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Effects of chain length and geometry on the activation of DNA damage bypass by polyubiquitylated PCNA

Diane T. Takahashi [1], Hans-Peter Wollscheid[2], Jonathan Lowther[3] and Helle D. Ulrich [2,*]

[1]Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, Université Paris-Saclay, 91198, Gif-sur-Yvette cedex, France, [2]Institute of Molecular Biology gGmbH (IMB), Ackermannweg 4, D–55128 Mainz, Germany and [3]Immunocore Ltd, 101 Park Dr, Milton, Abingdon OX14 4RY, UK

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ABSTRACT

Ubiquitylation of the eukaryotic sliding clamp, PCNA, activates a pathway of DNA damage bypass that facilitates the replication of damaged DNA. In its monoubiquitylated form, PCNA recruits a set of damage-tolerant DNA polymerases for translesion synthesis. Alternatively, modification by K63-linked polyubiquitylation triggers a recombinogenic process involving template switching. Despite the identification of proteins interacting preferentially with polyubiquitylated PCNA, the molecular function of the chain and the relevance of its K63-linkage are poorly understood. Using genetically engineered mimics of polyubiquitylated PCNA, we have now examined the properties of the ubiquitin chain required for damage bypass in budding yeast. By varying key parameters such as the geometry of the junction, cleavability and capacity for branching, we demonstrate that either the structure of the ubiquitin-ubiquitin junction or its dynamic assembly or disassembly at the site of action exert a critical impact on damage bypass, even though known effectors of polyubiquitylated PCNA are not strictly linkage-selective. Moreover, we found that a single K63-junction supports substantial template switching activity, irrespective of its attachment site on PCNA. Our findings provide insight into the interrelationship between the two branches of damage bypass and suggest the existence of a yet unidentified, highly linkage-selective receptor of polyubiquitylated PCNA.

INTRODUCTION

Ubiquitylation as an important posttranslational protein modification impinges on many cellular pathways in eukaryotes. Conjugation of ubiquitin generally involves the attachment of ubiquitin's carboxy (C)-terminus to an amino group within the substrate. Repeated conjugation to ubiquitin itself thus results in the formation of a polyubiquitin chain. As ubiquitin contains seven lysines in addition to its amino (N)-terminus, all of which can serve as ubiquitin acceptors, ubiquitin chains can adopt structurally distinct linkages depending on the residue that is used for polymerization (1,2). Both mono- and polyubiquitylation alter the properties of the modified proteins, predominantly via interactions with so-called ubiquitin receptors that harbour ubiquitin-binding domains (UBDs) and mediate the biological effects of the modification (3). Consequences of ubiquitylation are manifold. While monoubiquitylation has been implicated in the regulation of endocytosis, nuclear import and export as well as chromatin structure (4), polyubiquitylation is best known for its contribution to proteasomal degradation (2,5). However, it also plays important roles in non-proteolytic pathways. As many ubiquitin receptors are able to discriminate between polyubiquitin chains of different geometries, the linkage of the ubiquitin chain is thought to determine the fate of the modified substrate (1). Thus, while chains linked via lysine (K) 48 of ubiquitin act as efficient proteasome targeting signals, K63-linked polyubiquitin chains modulate such diverse processes as endocytosis, inflammatory signalling and various aspects of the DNA damage response (6–8).

One of the most prominent examples of how both mono- and polyubiquitylation promote the resistance to genotoxic insults is the pathway of DNA damage bypass, also called DNA damage tolerance. This pathway regulates the replication of damaged templates and ensures complete genome duplication in the presence of lesions (9,10). Exposure of replicating cells to DNA damage or replication stress results in the modification of the replication clamp, PCNA, at a conserved lysine, K164, by a set of dedicated ubiquitin conjugation factors (11). Monoubiquitylation of PCNA by the ubiquitin-conjugating enzyme (E2), Rad6, in cooperation with the ubiquitin protein ligase (E3), Rad18, promotes the activation of a set of damage-tolerant DNA polymerases for a mutagenic pathway named translesion synthesis (TLS) (12–14). Although some ubiquitin-independent activity of TLS polymerases has been reported, these enzymes are

*To whom correspondence should be addressed. Tel: +49 6131 3921490; Fax: +49 6131 3921499; Email: h.ulrich@imb-mainz.de

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thought to gain preferential affinity for the ubiquitylated form of PCNA through one or more UBDs, which allows their recruitment to stalled replication intermediates (15–17). Alternatively, monoubiquitylated PCNA can be further modified by the heterodimeric E2, Ubc13-Mms2, with its cognate E3, Rad5 (in *Saccharomyces cerevisiae*) or SHPRH and HLTF (in humans), resulting in a uniform K63-polyubiquitin chain on K164 of PCNA (11,18–19). This modification activates a largely error-free pathway called template switching (TS) that involves transient use of the undamaged sister chromatid as a replication template in a recombination-like reaction.

Compared to the TLS pathway, the mechanism by which polyubiquitylation of PCNA activates TS and the significance of the K63-linkage for this process are poorly understood. Although there are cases where K63-linked polyubiquitin chains have been implicated in proteasomal degradation (20,21), a contribution of the proteasome to TS activation was ruled out by our previous work (22). A number of factors interacting preferentially with polyubiquitylated PCNA have been reported, such as yeast Mgs1 or its human homolog WRNIP1 (23,24) and human ZRANB3 (25–27). However, their relevance as key mediators of TS is still debated. Deletion of *MGS1* does not cause any DNA damage sensitivity (28,29), and in light of the overall conservation of the factors involved in DNA damage bypass, the absence of a convincing homologue of ZRANB3 in fungi suggests an alternative, conserved mechanism of TS activation. Based on interaction studies of TLS polymerases with polyubiquitin chains, it has been speculated that the K63-chain on PCNA might promote TS by suppressing TLS, possibly by directing the TLS polymerases away from the stalled primer terminus (17,30). In contrast, Coulon *et al.* (31) have postulated a positive contribution of polyubiquitylated PCNA to TLS in *Schizosaccharomyces pombe*. Further complication arises from the multifunctional nature of Rad5 and its homologues. This E3 not only mediates PCNA polyubiquitylation, but also harbours a DNA-dependent adenosine triphosphatase (ATPase) activity that contributes to protection from replication stress (32). However, the latter activity is separable and independent of Rad5′s involvement in ubiquitylation-dependent TS (33–35). Moreover, physical interactions with TLS polymerases have implicated Rad5 in mutagenesis (36–38), indicating a structural role of the protein in TLS, again independent of its function in K63-polyubiquitylation.

