconstituted the poly-ubiquitylation of budding-yeast PCNA with purified components in order to differentiate between these models. In addition, we have analysed the relevance of DNA

and the ubiquitin attachment site for the process. Our results lend support to model A where Rad6–Rad18 and Ubc13–Mms2–Rad5 act sequentially and mediate PCNA poly-ubiquitylation by stepwise addition of ubiquitin monomers.

Results

In vitro reconstitution of PCNA poly-ubiquitylation

In order to analyse the mechanism of PCNA poly-ubiquitylation, Rad5 and the Rad6–Rad18 complex were purified from *Saccharomyces cerevisiae* strains overexpressing the relevant genes. Bovine ubiquitin and recombinant human E1 were obtained from commercial sources, and PCNA, Ubc13 and Mms2 were produced in *Escherichia coli*. Rad5, Rad18 and Ubc13 were produced with an N-terminal His₆-epitope to aid purification. Previous studies had indicated that budding-yeast Rad18 is active only towards PCNA that is loaded onto DNA (Garg and Burgers, 2005). We, therefore, included a nicked plasmid and recombinant clamp loader, Replication Factor C (RFC), in our reactions. Figure 2A shows efficient poly-ubiquitylation of PCNA in the presence of all components, in accordance with analogous experiments using human enzymes (Unk *et al.*, 2006, 2008). High-molecular-weight species of PCNA were produced under these conditions, indicating assembly of long poly-ubiquitin chains on the loaded clamp. As expected from the behaviour of the respective mutants *in vivo*, Rad6–Rad18 alone produced mono-ubiquitylated PCNA, and omission of Rad6–Rad18 from the reaction prevented both mono- and poly-ubiquitylation. The confinement to mono-ubiquitylated PCNA in reactions containing a K63R mutant of ubiquitin confirmed the

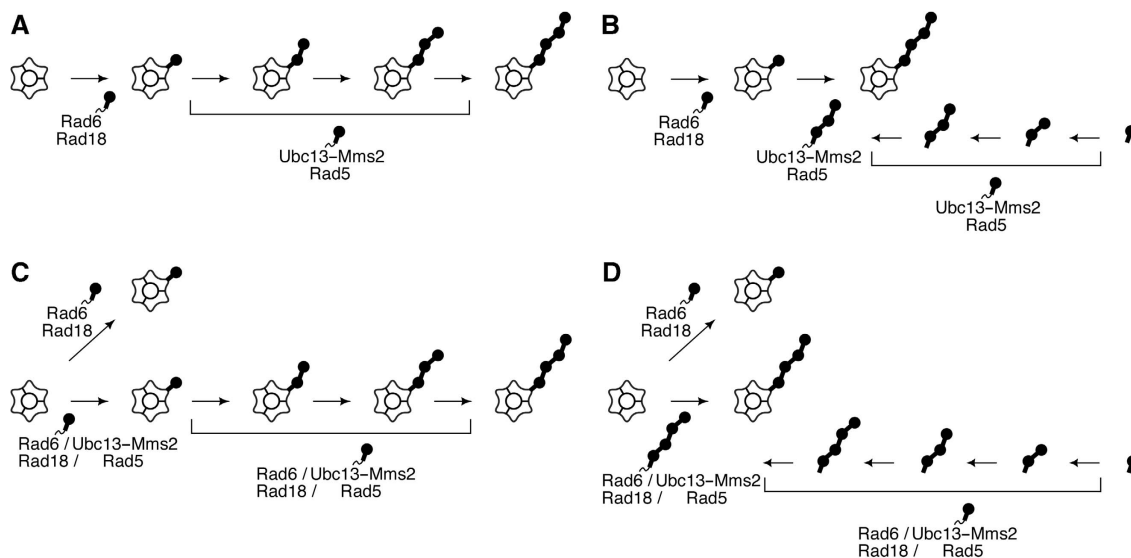


Figure 1 Alternative models for the mechanism of PCNA poly-ubiquitylation by Rad6–Rad18 and Ubc13–Mms2–Rad5. (A) Sequential action, stepwise assembly: Rad6–Rad18 and Ubc13–Mms2–Rad5 act independently and sequentially, each attaching the ubiquitin moieties in a stepwise manner. After conjugation of the first ubiquitin, Rad6–Rad18 is no longer required, and Rad5 recognises the mono-ubiquitylated PCNA as a substrate for chain elongation through K63. (B) Sequential action, preformed chains: Rad6–Rad18 and Ubc13–Mms2–Rad5 act independently and sequentially. A K63-linked poly-ubiquitin chain is assembled by Ubc13–Mms2–Rad5 and transferred en bloc to the mono-ubiquitylated PCNA. The chain may be assembled either free in solution or as a thioester on the active-site cysteine of Ubc13. (C) Separate complexes, stepwise assembly: A dedicated Rad6–Rad18 complex mono-ubiquitylates PCNA. Independently, a complex containing Rad6, Rad18, Ubc13, Mms2 and Rad5 mediates K63-poly-ubiquitylation. Rad6–Rad18 within this complex attaches the first ubiquitin moiety and enhances the contact of PCNA to Ubc13–Mms2–Rad5, which catalyses chain elongation in a stepwise manner. (D) Separate complexes, preformed chains: As in model C, a dedicated Rad6–Rad18 complex mediates PCNA mono-ubiquitylation. A separate Rad6–Rad18–Ubc13–Mms2–Rad5 complex poly-ubiquitylates PCNA by the assembly of a K63-linked poly-ubiquitin chain through Ubc13–Mms2–Rad5, which is then transferred en bloc to PCNA by Rad6–Rad18.

