nature portfolio

Peer Review File

USP1/UAF1 targets polyubiquitinated PCNA with an exo-cleavage mechanism that can temporarily enrich for monoubiquitinated PCNA

Corresponding Author: Professor Titia Sixma

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The paper by Keijzer and coworkers presents a focused study of USP1/UAF1 in which the authors uncover the mechanism that dismantles polyubiquitin chains from PCNA in vitro.
They use a combination of biochemistry, kinetic modeling and cryo-EM to argue that USP1/UAF1 prefers to remove ubiquitin from ubiquitin chains rather than removing mono-ubiquitin from PCNA. Although this seems to be supported by kinetic modeling, I am not convinced that this claim holds up with the results of the DUB assays in Figure 1. The authors also present a cryo-EM structure of USP1/UAF1 bound to K63-linked di-ubiquitin and identify the negatively charged patch in USP1 as the main interface that drives exo-cleavage of K63- and K48-linked ubiquitin chains. The K63-linkage is mimicked by a diubiquitin vinyl amide, which conjugates to USP1 when USP1/UAF1 attempts to cleave the Ub-Ub bond. These assays are interesting and shed new light on the deubiquitination mechanim. The studies in Figure 4 designed to support the notion that UAF1 obstructs USP1's exo activity appear to reveal that mutations in UAF1 limit the deubiquitination to exo- and endo mechanism and restrict the base cleavage of mono-ubiquitinated PCNA. I find this section in the Results rather confusing.

The authors conclude the study with a bold and speculative model that takes into account other published data but overstates their findings. For example, the claim that USP1/UAF1 controls the onset of DDT by "stabilizing" mono-ubiquitinated PCNA is far-fetched and not addressed in the paper.

Specific concerns:

Figure 1: I agree with the interpretation of panels B-D, but not with that of panel 1E. The time course very clearly shows that monoubiquitinated PCNA is a transient product in the reaction, and that there is quite a significant amount of base-cleavage, as unmodified PCNA is the end product of the reaction. The same is true for 1F.

Figure 2: I am concerned here that the kinetic modeling is not in agreement with the kinetics shown in Figures 1E and F. The simulation in panel C is poorly described. If this simulation were correct, one would expect to see a much more stable accumulation of mono-ubiquitinated PCNA in Figures 1E and F.

Figure 3: Structures of USP1/UAF1 have been reported previously. What's new here is the conjugation with di-ubiquitin. For the reader to appreciate the differences, it would have been nice to contrast the structures of free vs. di-ubiquitin bound USP1/UAF1.

Figure 4: The UAF1 SLD/Anc mutant appears to inhibit base cleavage, as mono-ubiquitinated PCNA accumulates significantly in this mutant. In other words, these domains facilitate the cleavage of mono-ubiquitin from PCNA. There is indeed a slight delay in the erosion of polyubiquitin, but the most compelling difference is the stark accumulation of mono-ubiquinated PCNA in the double mutant. The gels have different degrees of exposure and if you follow the accumulation of free ubiquitin, there is a slight delay in the mutant, but it is not clear to me that a slightly longer exposure of the gels in the middle and right panels wouldn't show a fairly similar signal for mono-ubiquitin to the WT reaction.

Figure 6: The model is solely based on the kinetic modeling and models proposed in other studies that have not been proven. In yeast, template switching is the preferred DDT pathway and TLS is restricted to G2 because Rev1 is only expressed in G2 phase of the cell cycle. While it is possible that cells deal differently with smaller and bulky lesions, the authors do not address this question in their paper and simply entertain it to provide a biological context to their claims that USP1/UAF1 has a strong preference for exo-cleavage of polyubiquitinated PCNA.

The discussion is strongly focused on DDT pathway choice, which the authors do not address experimentally. To make a strong case for pathway choice, cellular assays are needed that assess the frequency of TLS and TS in USP1 KO mutants.