In-frame fusions of ubiquitin to potential substrates as mimics of monoubiquitylation have given valuable information about TLS activation. Although in one case a TLS-independent effect of a non-cleavable Ub-PCNA fusion was reported (39), studies in *S. cerevisiae*, *S. pombe* and human cells agree that a monoubiquitin moiety, irrespective of its attachment site, confers TLS activity by recruiting damage-tolerant DNA polymerases (40–43). The use of ubiquitin fusions to investigate polyubiquitylation is less straightforward, due to the conformational diversity of chain linkage. Structural analysis suggests that a linear head-to-tail arrangement closely resembles the K63-linkage (44). Indeed, in the context of membrane protein sorting, where K63-polyubiquitin chains feature prominently, even the non-covalent association of a head-to-tail ubiquitin trimer triggered uptake of a membrane protein into the vacuole (45). In the context of damage bypass, however, fusion of a linear, non-cleavable polyubiquitin chain to the N- or C-terminus of PCNA failed to rescue the TS defect of a *rad18* deletion mutant (22).

We have now explored features of the polyubiquitin chain that either promote TS in a native K63-linked chain or prevent activity of the linear mimics. Using variations of parameters such as linker structure, cleavability and capacity for branching, we have examined a panel of polyubiquitin chain mimics for their ability to restore DNA damage bypass activity in a *rad18Δ* background. We provide evidence that the structure of the ubiquitin-ubiquitin junction may be critical for TS, even though known interactors of polyubiquitylated PCNA do not discriminate between related arrangements. Alternatively, the dynamic assembly or disassembly of a chain at the site of its action may determine its functionality in TS. While our results are consistent with a minor negative effect of the polyubiquitin chain on the activity of TLS polymerases, its main impact was independent of TLS. Finally, we report that a single native K63-junction within the ubiquitin-PCNA conjugate confers significant TS activity, indicating that the total number of ubiquitin moieties in the chain is less important for biological function in DNA damage bypass than their connectivity. Our findings therefore suggest that a yet unidentified receptor of polyubiquitylated PCNA harbouring a highly linkage-selective UBD might be responsible for activating the TS pathway.

MATERIALS AND METHODS

Plasmids and yeast strains

Unless otherwise noted, yeast strains are derived from the haploid strain DF5 (46) and are listed in Supplementary Table S1. All plasmids, including those for expression of recombinant proteins, are listed in Supplementary Table S2. Yeast strains were cultured at 30°C unless otherwise noted, either in YPD or in synthetic complete medium supplemented with the relevant amino acids. Gene deletions were accomplished via transformation of polymerase chain reaction-based cassettes. Ub*-PCNA* constructs were introduced into the relevant strains via integration into the *LEU2* or *URA3* locus. If needed, deletion of the *URA3* marker from *hisG-URA3*-containing strains was achieved by selection on 5-fluoro-orotic acid. For two-hybrid assays, fusions with the *GAL4* activation and DNA-binding domains were generated in pGAD424 and pGBT9 (Clontech).

DNA damage sensitivity and mutagenesis assays

Quantitative survival assays were performed by plating a defined number of cells from exponential cultures onto YPD medium in triplicate, followed by ultraviolet (UV) irradiation (254 nm) with the indicated doses. Colonies were counted after incubating the plates at 30°C for 3 days. Averages and standard deviations were generated from at least three biological replicates. For comparative spot assays, samples from exponential cultures at equal densities were spotted in 5-fold serial dilutions onto freshly prepared YPD plates. These were then exposed to the indicated doses

of UV radiation, and images were scanned after incubation at 30°C for 2 days.

Mutation rates were determined in the *CAN1* gene. For each strain, 11 independent cultures were inoculated with about 10^3 cells in 2 ml of YPD each and grown at 30°C for 3 days. Cell density was measured by plating dilutions on YPD agar plates and colony counting after 2 days at 30°C. Numbers of canavanine-resistant mutants (Can^R) were determined by plating on selective YNBD medium containing 60 mg/l L-canavanine (47). Colonies were counted after 3–4 days at 30°C. All experiments were repeated independently at least three times. Mutation rates and standard deviations were calculated from the number of Can^R colonies by the method of the median (48).

Detection of proteins

A home-made, affinity-purified rabbit polyclonal antibody was used for detection of PCNA in western blots (34). Detection of ubiquitin was achieved with mouse monoclonal antibody P4D1 (Cell Signaling Technology). Fluorescently labelled ubiquitin constructs were detected in-gel on an Odyssey CLx system (LI-COR). Biotinylated ubiquitin variants were transferred to nitrocellulose membrane, probed with Streptavidin IRDye® 800CW (Licor Biosciences) and analysed on an Odyssey CLx system.

Detection of PCNA modifications in yeast

Linear fusions of ubiquitin and PCNA were detected in total cell lysates by western blotting with PCNA-specific antibodies. For detection of native K63-polyubiquitylation of PCNA, the relevant PCNA construct was expressed as an N-terminally His_6-tagged allele. In order to induce replication stress, exponentially growing cultures of the relevant strains were treated with 0.02% methyl methanesulfonate (MMS) for 90 min. Total cell extracts were prepared as described (49). ^{His}PCNA was then isolated by Ni-NTA affinity purification under completely denaturing conditions as described previously (11,50), and ubiquitylated forms were detected by western blotting against ubiquitin.

Protein production, purification and labelling

Recombinant proteins were produced in *Escherichia coli*. Murine ^{His}Uba1 (E1) and budding yeast ^{His}Ubc13 and Mms2 were purified as described (19). *In vivo* biotin-labelled proteins (see below) were purified by Ni-NTA affinity chromatography. The UBZ domain from Mgs1, the NZF domain from ZRANB3 and E2-25K were purified as GST fusion proteins via glutathione Sepharose affinity chromatography. Untagged Ub^{G76C} and linear diubiquitin constructs bearing a G76C mutation were produced as GST fusions and purified as above, followed by cleavage of the GST moiety with PreScission Protease (GE Healthcare) at 4°C overnight. After renewed passage over glutathione Sepharose, they were subjected to size exclusion chromatography in labelling buffer (50 mM HEPES pH 7.5, 100 mM NaCl and 1 mM TCEP) on a Superdex 75 10/300 GL column. Untagged Ub^{K48R} and Ub^{K63R} were purified by acid precipitation (51).