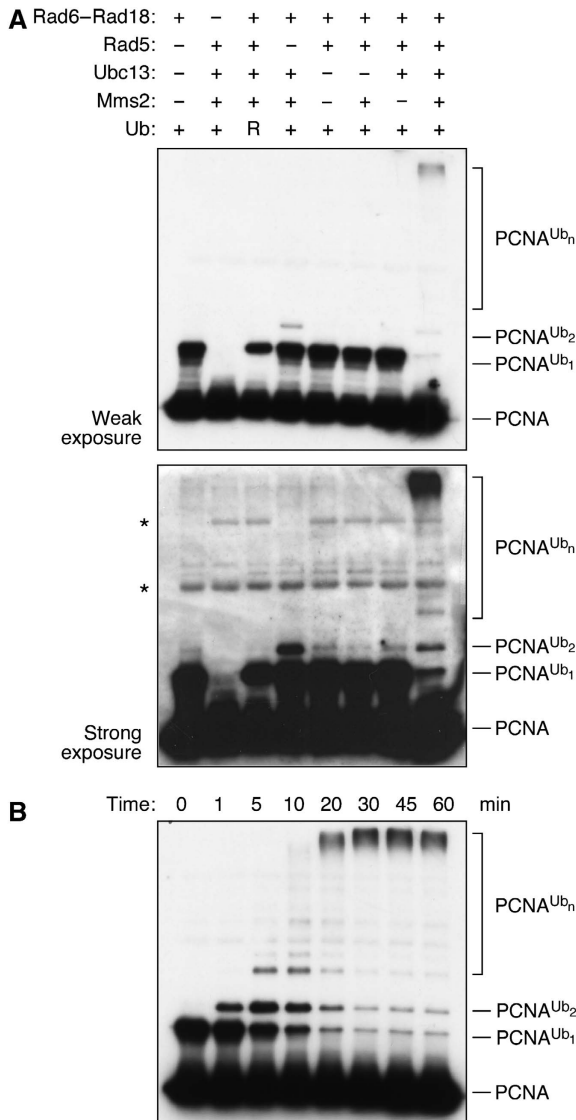


Figure 2 *In vitro* reconstitution of PCNA poly-ubiquitylation. (A) Modification of PCNA by mono- and poly-ubiquitylation using purified enzymes. Reactions were performed in the presence of a nicked plasmid and the clamp loader RFC in order to provide loaded PCNA as a substrate. All reactions contained E1 and ATP. Enzymes for PCNA mono-ubiquitylation (Rad6–Rad18) were added first where indicated and the reaction mixture was incubated for 40 min at 30°C before addition of the poly-ubiquitylation factors (Ubc13–Mms2–Rad5) where indicated and further incubation for 40 min. Replacement of ubiquitin by a K63R mutant is indicated as ‘R’. Products were detected by Western blotting with a PCNA-specific antibody. Asterisks indicate cross-reactive bands visible upon prolonged exposure of the blots. (B) Time course of the poly-ubiquitylation reaction. Reactions were set up with the mono-ubiquitylation enzymes as described above, and Ubc13, Mms2 and Rad5 were added after a 60 min incubation at 30°C. Starting from this point, samples were taken at the indicated times and analysed by Western blotting as above.

linkage specificity of Ubc13–Mms2. Moreover, we observed a low amount of di-ubiquitylated PCNA in the absence of Rad5, indicating that Ubc13–Mms2 was marginally active towards mono-ubiquitylated PCNA even without its cognate E3. Taken together, these results are consistent with *in vivo* data indicating requirement of Rad6–Rad18 for K63 poly-ubiquitylation of PCNA by Ubc13–Mms2–Rad5, but they do not allow

distinction between the different models depicted in Figure 1. In order to assess the kinetics of chain formation on PCNA, we, therefore, followed a time course of poly-ubiquitylation in reactions that had been preincubated with Rad6–Rad18. Figure 2B shows chains of intermediate lengths at early time points that were chased into higher molecular weight species in the course of the reaction. This pattern appears to indicate a stepwise addition of ubiquitin monomers according to models A and C. However, we cannot exclude a combination of stepwise and en-bloc transfer, as the di- and tri-ubiquitylated forms of PCNA may well have been converted to higher forms by the addition of a chain instead of monomers.

Continued presence of Rad18 is not required for poly-ubiquitin chain extension on PCNA

The interaction between Rad18 and Rad5 suggests that they might act as a complex in PCNA poly-ubiquitylation, according to models C and D (Ulrich and Jentsch, 2000). In this case, presence of Rad18 would be required throughout the poly-ubiquitylation reaction, either to enhance the contact between PCNA and Ubc13–Mms2–Rad5 (model C) or for catalytic transfer of an entire poly-ubiquitin chain (model D). Alternatively, Rad6–Rad18 might solely be required to attach the first ubiquitin moiety onto PCNA. In the latter case, the complex would be dispensable for the subsequent action of Ubc13–Mms2–Rad5 (models A and B). In order to distinguish between these possibilities, we used a purified preparation of partially mono-ubiquitylated PCNA in chain extension reactions either containing or lacking Rad6–Rad18. Figure 3A shows that the extent of poly-ubiquitylation as judged by the disappearance of mono-ubiquitylated PCNA and the appearance of higher molecular weight forms was unaffected by the Rad6–Rad18 complex. This was true both in the presence of DNA and RFC, where Rad6–Rad18 is in principle capable of modifying PCNA, and in their absence, where Rad18 would interact with PCNA and Rad5 without being able to modify the clamp (Figure 3A). Titration of the concentration of Rad6–Rad18 in the reaction confirmed that the complex had no effect on the activity of Ubc13–Mms2–Rad5 towards mono-ubiquitylated PCNA (Figure 3B). Intriguingly, the unmodified PCNA in the preparation was not further ubiquitylated by the newly added Rad6–Rad18. We suspect that this may be due to either a failure to modify PCNA within a trimer already bearing one or two ubiquitin moieties, or simply due to the low concentration of unmodified PCNA in the reaction. Along a similar line of observation, Rad6–Rad18 was previously reported to be rather sensitive to the scale of the reaction (Garg and Burgers, 2005). Overall, however, our data suggest that despite their physical interactions, the two E2–E3 pairs, Rad6–Rad18 and Ubc13–Mms2–Rad5, act sequentially and independently of each other, in support of models A and B (Figure 1).