Reviewer #2

(Remarks to the Author)
This manuscript describes a kinetic comparison of PCNA poly and mono deubiquitination in detail. The authors used chemically modified VA to capture Endo Ub and the fluorescent tag TMR on the distal Ub to follow Exo deUb. By employing these two different techniques they argue USP1/UAF1 prefers exo-cleavage, removing the distal ubiquitin preferentially. Then they took this further with carefully designed kinetic modelling to quantify kinetics at different steps of the deUbiquitination reaction. A CryoEM study was also carried out with USP1/UAF1 in complex with K63 diUb and this structure gives some new insight of how USP1/UAF1 cleaves the Exo bond. The validation report looks fine apart from a couple of minor points. Interestingly the authors noticed a conserved negative patch for the proximal ubiquitin and mutants on this patch will actually give higher deubiquitin activity. These lead the authors to conclude the different kinetics of the deUb allow enrichment of monoUb PCNA to have a regulatory role in DDR.

This is a well written manuscript with clear figures. I have 3 major points and some minor points to mention here:

1. The study doesn't demonstrate how USP1-UAF1 specifically deubiquitinates polyUb PCNA. There are some issues with the data presented in figure 1 - figure 1D the right hand panel doesn't show an increase in the amount of USP1-PCNA-Ub2 despite the legend and text asserting that it does. It shows a decrease in the amount of USP1, which lends some support, but where has the USP1 gone if it is not visible in the conjugate lane? It is clear in figure 1B, so why not in this panel? Figure 1 D left panel and the data in figure 5 are not consistent - the 1D left panel shows almost no fluorescence signal after 20s, while figure 5 shows the same experimental reagents (third panel) retains signal after 1000 seconds. Thus figure 5 doesn't support the first turnover conclusion put forward in the interpretation of the data in figure 1. It would be good to compare PolyUb PCNA deubiquitination with another polyubiquitinated protein or even the PolyUb in Fig1 without PCNA in the gel based assay as a negative control.

Also it’s nice to see how USP1/UAF1 cleave diUb on it’s own, the Cryo-EM structure of USP1/UAF1 in complex with polyUb PCNA will help us to understand how PCNA binding will change from cleavage of Exo and Base Ub. A recent paper demonstrates ATAD5 plays a scaffold role in PCNA deUb may help explain where the specificity comes from and probably needs to be considered in the experiment setup (https://www.pnas.org/doi/10.1073/pnas.2315759121), or at least discussed as something that may impact the interpretations of the data.

2. The kinetic analysis is interesting but requires more work to improve robustness of the conclusions:
a. The authors seem to imply that a model with only exo-cleavage fits better than a general model with option for exo-, endo- and base cleavage. A more general model should provide better agreement with experimental data even if it is overfit and so the authors description suggests that the fitting process for the general model did not converge. The authors should clarify or correct this. In particular the fact that the errors are symmetric suggests the error analysis may not be robust enough for the conclusions drawn in such a multi-parameter model.
b. Following a, Given that the kinetic models have many parameters a thorough parameter uncertainty analysis should be performed and reported to identify if there are any correlations between the fitted parameters (I believe this is Fitspace in the Kintec software).
c. Several different models have been compared throughout the kinetic analysis and so a more robust description of the comparisons would be suitable, for example using the Bayesian Information Criterion (BIC).
3. The idea of different deUb kinetics regulate mono or poly Ub PCNA therefore fine tune different DDR pathway is too speculative and needs cell biology to back it up. Does the DE/KK mutant lead to less monoUb in cells, for example? It may also be useful to test whether the DE/KK mutation has the same increase in deubiquitination of a different USP1 substrate before reaching the conclusion that the difference in the impact of mutations on chains is to preserve or enrich monoubiquitinated PCNA. It's certainly a possibility (although the data in figure 1 suggest that PCNA is fully deubiquitinated very quickly), but there are other possible interpretations of this experiment. Again ATAD5 might be important here.
Minor points:
1. In Fig 1 E and F why not only aim one Ub per PCNA trimer as in B to D to have a more direct comparison? Result 1 paragraph 3 last sentence “USP1 does not conjugate to K63-PCNA-UbVAUb-UbTMR,indicating that USP1 exclusively uses exo-cleavage in the first turnover and thus has a strong preferencefor trimming ubiquitin chains on PCNA. “ but in Fig1C and 1D we can see USP1+PCNA-Ub2 at 0.5 min so not much difference. Also it will be good to compare UbVAUb vs UbUbTMR to show if VA slows down the deUb reaction.
2. In my opinion Fig 1E suggest USP1/UAF1 prefer Exo Ub or cleave Exo Ub quicker as PCNA only band is constantly increasing.
3. Fig5E suggest the negative patch mutation has greater effect on PCNA Ub bond than UbUb bond. It’s strange to see in