K48- and K63-linked ubiquitin dimers were prepared from Ub^{G76C} in combination with Ub^{K48R} or Ub^{K63R} at a 1:1 ratio (50 μM each), using ^{GST}E2-25K and Ubc13-Mms2 as E2s, respectively. Reactions were performed in a buffer containing 40 mM HEPES pH 7.4, 8 mM magnesium acetate, 50 mM NaCl, 100 μM ATP and 0.1 μM E1. Ubc13-Mms2 were used at 0.2 μM, E2-25K at 2 μM. Following overnight incubation at 30°C, reactions were diluted in 50 mM ammonium acetate pH 4.5, and dimers were purified by cation exchange chromatography (Resource S, GE Healthcare).

Ub^{G76C}, linear di-Ub^{G76C} and the K48- or K63-linked ubiquitin dimers containing Ub^{G76C} at the proximal position (see above) were labelled with the thiol-reactive probe Alexa Fluor 647 C2 Maleimide (Life Technologies) in a buffer containing 50 mM HEPES pH 7.5, 100 mM NaCl and 1 mM TCEP, following the manufacturer's instructions. Unconjugated dye was removed by gel filtration through PD-10 columns (GE Healthcare). Linear ubiquitin dimers (M1, M1-G76V and M1-GSGS) were cloned in fusion with a C-terminal Avi-His_6-tag (Avidity) and co-expressed in *E. coli* with the biotin ligase BirA (52) by induction with 50 mM IPTG in the presence of 50 μM biotin (Sigma-Aldrich) for *in vivo* labelling.

Protein–protein interaction assays

Interactions between UBDs and linear ubiquitin-PCNA fusion constructs were analysed in the two-hybrid system using strain PJ69-4A as described previously (24). For *in vitro* interaction assays, GST fusion proteins (2 μM coupled to 10 μl glutathione Sepharose) were incubated with 2 μM of relevant ubiquitin dimer for 3 h at 4°C in a buffer containing 50 mM HEPES pH 7.5, 50 mM NaCl, 1.5 mM $MgCl_2$, 5 mM EGTA, 5% glycerol and 1% Triton X-100 in a total reaction volume of 150 μl. After three washes with the same buffer, proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and bound proteins were detected in the gel or after transfer to nitrocellulose membrane, according to their labelling (see above). The bound fraction was quantified by averaging the relative signals from three replicate experiments.

RESULTS

A K63-polyubiquitin chain can be assembled and promotes TS at a non-native position on PCNA

In an attempt to create mimics of polyubiquitylated PCNA for *in vivo* functional analysis, we had previously constructed a series of head-to-tail fusions of ubiquitin to PCNA (22). Branching or further modification was prevented by mutation of relevant lysine residues in both the ubiquitin (Ub^*: K29,48,63R) and the PCNA moiety ($PCNA^*$: K127,164R). In addition, the constructs were rendered resistant to DUB cleavage by mutation of ubiquitin's C-terminal glycine (G76V). Considering the structural similarity of the K63- to the M1-linkage (44), we expected that these constructs might promote TS activity *in vivo*. However, while fusion of a single ubiquitin moiety to PCNA (Ub^*-$PCNA^*$) was sufficient to restore TLS, head-to-tail polyubiquitin mimics fused to PCNA (Ub^*_4-$PCNA^*$ or

PCNA*-Ub*$_4$) failed to complement the TS defect of mutants deficient in PCNA ubiquitylation (22).

Failure to promote TS could be due to the non-native attachment site of the chain on PCNA (M1 rather than K164). We addressed this possibility using a fusion of a monoubiquitin unit to PCNA, Ub^{K63*}-PCNA*, where K63 of ubiquitin was available for chain extension (Figure 1A). When this construct was expressed in a *rad18Δ* mutant, it afforded resistance to UV irradiation beyond Ub*-PCNA* in a manner that was independent of the presence of the three TLS polymerases, Rev1, Rev3 and Rad30 (Figure 1B and C) (22). This suggested an activation of error-free TS via bypass of the PCNA monoubiquitylation reaction. Consistent with this model, we found that the rescue of UV resistance required K63-polyubiquitylation, as the effect was abolished by deletion of *UBC13* (Figure 1D). A rescue by Ub^{K63*}-PCNA* was also observable in a non-modifiable PCNA mutant background, *pol30*$^{K127/164R}$ (Supplementary Figure S1). Further evidence that the rescue resulted from genuine TS activity came from a suppression of the *rad18Δ* hypermutation phenotype by Ub^{K63*}-PCNA*, which indicated that cells regained a wild-type (*WT*)-like balance between the mutagenic TLS and the error-free TS pathway (Figure 1E). Finally, we examined whether the N-terminal ubiquitin moiety of the Ub^{K63*}-PCNA* construct was used for the extension of a K63-polyubiquitin chain *in vivo*. As predicted based on the genetic data, we observed a damage-induced polyubiquitylation of the fusion protein *in vivo* that depended on the presence of K63 in the ubiquitin moiety (Figure 1F). Thus, a K63-chain can be assembled and is functional at a non-native position. The moderate UV sensitivity of *rad18Δ* and *pol30*$^{K127/164R}$ mutants harbouring Ub^{K63*}-PCNA* compared to *WT* cells (Figure 1B and Supplementary Figure S1) indicated that the fusion construct cannot fully complement a defect in PCNA modification at K164. This may likely be a consequence of a sub-optimal positioning of the chain.

Taken together, these results indicate that the attachment site of a polyubiquitin chain on PCNA affects the efficiency of TS to a minor extent, but is not critical for activity, similar to the effect of the monoubiquitylation site for TLS (39–43). This relative flexibility with respect to the site of modification on PCNA is surprising because K164 is one of the most highly conserved ubiquitylation sites, which would normally implicate a functional relevance of this position. It suggests that any potential downstream effector would likely recognize ubiquitin and PCNA in a modular fashion, as postulated for the TLS polymerases (40), rather than via bipartite recognition motifs observed for other ubiquitin-dependent DNA damage signalling proteins (53). Importantly, the data also clearly demonstrate that K63-polyubiquitylation of PCNA can trigger TS independently of TLS polymerases, arguing against models that view the polyubiquitin chain predominantly as a—positive or negative—regulator of the TLS polymerases (17,30–31).