Loading of PCNA onto DNA enhances the efficiency of PCNA poly-ubiquitylation

A second important conclusion from the experiments shown in Figure 3A and B is that chain elongation by Ubc13–Mms2–Rad5—in contrast to Rad6–Rad18-dependent mono-ubiquitylation—does not require PCNA to be loaded onto DNA. Nevertheless, comparison of the modification efficiencies in the presence and absence of DNA and RFC (Figure 3A) indicated that loading of PCNA might stimulate the poly-

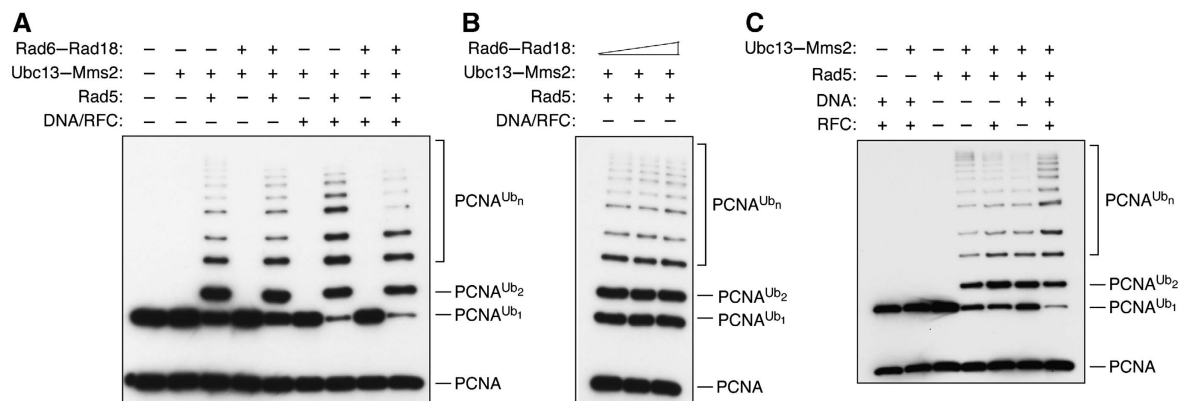


Figure 3 Influence of Rad6-Rad18 and DNA/RFC on PCNA poly-ubiquitylation. (A) Rad6-Rad18 is dispensable for chain extension by Ubc13-Mms2-Rad5. Purified, partially mono-ubiquitylated PCNA was used in reactions containing Rad5, Ubc13 and Mms2 where indicated. All reactions contained E1 and ATP, and Rad6-Rad18 was added where indicated. The assay was performed both in the presence and absence of nicked plasmid DNA and RFC. (B) Titration of the concentration of Rad6-Rad18 in poly-ubiquitylation reactions on purified, partially poly-ubiquitylated PCNA confirms that Rad6-Rad18 is dispensable for chain extension. Rad6-Rad18 concentration was varied from 200 to 800 nM. (C) Loading of PCNA enhances the efficiency of chain extension by Ubc13-Mms2-Rad5. Poly-ubiquitylation reactions on purified, partially mono-ubiquitylated PCNA were performed as described above, but adding DNA and RFC separately as indicated.

ubiquitylation reaction to some extent. Considering that Rad5, like Rad18, is a DNA-binding protein (Johnson *et al.*, 1994), a similar behaviour would not be surprising. In order to verify that the observed stimulation was really due to PCNA loading and not to the mere presence of either DNA or RFC, we repeated the chain extension reactions, adding DNA and RFC separately (Figure 3C). Based on the depletion of the mono-ubiquitylated substrate, we found that stimulation of the reaction required both DNA and RFC, suggesting that mono-ubiquitylated PCNA is a better substrate for Ubc13-Mms2-Rad5 when residing on DNA than free in solution. Surprisingly, while RFC alone had no effect, addition of DNA alone reproducibly resulted in slight inhibition of PCNA poly-ubiquitylation. This effect might be due to sequestering of Rad5 on DNA, which could render modification of soluble PCNA less efficient. Importantly, however, the scaffolding function of DNA that was found indispensable for mono-ubiquitylation by Rad6-Rad18 appears to be beneficial, but not essential, for the activity of Rad5 towards PCNA.

Rad5 tolerates variations in the site of ubiquitin attachment on PCNA

In the absence of PCNA, Rad5 strongly stimulates the synthesis of unanchored poly-ubiquitin chains by Ubc13-Mms2 (Figure 4A). By means of its interaction with PCNA, Rad5 may therefore simply act as an enhancer of E2 activity that mediates proximity of the Ubc13-Mms2 complex to the substrate. Alternatively, Rad5 may be involved in the specific recognition of PCNA mono-ubiquitylated at K164. In order to determine to what extent Rad5 is selective with respect to the site of modification on PCNA, we generated fusions of ubiquitin to the N- or C-terminus of PCNA as artificially 'mono-ubiquitylated' substrates. As shown in Figure 4B and C, both constructs were modified by Ubc13-Mms2-Rad5. This indicates that Rad5 does not require the first ubiquitin moiety to be attached to a specific site on PCNA. However, the two constructs were modified with very different efficiencies. The fusion protein bearing ubiquitin at its N-terminus was virtually depleted in the course of a 30-min reaction, thus exhibiting substrate qualities comparable to or even better

than the physiologically K164-modified PCNA (Figure 4B). In contrast, modification of the C-terminal fusion was rather inefficient (Figure 4C). Although the inefficiency of Ubc13-Mms2-Rad5 towards the C-terminal fusion construct could possibly indicate some preference with respect to the modification site, it is more likely due to the arrangement of the fusion partners: as K63 is spatially adjacent to the N-terminus of ubiquitin, modification at this site could easily be impeded by a partial obstruction by means of the fusion. Time-course and titration experiments with the N-terminal fusion protein again showed a stimulatory effect of PCNA loading (Figure 4D and E). Interestingly, we also observed inhibition of poly-ubiquitylation by concentrations of RFC approaching stoichiometric amounts in the absence of DNA (Figure 4E). It is unclear whether this effect was due to an inability of Ubc13-Mms2-Rad5 to modify PCNA in the ring-opened conformation or whether RFC simply sequestered the clamp away from the modifying enzymes.

Rad18 and Rad5 can catalyse the transfer of preassembled poly-ubiquitin chains onto PCNA

Models B and D (Figure 1) postulate a Ube2g2-like mechanism in which the E2 transfers an entire poly-ubiquitin chain en bloc to a substrate (Li *et al.*, 2007). In the case of Ube2g2, the chain is built as a thioester upon the active-site cysteine of the E2 (Li *et al.*, 2007); however, other E2s such as E2-25K are also known to accept activated preassembled chains from E1 (Piotrowski *et al.*, 1997). In order to establish whether a similar mechanism could apply to mono- and/or poly-ubiquitylation of PCNA, we assessed the ability of the two E2-E3 pairs to accept and transfer ubiquitin dimers and tetramers of different linkages. A prerequisite for this activity is the ability of the E2 to form a thioester with the respective ubiquitin derivatives. Figure 5A shows that Rad6 formed thioesters with wild-type (WT) ubiquitin, a K63R mutant and di-ubiquitin of K48- and K63-linkage with comparable efficiency. Tetra-ubiquitin of K48 linkage was accepted equally well, and only K63-linked tetra-ubiquitin was used less efficiently for thioester formation. The presence of Rad18 did not significantly affect this pattern (Supplementary Figure S1A).