Fig5B a nice interaction between Ub and USP1 but has very little effect with the DE mutations. Have the authors looked at the alphafold prediction on PCNA-Ub or PCNA bind to USP1/UAF1 to identify a possible interaction region on PCNA? Possibly a negative patch?
4. Can authors describe local resolution, especially on chain D and insert 3? Q score suggest fitting is poor, is this because of poor resolution or flexibility? It's hard to claim insert 3 contact UAF via hydrogen bonds at that resolution (last sentence of structure description section)
5. There are a few issues with references - 2021a for one, when there is no b (Rennie), some duplications listed as a and b manuscripts (eg Hibbert, Hoege, Huang, Joosten), please check carefully..

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
USP1 is an important DUB reglating the response to DNA damage, and a key substrate of USP1 is ubiquitinated PCNA. The type of ubiquitin modification on PCNA dictates pathway choice of DNA damage repair and hence it is important to understand the contribution of USP1 to this process. In this study, the authors use biochemistry, biophysical and structural approaches together with some cleverly designed chemical biology tools to analyse the recognition and cleavage mechanism of USP1/UAF1 of monoubiquitinated PCNA versus PCNA modified with K48 or K63 linked chains.

My major concern pertains to the authors' interpretation of USP1 being an exo-DUB and not examining the contribution of PCNA to substrate recognition and cleavage. Here are my specific points and suggestions:
1. In figure 1, the cleavage of PCNA carrying K48 or K63 chains is analysed. If USP1 is an exo-DUB (as concluded by the authors), then the only product generated should be monoUb. The presence of short chains Ub2-Ub5 at the first time point suggests that endo-cleavage occurs as efficiently as exo-cleavage (monoub product) and hence USP1 is not strictly an exo-DUB. To make the claim that USP1 is an exo-DUB, the authors will have to use polyubiquitinated PCNA with a tag or fluorescent label only on the distal most ubiquitin to analyse the products formed.
2. How does the cleavage of polyubiquitinated PCNA compare with cleavage of free ubiquitin chains? In other words, does PCNA dictate the mode of cleavage?
3. While the authors use the minimally diUb linked substrate to infer hte endo/exo preference of cleavage, anlaysing how the off rates would vary in a PCNA modified with trimers or longer chains will strengthen the claim. I believe this would add significant insights on the selective mechanism USP1/UAF1 uses to cleave in a linkage specific manner as PCNA is not modified by dimers but rather longer chains in cells.
4. In figure 3, can the authors test if an additional S2' or S3' is present for Ub binding, say using AlphaFold and sequence conservation? This would help explain the endo-cleavage observed.
5. In relation to point 2, can the authors comment on where PCNA interacts with USP1/UAF1 and show this on the structure in figure 3. This will help establish why PCNA-Ub is a poorer substrate compared to PCNA-polyUB.
6. If PCNA has no role, then a kinetic model would show Kcut to be the same for both PCNA-Ub and free ubiquitin chains.
7. Since there are 3 ubiquitination sites on PCNA, does USP1 cleave in cis or trans?
8. the modifications seen in figure 1 are difficult to see as they are very faint on the gel, could the authors please quantify the band intensities of the products.

The following comments are intended to help the authors clarify their message or provide additional information for the reader:

1. To characterize the cleavage reaction, the authors generate a global kinetic model. However, it is not always clear how this model was generated. The conceptual difference between the distributive and processive reaction described in Figure 2B and Supplementary Table 1 is clear, but it lacks the description on how they affect the model. In addition, they state the results did not fit either model but rather a mixture of both, while not showing any fitting. I would suggest including a supplementary figure with fittings to each model so the reader can understand better and a reference or detailed methods section explaining the global model, perhaps highlighting the overall mathematical differences between the processive and distributive reaction.