Polyubiquitin chain structure impinges on TS and TLS

In our previous study (22) we had constructed two versions of linear chain mimics, either by fusing the ubiquitin moieties seamlessly in a head-to-tail arrangement or by in-

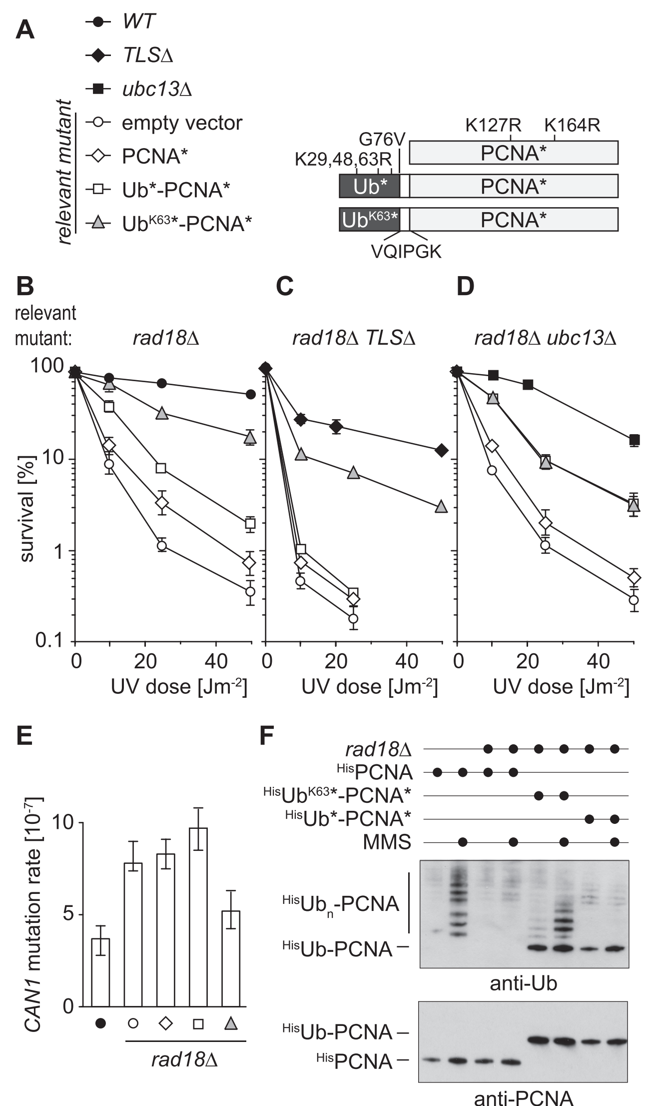

Figure 1. K63-polyubiquitin chains can be assembled and promote TS at a non-native position on PCNA. (**A**) Schematic view of the constructs. Peptide linkers (VQIPGK) and relevant mutations in the ubiquitin and PCNA moieties are indicated (Ub*: K29,48,63R, G76V; PCNA*: K127,164R). (**B**) Expression of Ub^{K63*}-PCNA* in *rad18Δ* provides enhanced UV resistance compared to expression of Ub*-PCNA*. Survival was quantified by colony formation after UV irradiation with defined doses on plates. Symbols correspond to those in panel A. Error bars indicate standard deviations. (**C**) Ub*-PCNA*-mediated UV resistance is abolished by deletion of the genes encoding TLS polymerases (*TLSΔ*: *rev1Δ rev3Δ rad30Δ*), whereas Ub^{K63*}-PCNA* is active in a TLS-independent manner. UV sensitivities were determined as described in panel B. (**D**) The increased UV resistance conveyed by Ub^{K63*}-PCNA* compared to Ub*-PCNA* depends on *UBC13*. UV sensitivities were determined as described in panel B. (**E**) The ability to undergo polyubiquitylation at the N-terminus of PCNA in Ub^{K63*}-PCNA* reduces the spontaneous mutation rate of *rad18Δ* cells. Symbols correspond to those in panel A. Error bars indicate standard deviations. (**F**) A polyubiquitin chain is formed on Ub^{K63*}-PCNA* in a K63- and DNA damage-dependent fashion. Western blots were prepared from material isolated via denaturing Ni-NTA pull-down from *rad18Δ* cells expressing His$_6$-tagged versions of the indicated constructs and exposed to 0.02% MMS for 90 min where indicated.