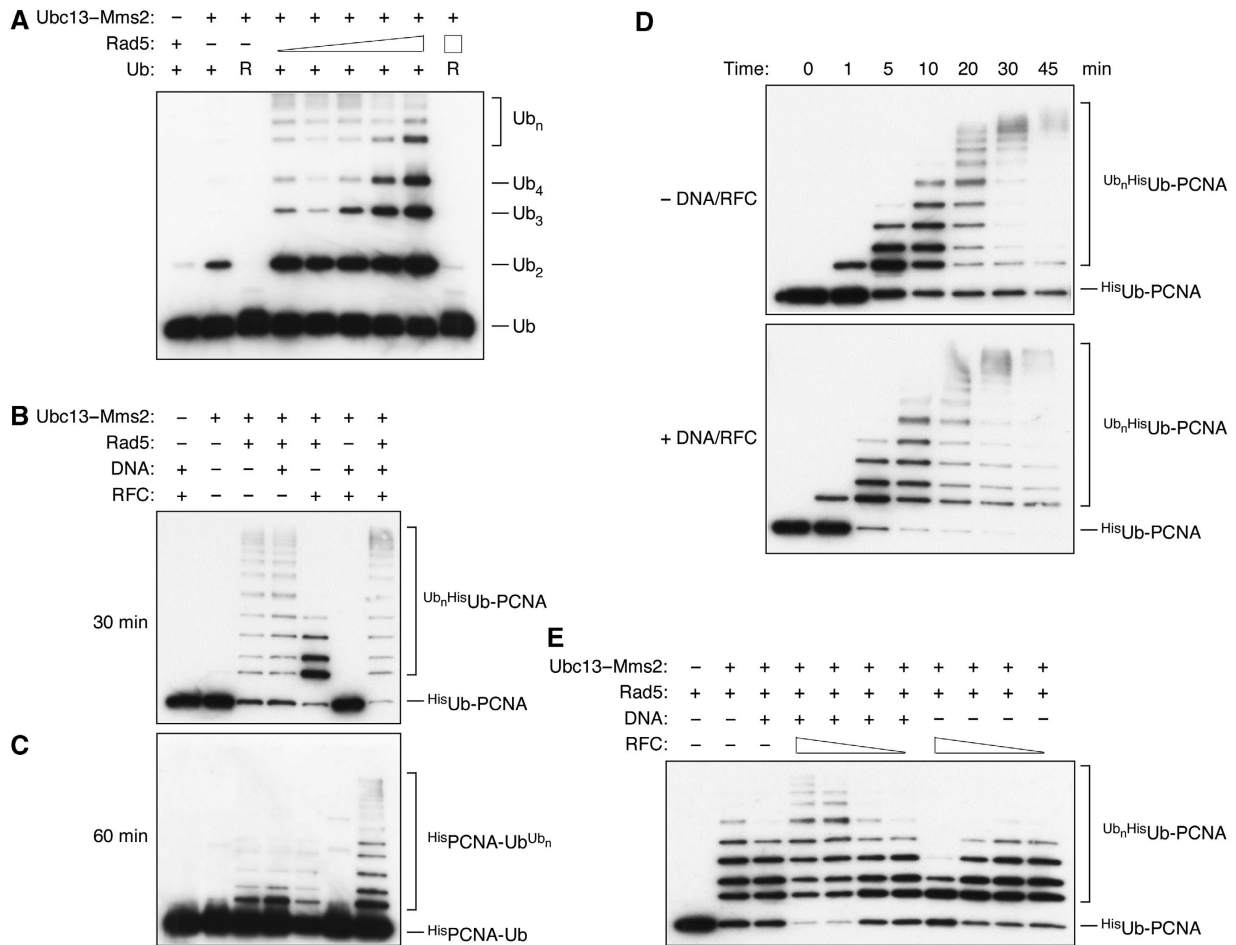


Figure 4 Influence of the ubiquitin attachment site on poly-ubiquitin chain formation. (A) Rad5 stimulates the polymerisation of free ubiquitin by Ubc13 and Mms2. Chain synthesis assays were performed in the absence of PCNA under our standard reaction conditions, using Ubc13–Mms2 at concentrations where chains above di-ubiquitin are formed inefficiently (200 nM). Rad5 concentration was varied from 25 to 200 nM. Replacement of ubiquitin by a K63R mutant is indicated as ‘R’. (B–E) Linear N- and C-terminal fusions of ubiquitin to PCNA can be poly-ubiquitylated by Ubc13–Mms2–Rad5. (B) Chain extension reactions with Rad5, Ubc13 and Mms2 as indicated were carried out for 30 min using the N-terminal ubiquitin fusion, ^{His}Ub–PCNA, as a substrate. DNA and RFC were added for loading of the fusion protein where indicated. (C) The same reactions as in panel B were performed with the C-terminal ubiquitin fusion, ^{His}PCNA–Ub, for 60 min. (D) Time-course experiments were performed with ^{His}Ub–PCNA in order to compare the efficiency of chain elongation in the loaded versus the unloaded state. (E) Titration of RFC in the presence and absence of DNA indicates that loading stimulates poly-ubiquitylation of ^{His}Ub–PCNA, whereas high concentrations of RFC in the absence of DNA inhibit the reaction. RFC concentration was varied from 2.5 to 20 nM.