2. When explaining the model, authors highlight the assumptions they make. Although most of them are well defined, I find it hard to comprehen that the Kcut going from PCNA_Ub_Ub_USP1 to PCNA_Ub_USP1_Ub and the one going from PCNA_Ub_USP1 to PCNA_USP1_Ub being same. Looking at the fluorescence polarization assays of USP1/UAF1 activity comparing PCNA-Ub and PCNA-Ub2 (Figure 2A) and the description of the results from the authors throughout the manuscript, it is clear the USP1 cleaves much more efficiently the distal Ub from PCNA-Ub2 than the Ub of a monoubiquitylated PCNA. Could the authors clarify how they calculated the Kcut and why it is the same for different substrates?

3. There are other DUBs that have been shown to have exo-DUB and chain length preferences. Could the authors discuss the activity observed here with those reported previously?

4. The authors generate mutations based on the cryo-EM structure that confer higher USP1 DUB activity. Could the authors comment if these residues (or this region) are mutated or have SNPs that may indicate a role in disease.

5. The authors suggest a model for USP1/UAF1's role in directing the DNA damage tolerance pathway choice. In the absence of cellular data supporting their model, I would advise that this be toned down. Alternatively, they could perform cellular experiments to support this hypothesis.

Overall, the results provided in this manuscript are interesting and of good quality but hard to follow in some areas. I would encourage the authors to revise the manuscript to make it more accessible to the non-specialist reader.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In general, the manuscript has been improved. There is still a strong focus on "pathway choice" (Figure 7) and this is decidedly not directly addressed in the manuscript. Although the legend to Figure 7 has more hypothetical language than in the original version, it is essentially the same.

1)The speculation about pathway choice as it is presented in the discussion is relatively pointless. I would much rather see the authors discussing their findings with those by Nusawhardana et al (which the authors cite), who showed that USP1 inhibition suppresses gap formation at replication forks in a manner that is at least partially dependent on REV1, and thus on the mono-ubiquitination of PCNA. These findings are seemingly at odds with the notion that USP1 has a strong preference for exo-cleavage in vivo, and would much more support a model in which USP1 does both, deubiquitinating PCNA and the polyubiquitination chains on PCNA. I think the reader will understand that in vitro findings and in vivo regulation are two different issues, but a discussion in the context of the above-mentioned in vivo findings is more valuable than a speculative model on pathway choice that is inconsistent with the current literature.

2)On page 11, lines 491, the authors claim that "The findings of this study reveal how the intrinsic.....a role in regulating DNA damage tolerance". I do not agree with this statement. The authors present biochemical and biophysical data that describe a strong preference for exo-cleavage in vitro, and only in vitro. We don't know whether this intrinsic biochemical property is modulated in vivo to work differently.

Reviewer #2

(Remarks to the Author)
For the most part the authors have addressed our previous concerns.

However, there are still a few points that I am a little confused about, and would appreciate some clarification.

1. the authors did not respond to the suggestion of comparing UbVAUb with UbUbTAMRA to see if VA slows down the deUb reaction - did they just miss the comment, or have I missed something in the revised manuscript?
2. There is no legend for figures 4D and 4E.
3. The authors state: "As USP1/UAF1 is highly efficient, all distal Ub-TMR is rapidly cleaved from PCNA-Ub3 in Fig. 2D after which USP1/UAF1 proceeds with cleavage of the remaining PCNA-Ub2 within 30 seconds." Perhaps a shorter time point would show exclusive exo-cleavage, but with the current data it's hard to draw that conclusion.
4. Our comment about concentrations - comparing figure 2D to figure 6E seems to show a different outcome - explicitly in fig 2D there is complete loss of fluorescent signal before 30s, while in 6E, there is still signal way longer than that. There is no concentration listed in the legend for figure 2D of the substrate (despite the authors stating in their response that the concentrations are in the text - I couldn't find it in the text or legend, and I can't tell from the methods section what the concentration of PCNA-Ub-VA-Ub-Ub-TMR is - is it 0.67, 1.33, or 2.67uM?), whereas in figure 6E, the substrate concentration is on the y axis, and I am still struggling to be able to compare the two experiments, can the authors please tell me how to do so? Would it be easier to interpret a ratio of enzyme:substrate for example? If the conc is 2.67uM that is comparable to the 4uM starting conc in fig 6E, in which case the differences in remaining signal after 30 secs is quite stark.
5. Our original review we raised the following concern: "figure 1D the right hand panel doesn't show an increase in the amount of USP1-PCNA-Ub2 despite the legend and text asserting that it does. It shows a decrease in the amount of USP1, which lends some support, but where has the USP1 gone if it is not visible in the conjugate lane? It is clear in figure 1B, so why not in this panel?" In the new 'improved' experiment in Fig 2D, there is still a loss of the USP1-PCNA-Ub2 signal, which is inconsistent with the earlier experiments (now Fig2C). We are not disputing the data, data are data! But we are still confused by the assertion in the text that this complex is being formed when it looks in the gel as though it is decreasing. The USP1 decreases but the complex does not increase, how do the authors interpret this in light of their preferred explanation?
6. Given how much of it has been toned down, due to the overstating in the original manuscript, I'm not sure that the use of 'enrichment' in the title and abstract is reasonable.