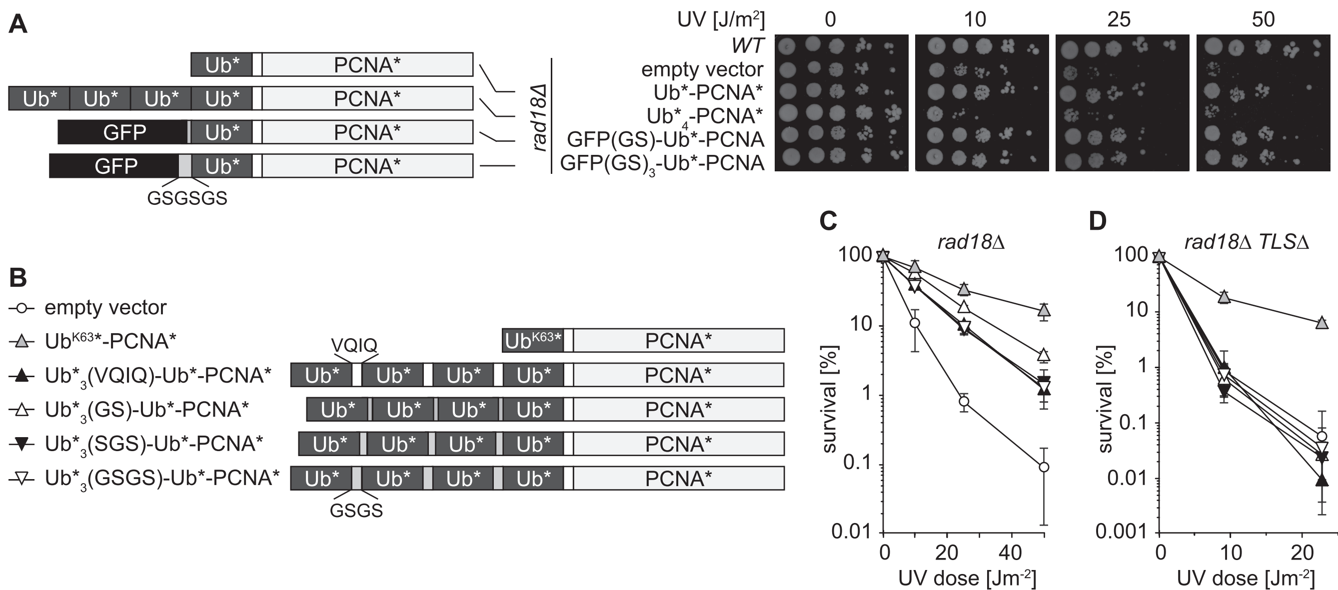

Figure 2. Polyubiquitin chain structure impinges on TS and TLS. (**A**) Fusion of GFP to the N-terminus of Ub*-PCNA* does not affect the ability of the construct to support TLS. Left: schematic view of the constructs, labelled as in Figure 1A; right: UV sensitivity assays of *rad18Δ* cells harbouring the indicated constructs, determined by monitoring growth on solid medium after exposure to the indicated doses of UV radiation. (**B**) Schematic view of Ub_4*-PCNA* constructs used in panels C and D, harbouring variations of the linker between the ubiquitin moieties. (**C**) Variation of the ubiquitin-ubiquitin junction in Ub_4*-PCNA* has little effect on the ability to promote UV resistance in *rad18Δ* cells. Damage sensitivities were determined as in Figure 1C. (**D**) The ability of the Ub_4*-PCNA* variants to promote UV resistance in *rad18Δ* is abolished in a TLS-deficient background.

serting a 4-amino acid linker peptide, VQIQ, at each junction. Neither of these constructs promoted TS. Intriguingly, however, while the 'extended' fusion containing the linker conferred a TLS-dependent UV resistance comparable to the monoubiquitin fusion (Ub*-PCNA*), the seamless construct did not afford any rescue beyond the level of unmodified PCNA* (22). This finding suggested an impact of polyubiquitin chain structure on the TLS polymerases. Alternatively, however, it could reflect an unspecific steric obstruction of the N-terminus of the proximal ubiquitin unit by the rest of the chain, thus preventing access of the TLS polymerases. In order to differentiate between these two scenarios, we fused an unrelated protein, GFP, to the N-terminus of the Ub*-PCNA* construct, separated by either a short or a longer linker peptide (GS or GSGSGS). According to the published structure of a GFP-ubiquitin fusion (54), the short linker spans a distance between the bulk of the two fusion partners comparable to that in a K63- or seamless linear ubiquitin-ubiquitin junction (Supplementary Figure S2). Both arrangements resulted in a UV resistance comparable to Ub*-PCNA*, indicating functionality in TLS despite the steric bulk at the N-terminus (Figure 2A). Thus, the inhibitory effect of the seamless head-to-tail linkage on TLS appears to be due to the chain itself rather than an N-terminal blockage of the proximal ubiquitin.

In order to further explore the relevance of the ubiquitin-ubiquitin junction for TLS as well as TS, we varied the linker sequence in the Ub_4*-PCNA* constructs (Figure 2B) and tested their ability to suppress the UV sensitivity of *rad18Δ* mutants in the presence and absence of the TLS polymerases (Figure 2C, D). We found that variations in the length of the linker and its flexibility had little effect on functionality, as merely the construct bearing the linker sequence 'GS' afforded a marginally enhanced UV resistance compared to Ub*-PCNA* in *rad18Δ* (Figure 2C). In a TLS-deficient background, none of the constructs had any effect (Figure 2D), indicating that the rescue observed in *rad18Δ* single mutants was all due to TLS, while none of the linear polyubiquitin mimics was able to imitate the structure of the K63-chain and afford TS. Yet, all but the seamless construct supported TLS to a degree comparable to the monoubiquitin fusion.

These findings demonstrate the importance of a correct geometry of the polyubiquitin chain on PCNA for functionality in TS. At the same time, they are consistent with a subtle inhibitory effect of PCNA polyubiquitylation on TLS activity that also depends on the structure of the chain and is abolished by the insertion of as few as two additional amino acids at the junction.

Known downstream effectors recognize both K63-linked and linear chains

The inability of the linear mimics to support TS might imply a receptor protein whose UBD differentiates between linear and K63-linked polyubiquitin chains on PCNA. This was unexpected, because we had shown that the ubiquitin-binding zinc finger (UBZ) domain of Mgs1, a protein that interacts with polyubiquitylated PCNA, can also associate with linear polyubiquitin mimics in the two-hybrid system (24). We now found the same to apply to the Npl4 zinc finger (NZF) domain of human ZRANB3 (Figure 3A), another factor implicated in the recognition of polyubiquitylated PCNA (25–27). Whilst both UBDs strongly prefer the K63- over the K48-linkage (24–25,27), *in vitro* interaction