Modification reactions with Rad6–Rad18 on loaded PCNA with the ubiquitin derivatives indicated that all forms were attached to the substrate with similar efficiency when present as the only source of ubiquitin. The reduced thioester formation with K63-tetra-ubiquitin was not limiting for PCNA modification. However, when an equimolar mixture of mono-, di- and tetra-ubiquitin of K63 linkage was used, di- and particularly mono-ubiquitin were strongly preferred over the tetramer. Likewise, minor contaminations of mono-ubiquitin in the K48-di- and tetra-ubiquitin preparations that had negligible effect in the thioester assay (Figure 5A) gave rise to noticeable quantities of mono-ubiquitylated PCNA (Figure 5B). These observations suggest that despite the ability to use polymeric ubiquitin for thioester formation and transfer, mono-ubiquitin is the preferred moiety for transfer to PCNA by Rad6–Rad18, arguing against model D (Figure 1).

In order to assess the validity of model B, we performed analogous experiments for the chain extension step. The

pattern of thioester formation by Ubc13 was very similar to that obtained with Rad6, in that the efficiency was reduced with K63-tetra-ubiquitin, but comparable for all other derivatives (Figure 5C). Addition of Mms2 and Rad5 did not significantly change this pattern (Supplementary Figure 1B). Chain extension reactions were initially performed with the ^{His}Ub–PCNA fusion, as this was modified by Ubc13–Mms2–Rad5 with similar efficiency as K164-mono-ubiquitylated PCNA. We found that all derivatives were efficiently attached to the substrate (Figure 5D), indicating that preformed chains of varying linkage can be used by Ubc13–Mms2–Rad5 on PCNA for chain extension. As expected, only one moiety of the K63R ubiquitin mutant was attached to ^{His}Ub–PCNA. Based on substrate depletion, K48 chains were less effectively used than K63 chains, and whereas multiple units of K48- and K63-di-ubiquitin were attached, ^{His}Ub–PCNA was modified by no more than a single unit of K48-tetra-ubiquitin. In the case of K63-tetra-ubiquitin, single modification predominated, but higher forms were detectable as well.

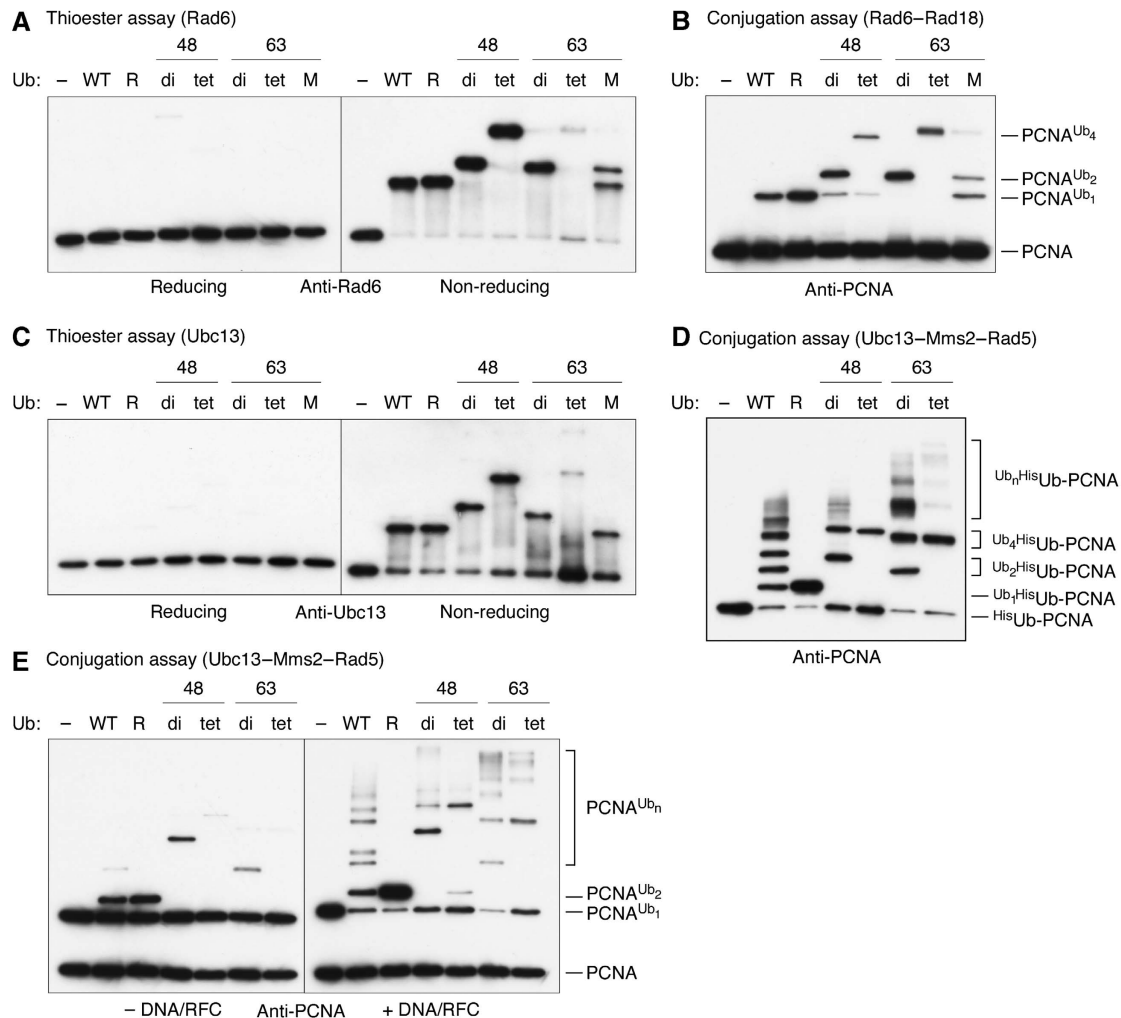


Figure 5 Transfer of preformed ubiquitin chains by Rad6-Rad18 and Ubc13-Mms2-Rad5. Abbreviations for ubiquitin derivatives are as follows: 'R', K63R mutant; 'di' and 'tet', di-ubiquitin and tetra-ubiquitin chains of K48- or K63-linkage as indicated; 'M', an equimolar mixture of mono-, di- and tetra-ubiquitin (K63 linkage). (A) Rad6 is capable of forming thioesters with di- and tetra-ubiquitin. Reactions containing E1, ATP, the indicated ubiquitin derivatives and Rad6 were analysed by Western blotting with a Rad6-specific antibody under reducing and non-reducing conditions in order to assess thioester formation. (B) Rad6-Rad18 can transfer preformed di- or tetra-ubiquitin moieties onto loaded PCNA. Mono-ubiquitylation reactions were performed under standard conditions in the presence of DNA and RFC with the indicated ubiquitin derivatives. (C) Ubc13 is capable of forming thioesters with di- and tetra-ubiquitin. Thioester assays were performed with Ubc13 as in panel A and analysed by Western blotting with a Ubc13-specific antibody. (D) Ubc13-Mms2-Rad5 can attach preformed di- or tetra-ubiquitin to the N-terminal ubiquitin-PCNA fusion, ^{His}Ub-PCNA. Chain extension reactions were performed under standard conditions with the indicated ubiquitin derivatives. (E) Ubc13-Mms2-Rad5 can attach preformed di- or tetra-ubiquitin to PCNA mono-ubiquitylated at K164. Chain extension reactions were performed with purified, partially mono-ubiquitylated PCNA as in panel D, either in the presence or in the absence of DNA and RFC.