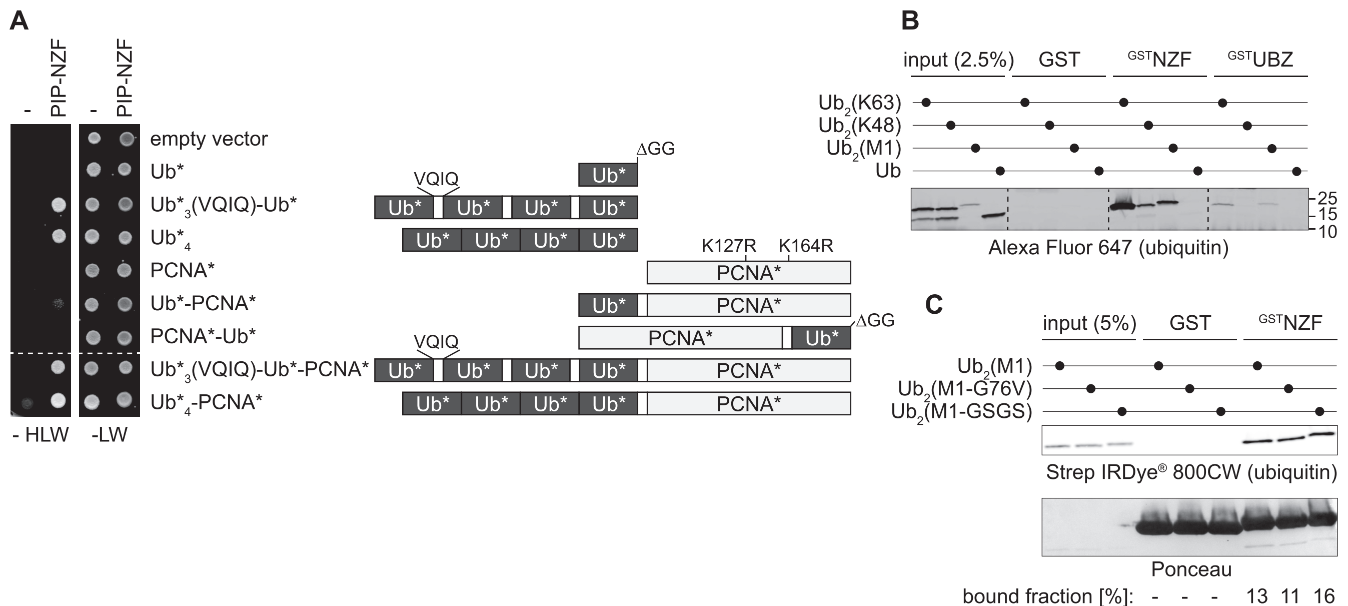

Figure 3. Known interactors of polyubiquitylated PCNA do not discriminate between similar chain geometries. (**A**) Two-hybrid assay showing the interaction of the UBD of human ZRANB3 with linear polyubiquitin mimics. A fragment of human ZRANB3, spanning the conserved PCNA-interaction peptide (PIP) and the ubiquitin-binding NZF domain (aa 141–311), was expressed as a fusion to the Gal4 activation domain and ubiquitin and PCNA constructs were fused to the Gal4 DNA-binding domain. Growth on medium lacking leucine and tryptophan (-LW) controlled for the presence of both constructs, and growth on medium additionally lacking histidine (-HLW) indicated positive interaction. Dashed lines indicates juxtaposition of different sections of the same plate. (**B**) The NZF domain of ZRANB3 and the UBZ domain of Mgs1 do not discriminate between K63- and M1-linkages, but bind inefficiently to monoubiquitin or K48-linked diubiquitin. Interaction was monitored via retention of fluorescently (Alexa Fluor 647) labelled ubiquitin constructs by GST-UBD fusions immobilized on glutathione sepharose. GST served as a control. Dashed lines indicate removal of intervening lanes of the same gel image. (**C**) The NZF domain of ZRANB3 tolerates variants of the linear chain geometry. Retention on immobilized ^{GST}NZF was compared for linear diubiquitin constructs connected either by a seamless *WT* head-to-tail linkage (M1) or by a junction containing steric bulk (G76V) or a hydrophilic linker (GSGS). The constructs carried a biotinylated Avi-His$_6$-tag on the C-terminus and were detected by a fluorescent probe (Streptavidin IRDye® 800CW) after transfer to a membrane. Ponceau staining of the same membrane served for detection of the GST constructs. The bound fraction was calculated as the average from three independent replicates.

assays confirmed that they measurably associated with both K63- and M1-linked diubiquitin (Figure 3B). In order to take into account a potential influence of subtle variations in the geometry of the ubiquitin–ubiquitin junction, we also compared the association of the NZF domain with variants of the head-to-tail (M1) linkage that introduced either steric bulk (G76V) or additional amino acids (GSGS) at the junction. However, *in vitro* association was insensitive to these changes (Figure 3C). Thus, a lack of recognition by one of the known effectors of polyubiquitylated PCNA is unlikely to be the cause of the inactivity of the linear chain mimics.

Permanent polyubiquitin chains on PCNA do not interfere with damage bypass

Permanent fusion of a polyubiquitin chain to a significant fraction of the total cellular pool of PCNA could in principle exert a dominant-negative effect by sequestering a low-abundance critical effector protein away from relevant chromatin sites *in vivo*. We addressed this issue by means of a Ub^{K63*}-Ub*-PCNA* construct, carrying a non-cleavable linear diubiquitin whose distal unit allowed extension via K63 (Figure 4A). Modification by Ubc13 upon replication stress would thus result in a polyubiquitin chain that is separated from PCNA by the permanent linear diubiquitin unit. As shown in Figure 4B, this construct suppressed the UV sensitivity of the *rad18Δ* mutant to almost the same degree as Ub^{K63*}-PCNA*, and western blot analysis confirmed its damage-dependent polyubiquitylation (Figure 4C). In addition, we expressed a linear tetraubiquitin fusion to PCNA* in an otherwise *WT* background, which is expected to form heterotrimers with *WT* PCNA *in vivo*. This construct did not confer any DNA damage sensitivity beyond the effect of PCNA* alone, confirming that permanent linear chains on PCNA do not interfere with DNA damage bypass (Supplementary Figure S3).

These results suggest that (i) productive TS can be achieved by a K63-polyubiquitin chain that is assembled at a considerable distance from PCNA, (ii) a linear ubiquitin-ubiquitin junction adjacent to a physiological K63-linked chain does not significantly interfere with TS and (iii) permanent modification of ∼50% of the total cellular pool of PCNA with a non-productive di- or polyubiquitin structure has no major effect on the ability of a physiological K63-chain to activate TS.

Cleavability by DUBs does not render linear chain mimics active in TS

A permanent fusion of a ubiquitin chain could interfere with TS not only because of a potential dominant-negative effect, but also if disassembly of the conjugate at a later step

Figure 4. Non-cleavable diubiquitin at the N-terminus of PCNA has little effect on polyubiquitin-mediated TS activity. (**A**) Schematic view of the constructs, labelled as in Figure 1A. (**B**) UV sensitivities of *rad18Δ* cells expressing the indicated variants of Ub*-PCNA* or Ub*-Ub*-PCNA, either permitting or preventing extension via K63 at the distal ubiquitin moiety. (**C**) DNA damage-dependent polyubiquitylation of Ub^{K63*}-Ub*-PCNA, detected in cells expressing His_6-tagged versions of the constructs as described in Figure 1F.

were required for proper function of the pathway. For example, deconjugation of monoubiquitylated human PCNA by the DUB, Usp1, has been reported to promote genome stability by protecting cells from the mutagenic action of unscheduled TLS (55). While in budding yeast a permanent fusion of monoubiquitin allows for a substantial activation of TLS and affords TS via K63-chain extension (Figure 1), we considered the possibility that the polymeric chain required disassembly by DUBs. In order to assess this, we created a series of variants of Ub^*_3(VQIQ)-Ub*-PCNA* with modified ubiquitin C-termini, inserting either glycine (G), alanine (A) or serine (S) at positions 76 (Figure 5A). These mutant versions indeed afforded variable degrees of stability *in vivo* (Figure 5B): whereas the *WT* residue, G76, resulted in complete cleavage and G76V was completely stable, G76A and G76S exhibited intermediate degrees of disassembly *in vivo*, thus resulting in a distribution of conjugates very similar to native K63-polyubiquitylated PCNA. However, none of the constructs suppressed the UV sensitivity of the *rad18Δ* mutant beyond the level of Ub*-PCNA* (Figure 5C). This indicates that a lack of cleavability of the polyubiquitin mimics *per se* was not the reason for their failure to promote TS. However, since deubiquitylation of such partially cleavable constructs might occur at random, prior to the encounter of replication stress, our observations do not rule out a requirement for coordinated chain disassembly *in situ*, i.e. at the site of action, as part of the TS process.