Time-course experiments confirmed the slight preference for mono-ubiquitin and for K63- over K48-di-ubiquitin, although the rate of tetra-ubiquitin attachment appeared similar for the two linkages (Supplementary Figure 2). The modification pattern on physiologically K164-mono-ubiquitylated PCNA was very similar and was not influenced by the presence of DNA and RFC, although the overall efficiency of the reaction was enhanced (Figure 5E). These results indicate some linkage specificity with respect to the use of preformed chains by Ubc13-Mms2-Rad5 and a slight preference for attachment of monomers over chains. The notion that K63-tetra-ubiquitin was used by Ubc13-Mms2-Rad5 for chain extension despite the inefficiency of thioester formation indicates that the latter was not rate-limiting in our reactions. Considering that this might be different under physiological conditions, transfer of

longer poly-ubiquitin units en bloc by Ubc13-Mms2-Rad5 is rather unlikely. Our data, thus, provide support for model A (Figure 1), although some use of short chains cannot be excluded, as the enzymes involved are in principle capable of transferring poly-ubiquitin units.

Discussion

Our efforts to reconstitute PCNA modification *in vitro* have given important insights into a mechanism of poly-ubiquitin chain formation that is distinct from previously analysed examples. In contrast to many reported cases where a single E2-E3 pair mediates both attachment of the first ubiquitin moiety to the substrate and extension to a polymeric chain, poly-ubiquitylation of PCNA involves cooperation of two

E2–E3 pairs with distinct properties, that is, Rad6–Rad18 and Ubc13–Mms2–Rad5. Our results indicate that there is a clear separation of tasks between the two complexes.

Mechanism of cooperation between Rad6–Rad18 and Ubc13–Mms2–Rad5

Previous information about the properties of the conjugation factors involved in PCNA modification gave rise to several alternative models of how poly-ubiquitylation might be mediated (Figure 1). On the one hand, physical interactions between the E3s and their cognate E2s suggested the existence of a complex in which both E2–E3 pairs are present (Bailly *et al*, 1994; Ulrich and Jentsch, 2000; Ulrich, 2003). In addition, Rad18 is known to dimerise and form a heterotetramer with Rad6 (Ulrich and Jentsch, 2000; Notenboom *et al*, 2007). These notions suggested that there might be dedicated complexes for mono- versus poly-ubiquitylation of PCNA, consisting of either Rad6–Rad18 alone or of all five components, according to models C and D (Figure 1). On the other hand, both Rad18 and Rad5 interact directly with PCNA and could, thus, in principle recognise their substrate independently (Hoege *et al*, 2002). It was, therefore, important to determine whether Rad6 both Rad18 was required for the chain extension step or whether purified mono-ubiquitylated PCNA could serve as a substrate for modification by Ubc13 both Mms2 both Rad5 alone. Our experiments have now shown that the latter is clearly the case, lending support to models A and B, and effectively ruling out models C and D (Figure 1). Hence, despite their interaction, Rad18 and Rad5 do not follow the strategy of heterodimeric RING E3s such as the BRCA1–BARD or the RING1b–BMI1 complex, where activity depends on dimerisation (Hibbert *et al*, 2009). The notion that Rad18 and Rad5 interact with each other by means of domains distinct from their RING fingers (Ulrich and Jentsch, 2000; Notenboom *et al*, 2007) argues against a BRCA1–BARD1-like mode of operation as well.

Mechanism of ubiquitin transfer

A second basic question about the mechanism of chain assembly concerned the nature of the ubiquitin species transferred by the E2s. We sought to assess whether Rad6 and Ubc13 are capable of transferring ubiquitin chains en bloc to their substrate in a Ube2g2-like manner (Figure 1B and D), or whether they work in a stepwise manner by transferring only monomers (Figure 1A and C). Self-interactions of both Rad18 and Rad5 suggested that they might bring two E2 molecules into close proximity, thereby allowing a ‘see-saw’ action where a chain could be built up on the active-site cysteine of the E2s by addition to the proximal end (Hochstrasser, 2006). Alternatively, free chains might be extended through their distal end and activated by E1, a mechanism supported by the high efficiency with which Ubc13 and Mms2 promote the assembly of unanchored poly-ubiquitin chains (Hofmann and Pickart, 2001; Ulrich, 2003). Without clarifying whether chains are synthesised on the active-site cysteine of the E2 or free in solution, our experiments indicate that although preformed chains can be accepted and transferred by both Rad6–Rad18 and Ubc13–Mms2–Rad5, both complexes—particularly Rad6–Rad18—prefer ubiquitin monomers. In addition, the kinetics of chain assembly indicated the formation of di- and tri-ubiquitylated PCNA as transient intermediates that were depleted in

the course of the reaction, again arguing for a stepwise chain assembly. Considering the independent action of the two E2–E3 pairs, our data, therefore, strongly support the validity of model A (Figure 1A), although they do not completely rule out the use of short ubiquitin oligomers to some extent.

Relevance of substrate recognition by Rad5

Our evidence in support of model A raises the question of how Rad5 recognises mono-ubiquitylated PCNA as a substrate for chain extension independently of Rad18. In contrast to the latter, Rad5 is only mildly responsive to the loading state of the clamp, as the addition of DNA and RFC to the reaction provided only minor enhancements to the efficiency of the Rad5-catalysed step. In addition, Rad5 has previously been shown to interact with (presumably) unmodified PCNA (Hoege *et al*, 2002). Moreover, the site of mono-ubiquitin attachment on the clamp is apparently not relevant for recognition by Rad5, as linear fusions of mono-ubiquitin to the N- or C-terminus of PCNA were both modified by Ubc13–Mms2–Rad5. It is, therefore, likely that Rad5’s task in chain extension is on the one hand a bridging function that brings the Ubc13–Mms2 dimer into close proximity to PCNA, and on the other hand a stimulation of E2 activity, for example by acceleration of either thioester formation or thioester discharge. Additional interaction between Rad5 and Rad18 *in vivo* could then guide the Ubc13–Mms2–Rad5 complex preferentially to loaded PCNA that has been mono-ubiquitylated by Rad6–Rad18 (Davies *et al*, 2008). According to this scenario, Rad5 would not be involved in the recognition of the ubiquitin moiety on mono-ubiquitylated PCNA, but it would be the main determinant that directs the specificity of Ubc13–Mms2 towards PCNA, while the E2 complex, by means of the Mms2 subunit, would be responsible for ubiquitin binding.

Strategies to catalyse poly-ubiquitin chain formation

It is instructive to compare the mechanism of Rad6–Rad18 and Ubc13–Mms2–Rad5 to that of other E2–E3 pairs capable of poly-ubiquitin chain assembly. The notion that enzymes such as the SCF^{Cdc4} complex with the E2–Cdc34 (Petroski and Deshaies, 2005) or human APC with the E2 UbcH10 (Jin *et al*, 2008) manage to catalyse both chain initiation and elongation on a physiological substrate raises the question of why separate complexes are needed for the two steps of PCNA modification. A clue to this problem may come from differences in substrate- versus ubiquitin-recognition: The SCF^{Cdc4} catalyses poly-ubiquitin K48-specific chain assembly in a highly processive reaction that depends on the presence of an acidic loop within Cdc34 and on a hydrophobic patch in ubiquitin adjacent to the K48-acceptor site (Petroski and Deshaies, 2005). In contrast, recognition of the substrate is less efficient, does not require the acidic loop in Cdc34 and is not restricted to a particular lysine. This arrangement facilitates processive synthesis of K48-linked chains on a variety of substrates, but it renders the first step, the initiation of the chain, rate-limiting. Human APC with UbcH10 appears to operate according to a different strategy, as it requires a distinct sequence motif, the TEK box, in both the substrate and ubiquitin for modification (Jin *et al*, 2008). TEK boxes are found on several APC substrates involved in cell-cycle control and were suggested to provide a specific

recognition feature for APC-mediated ubiquitylation. Within ubiquitin, a TEK box surrounds K11, the residue used for chain extension. Chain initiation and elongation, therefore, appear to obey the same criterion, even though additional substrate lysines outside the TEK boxes may also function as acceptor sites.

Finally, there are several examples of E3 enzymes that can use distinct E2s to achieve mono- or poly-ubiquitylation, respectively. Budding-yeast APC, which catalyses the formation of K48-linked poly-ubiquitin chains on its substrates, uses two distinct E2s for chain initiation and elongation, respectively (Rodrigo-Brenni and Morgan, 2007). While Ubc4 mediates rapid, non-selective modification of substrate lysines, Ubc1 specifically mediates chain extension on pre-attached ubiquitin moieties. This activity is supported by the presence of a ubiquitin-binding UBA domain within Ubc1, indicating that Ubc1 specialises in ubiquitin recognition, whereas Ubc4 is responsible for substrate recognition. A similar strategy is followed by the viral ubiquitin ligase K3, which employs members of the Ubc5 E2 family for mono-ubiquitylation of cell-surface MHC class-I molecules and subsequently cooperates with Ubc13–Mms2 in the assembly of a K63-linked poly-ubiquitin chain for downregulation of the receptor by endocytosis (Duncan *et al*, 2006). The BRCA1–BARD1 complex is capable of cooperating with several different E2s in auto-ubiquitylation reactions *in vitro*, the identity of the E2 determining either attachment of mono-ubiquitin or extension to poly-ubiquitin chains of either K48- or K63-linkage (Christensen *et al*, 2007).

PCNA poly-ubiquitylation appears to proceed in a manner most similar to that of yeast APC, K3 and BRCA1–BARD1 in that substrate selection and ubiquitin recognition are achieved by distinct enzymes. Whereas Rad6–Rad18 is highly selective for a single lysine on PCNA, K164 (Hoegge *et al*, 2002; Garg and Burgers, 2005), Ubc13–Mms2, aided by Rad5, exclusively attaches ubiquitin to other ubiquitin moieties. Ubiquitin recognition in this case is mediated by the Mms2 subunit and requires the hydrophobic patch on the ubiquitin surface (McKenna *et al*, 2001). A major difference between E3s like SCF or APC and the enzymes involved in PCNA modification, however, is their range of physiological substrates. Whereas SCF and APC target a variety of proteins for degradation, Rad6–Rad18 has an extremely narrow substrate range. Its exclusive selectivity for a unique site on PCNA may explain why the complex is not only inefficient, but apparently completely unable to attach more than a single ubiquitin moiety to its substrate. It may also be the reason for why Ubc13–Mms2—in contrast to Ubc1—requires a second E3 for stimulation of chain formation. Finally, the notion that mono-ubiquitylated PCNA has a physiological function distinct from that of the poly-ubiquitylated form and may exhibit different regulatory requirements, might also have favoured the evolution of a seemingly complicated two-step modification system.

Poly-ubiquitylation by dedicated conjugation factors is not an isolated phenomenon *in vivo*. Chain elongation factors such as E4 enzymes can redirect the linkage of a poly-ubiquitin chain to one that is suitable for mediating degradation (Koegl *et al*, 1999), and proteasome-associated ubiquitin ligases such as Hul5 can extend the poly-ubiquitin chains on pre-modified substrates, thus apparently enhancing the efficiency of proteolysis (Leggett *et al*, 2002; Crosas *et al*, 2006).

Their mechanistic analysis will most likely reveal an even wider variety of possible mechanisms of ubiquitin chain assembly.