A single K63-junction confers TS activity

The question of how long a polyubiquitin chain needs to be to support *in vivo* function is rarely addressed, due to difficulties in controlling this property in enzymatically assembled chains. Using monoubiquitylated PCNA as a substrate, Ubc13-Mms2 and Rad5 or its human homologue, HLTF, are capable of generating chains of significantly more than ten ubiquitin moieties *in vitro* (19,56–57). When native K63-polyubiquitylation of PCNA is monitored *in vivo*, chains longer than four ubiquitin units are rarely observed (11,13); however, this might be a limitation of the detection system and does not rule out the possibility that the tetraubiquitin mimics were too short to trigger efficient TS. Rather than constructing further extended linear chain mimics, we addressed the issue of chain length in the context of native, K63-linked chains. We reasoned that expressing Ub^{K63*}-PCNA* in a strain background carrying a K63R mutation of endogenous ubiquitin (58) would generate a situation where chain extension is restricted to a single K63-junction (Figure 6A). Western blot analysis confirmed the addition of exactly one ubiquitin to Ub^{K63*}-PCNA* in the *rad18Δ* ubi^{K63R} background. Surprisingly, the modification occurred even in the absence of exogenous DNA damage, possibly indicating spontaneous replication problems in this strain or a more relaxed regulation at the level of polyubiquitylation. Importantly, however, analysis of UV sensitivities in the ubiquitin mutant background revealed a significant rescue by Ub^{K63*}-PCNA* compared to Ub*-PCNA* (Figure 6C), indicating that even a single K63-junction supports TS to some degree. Expression of the same construct in an isogenic strain harbouring *WT* ubiquitin afforded a higher UV resistance, suggesting that longer chains are superior. Rescue by Ub^{K63*}-PCNA* was still observed in a TLS-deficient strain background and therefore clearly ascribable to TS (Supplementary Figure S4A and B). As expected, the analogous linear diubiquitin fusion, Ub*-Ub*-PCNA*, remained unaffected by the presence or absence of the K63R mutation in endogenous ubiquitin (Supplementary Figure S4A and B). We conclude from these results that a single K63-junction provides a substantial activation of TS. Failure of the polyubiquitin mimics bearing three M1-junctions is therefore unlikely to be due to insufficient chain length.

DISCUSSION

How does polyubiquitylated PCNA activate template switching?

In this study we have taken a systematic approach toward identifying features of polyubiquitylated PCNA that are required for activation of TS. Starting from our observation that linear, non-cleavable polyubiquitin chain mimics do not support the pathway, we asked what properties prevented their activity when compared to a native K63-linked chain. We were able to exclude their non-native attachment site (Figure 1), the lack of susceptibility to DUB cleavage (Figure 5) as well as insufficient chain length (Figure 6) as factors preventing TS activation. We also showed that a permanent linear di- or polyubiquitin structure does not inter-

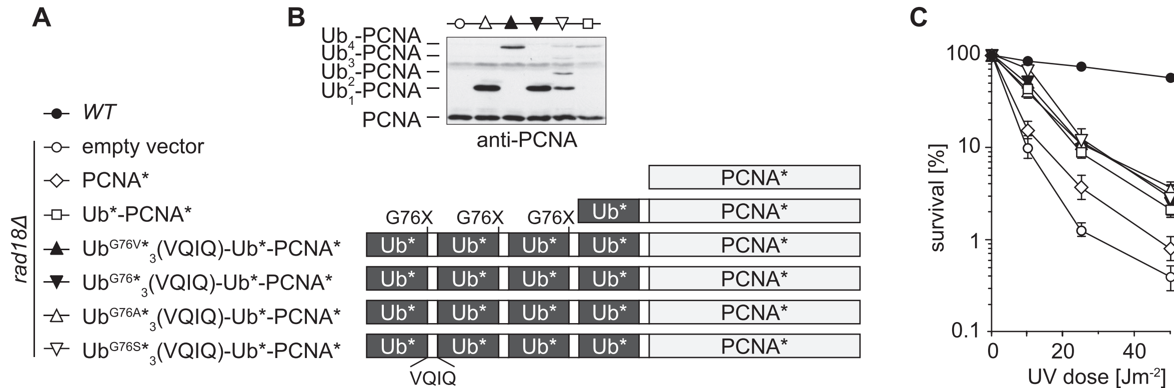

Figure 5. Failure of the linear polyubiquitin chain mimics on PCNA to activate TS is not due to their DUB resistance. (**A**) Schematic view of the constructs, showing variations in the C-terminal residues of their three distal ubiquitin moieties (G76X), labelled as in Figure 1A. (**B**) Western blot of total extracts from cells expressing the indicated constructs. The blot reveals cleavage products along with endogenous PCNA. (**C**) UV sensitivities of *rad18Δ* cells expressing the indicating constructs.

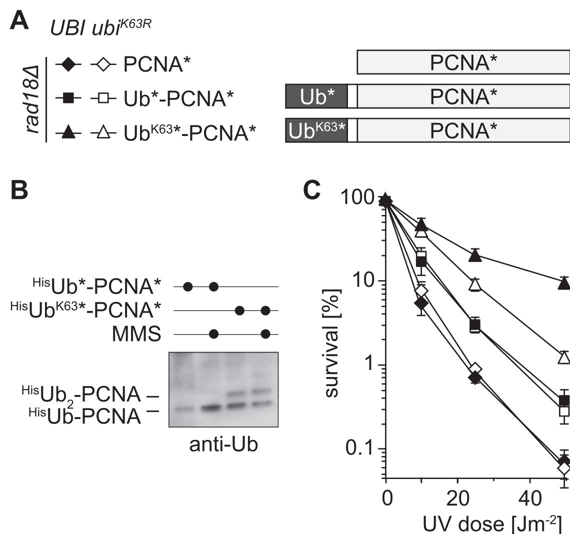

Figure 6. A single ubiquitin-ubiquitin junction of K63-linkage on PCNA affords partial rescue of UV resistance in *rad18Δ*. (**A**) Schematic view of the constructs, labelled as in Figure 1A. (**B**) Western blot analysis of His_6-tagged versions of the constructs in the ubi^{K63R} background, performed as in Figure 1F, indicates the addition of a single ubiquitin onto Ub^{K63*}-$PCNA^{*}$. (**C**) UV sensitivities of isogenic *rad18Δ* strains harbouring either a *WT* allele (*UBI*) or a K63R mutant as the sole source of ubiquitin (ubi^{K63R}) and expressing the indicated constructs.

fere with K63-chain assembly and TS, thus effectively ruling out a dominant-negative effect of the mimic (Figure 4, Supplementary Figure S3). Taken together, these findings all point to a critical role of the linkage itself in activating TS. Thus, if the polyubiquitin chain on PCNA functions in a 'conventional' way, i.e. by recruiting a specific receptor protein, the notion that variations of the ubiquitin–ubiquitin junction did not improve performance (Figure 2) suggests that the UBD of this putative receptor must be highly selective for the K63-linkage. The two known interactors of polyubiquitylated PCNA, Mgs1 and ZRANB3, do not fit this criterion, as they efficiently bind to the linear mimics (Figure 3) (24).

This raises the possibility that a yet unidentified effector of polyubiquitylated PCNA triggers the activation of TS. Numerous studies have discovered important mediators of the reaction, ranging from recombination proteins, nucleases and helicases to factors impinging on chromatin architecture (59–65). These factors were reported to either contribute to PCNA ubiquitylation or support the DNA resection and strand exchange reactions, but none of them was shown to act as a direct sensor of modified PCNA. Thus, their functional relationship to PCNA polyubiquitylation remains unclear.

We have not tested the performance of the polyubiquitin mimics in a human system; therefore, we cannot exclude that human cells are more tolerant towards alternative chain structures than yeast, and ZRANB3 might be a critical effector of TS despite its lack of linkage selectivity. Its ability to promote fork reversal is consistent with initiating strand exchange at stalled replication forks, and PCNA polyubiquitylation was shown to be required for this activity (26). In the budding yeast, however, fork reversal appears to be associated mainly with defective damage signalling (66), and TS is not limited to stalled forks, but can operate at postreplicative daughter-strand gaps that would not require a fork regression step (67,68). Thus, the absence of an obvious ZRANB3 homologue in fungi could indicate a divergence of the TS mechanisms between the species, despite the considerable conservation of the PCNA modification system and its enzymes.

As an alternative model that would not require a UBD-containing receptor of polyubiquitylated PCNA at all, a recent report postulated that K63-linked polyubiquitin chains directly and specifically interact with DNA, and that this might be responsible for activating the TS pathway (69). While we were unable to reproduce this activity (unpublished data), we also consider it unlikely because DNA binding was reported to require a chain length of at least four ubiquitin units, whereas we were able to achieve significant TS activation with a single K63-linkage (Figure 6).

We therefore consider it more likely that a protein with a highly K63-selective UBD and a basal affinity for PCNA transmits the polyubiquitin signal in a conventional man-

ner. The modular nature of many UBDs is consistent with the flexibility of the ubiquitin attachment site on PCNA, and the variety of structures capable of recognising ubiquitin (3) suggests that additional UBDs might still be identified. It should be noted, however, that our data do not rule out a requirement for *in situ* chain assembly or disassembly, i.e. a dynamic mode of ubiquitin conjugation or deconjugation at the site of action, as the underlying cause for our failure to rescue TS with preformed chains. Experimental differentiation between this scenario and the more conventional model involving a linkage-selective effector is not straightforward, as it would require the reversible modification of PCNA with alternative linkages *in vivo*, an approach that is currently unavailable due to the inherent selectivity built into the enzymes involved in PCNA polyubiquitylation.

How does PCNA polyubiquitylation impinge on TLS?

Synergistic interactions between mutants of *UBC13* or *MMS2* and genes encoding TLS polymerases argue for a largely independent action of TS and TLS (67,70–73). Our observation that a K63-chain assembled on a permanent ubiquitin-PCNA fusion promotes TS in the absence of any of the TLS polymerases support this view (Figure 1). Nevertheless, several reports have suggested an impact of PCNA polyubiquitylation factors on TLS. A positive effect of Rad5 and its human homologue, HLTF, has largely been ascribed to a structural role in TLS polymerase recruitment, independent of their E3 function (36,38,74). However, deletion of *UBC13* or *MMS2* were also found to reduce polymerase η-dependent TLS activity in a plasmid-based reporter system in *S. pombe*, suggesting that PCNA polyubiquitylation might contribute to the pathway to some extent (31). In contradiction to this model, Plosky *et al*. (17) proposed that the polyubiquitin chain on PCNA could promote a dissociation of the TLS polymerases from the primer terminus, thus inhibiting rather than promoting TLS. This notion was supported by the observation that polyubiquitylation of PCNA - in contrast to monoubiquitylation—inhibits polymerase η activity in an *in vitro* reaction, raising the idea that the chain locks the polymerase in an unproductive mode (30). Our observation that TLS is not supported by the seamless polyubiquitin chain mimic on PCNA is consistent with such model (Figure 2) (22), although the notion that insertion of as little as two amino acids at the ubiquitin–ubiquitin junction abolishes this effect is puzzling. It should also be noted that the *in vitro* study made use of a chemical ubiquitylation strategy, resulting in a disulfide-linked junction structure that is similar, but not identical to the native isopeptide bond. Thus, neither approach truly matches the physiological situation, which limits their informative value with respect to the impact of genuine K63-chains on TLS polymerases *in vivo*.

Overall, our study has provided evidence that in the context of DNA damage bypass, chain geometry is more important than the actual number of ubiquitin units within the polyubiquitin chain. This argues against the notion that the K63-linkage mainly functions as a non-K48-linked assembly of ubiquitin, designed to avoid recognition by the proteasomal degradation system. It highlights the complexity of ubiquitin as a signalling molecule and calls for the development of novel tools that would permit a direct manipulation of polyubiquitin chain linkage in an *in vivo* setting.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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