Materials and methods

Proteins

Recombinant budding-yeast PCNA (untagged), His₆Ubc13, Mms2 and RFC were produced as previously described (Ulrich, 2003; Franco *et al*, 2005; Parker *et al*, 2008). All of them were subjected to a final gel-filtration step for purification. Human His₆Uba1 (E1) was purchased from BioMol and ubiquitin and its derivatives were from Boston Biochem. The Rad6–Rad18 complex was produced by overexpression of His₆RAD18 from the vector pYES2 (Invitrogen) in the protease-deficient *S. cerevisiae* strain BJ5460 (ATCC no. 208285), which additionally carried an overexpression construct for RAD6 under control of the ADH1 promoter. Expression of His₆RAD18 was induced in cultures growing exponentially in uracil-free medium containing 0.1% glucose through the addition of galactose to 2% and further incubation for 20 h. The pellet from an 8-l culture was resuspended in buffer A (50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 10% glycerol) containing 15 mM imidazole and Complete protease inhibitors (Roche) and lysed by high-pressure cell disruption. All steps were carried out at 4°C. The lysate was cleared by centrifugation at 40 000 g for 20 min and then at 150 000 g for 45 min. The cleared lysate was subjected to Ni–NTA agarose affinity purification. Bound protein was eluted in buffer B (40 mM HEPES (pH 7.4), 10% glycerol) containing 200 mM imidazole and 200 mM NaCl. Fractions containing Rad6–Rad18 were pooled and applied to a 5 ml Hi Trap Heparin column (GE Bioscience) equilibrated in buffer B + 200 mM NaCl. Proteins were eluted with a gradient from 200 to 500 mM NaCl. Following MonoQ chromatography (1 ml column), fractions containing Rad6–Rad18 were pooled and subjected to filtration a Superdex 200 gel-filtration column equilibrated in buffer B + 150 mM NaCl. The purified protein was stored at –80°C. Rad5, bearing an N-terminal His₆ epitope, was purified from BJ5460 essentially like Rad6–Rad18, but the MonoQ column chromatography step was omitted. N- and C-terminal fusions of ubiquitin to PCNA (His₆Ub–PCNA and His₆PCNA–Ub) were constructed as described previously (Parker *et al*, 2007), but using WT ubiquitin. K127 and K164 of PCNA were mutated to arginine. G76 of ubiquitin was mutated to valine in the N-terminal fusion, and the two C-terminal glycines were deleted in the C-terminal fusion. Proteins were produced in *E. coli* and purified by Ni–NTA agarose chromatography and subsequent gel filtration into a buffer containing 40 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol and 1 mM DTT. Rad6 used for thioester assays was produced and purified from *E. coli* using an intein fusion system based on the vector pTYB12 (New England Biolabs) according to the manufacturer's instructions.

PCNA mono-ubiquitylation reactions

In vitro ubiquitylation assays were performed essentially as described previously (Garg and Burgers, 2005). A 10- μ l standard assay contained 40 mM HEPES (pH 7.4), 50 mM NaCl, 8 mM magnesium acetate, 1 mM ATP, 30 fmol of nicked pBluescript plasmid DNA, 0.5 pmol of PCNA trimer, 0.2 pmol of RFC, 2 pmol of Rad6Rad18, 0.5 pmol of His₆Uba1 (E1) and 10 pmol of ubiquitin. Reactions were incubated at 30°C for 60 min unless stated otherwise, stopped through addition of SDS loading buffer and denatured at 95°C for 3 min. Products were analysed by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting with a polyclonal PCNA-specific antibody.

Purification of mono-ubiquitylated PCNA

PCNA was ubiquitylated in a 2-ml reaction as described above, but using human His₆-tagged ubiquitin. Benzoylase (Novagen) was added for 15 min to degrade the DNA. The reaction was diluted 1:1 in buffer A (40 mM HEPES (pH 7.4), 10% glycerol) and passed through a 1 ml HiTrap Heparin column equilibrated in buffer A + 100 mM NaCl. Imidazole was added to the eluate to a final concentration of 30 mM, and the preparation was subjected to Ni–NTA agarose purification. Free ubiquitin was removed by binding the modified PCNA to a 1 ml HiTrap Q column and washing extensively with buffer A + 200 mM NaCl. Modified PCNA was

eluted with a NaCl gradient (0.2–1 M) and fractions were pooled, dialysed against buffer A + 100 mM NaCl and stored at -80°C .

Chain extension assays

Poly-ubiquitylation of unmodified PCNA was carried out essentially like the mono-ubiquitylation reactions, but 2 pmol each of Rad5, Ubc13 and Mms2 were added after a 40-min incubation with Rad6–Rad18. The reactions mixtures were incubated for a further 40 min. For chain extension assays with purified mono-ubiquitylated PCNA or the ubiquitin-PCNA fusions, the reaction mixtures were incubated at 30°C for 60 min (30 min for $^{\text{His}}\text{Ub-PCNA}$).

Free ubiquitin chain synthesis

Ubiquitin chain synthesis was analysed in 10- μl reactions in 40 mM HEPES (pH 7.4), 50 mM NaCl, 8 mM magnesium acetate, 1 mM ATP, 0.5 pmol of E1 and 100 pmol of ubiquitin. 2 pmol each of Ubc13 and Mms2 were added and 0.25–2 pmol of Rad5. Reactions were incubated at 30°C for 60 min, stopped through addition of SDS loading buffer and denatured at 95°C for 3 min. Products were analysed by SDS–PAGE and Western blots using a monoclonal ubiquitin-specific antibody.

Thioester assays with Rad6 and Ubc13

Standard reactions of 20 μl were performed in 40 mM HEPES (pH 7.4), 50 mM NaCl, 8 mM magnesium acetate, 1 mM ATP, containing

2 pmol of $^{\text{His}}\text{Uba1}$ and 20 pmol of ubiquitin or ubiquitin chains. The relevant E2 (4 pmol) was added and reactions were incubated at 30°C for 20 min. Aliquots of 9 μl each were added to 9 μl of HU loading buffer without a reducing agent (8 M urea, 200 mM Tris–HCl (pH 6.8), 1 mM EDTA, 5% SDS, 0.1% bromophenol blue) or 9 μl of $2\times$ SDS loading buffer containing 200 mM dithiothreitol as reducing agent. Reduced and non-reduced samples were incubated at 95°C for 5 min and at 50°C for 15 min, respectively. Samples were analysed by 12% SDS–PAGE and Western blotting with polyclonal antibodies specific to Rad6 or Ubc13.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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