This review was prepared by both reviewers 2 and 3.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
The authors have addressed all the comments and concerns of the reviewers and the manuscript is significantly improved. I am happy for it to accepted for publication.

We thank all three reviewers for their serious and constructive reviews, that helped us improve the manuscript significantly.

Here we list major changes, with more details in the point-by-point response below

- We have moved Fig. S1 to the main figures (now Fig. 1), to facilitate interpretation of the data.
- There was some confusion on the nomenclature of base-cleavage. We intended its use for removal of a chain from ubiquitin, and therefore now introduce mono-cleavage for removal of the last ubiquitin from PCNA (updated Fig. 1A, (now Fig 2A)
- Further confusion arose over the small amount of endo-cleavage that occurs on PCNA-Ub_N in Fig 2E. We describe this now better.
- We added a new experiment where the final, most distal Ub is fluorescent, to show in a different way that exo-cleavage is the main mechanism (New figure S2A, updated Fig S1 (now Fig. 1)).
- We added an experiment on free chains, to show that PCNA does not drive the exo-cleavage aspect of the mechanism (New Fig. S2B).
- Several reviewers wanted a better explanation of the development of the kinetic model, so we added a figure to explain the process (New Fig. S4), with the statistical analysis in (New Fig. S12, Table S1), and simulations at different USP1 concentrations in (New panel Fig. S6C).
- One reviewer asked for comparisons to existing USP1/UAF1 structures. We've added figures showing this comparison and explaining the similarities and differences (New panels S8CDE).
- To address the question about the resolution of insert 3, we have added a local resolution figure (New panel S8B) and now display insert 3 without sidechains in the cryo-EM density (New. Fig 4E).
- We have also improved Fig. 4 to more clearly display the cryo-EM densities and the cartoon depiction of the atomic model.
- All reviewers felt we were too explicit on the consequences of our data for DNA damage tolerance and we fully agree that we have not shown these consequences. Therefore we have toned down the writing on the biological effects, simplified the rather suggestive final model (schematic) and discussed it in a more tentative fashion, by clearly separating the legend between literature and our findings. We still feel it is important to spell out this consequence, as it will affect interpretation of many different types of experiments (Updated Fig 6 (now Figure 7).

Reviewer #1 (Remarks to the Author):

The paper by Keijzer and coworkers presents a focused study of USP1/UAF1 in which the authors uncover the mechanism that dismantles polyubiquitin chains from PCNA in vitro. They use a combination of biochemistry, kinetic modeling and cryo-EM to argue that USP1/UAF1 prefers to remove ubiquitin from ubiquitin chains rather than removing mono-ubiquitin from PCNA. Although this seems to be supported by kinetic modeling, I am not convinced that this claim holds up with the results of the DUB assays in Figure 1. The authors also present a cryo-EM structure of USP1/UAF1 bound to K63-linked di-ubiquitin and identify the negatively charged patch in USP1 as the main interface that drives exo-

cleavage of K63- and K48-linked ubiquitin chains. The K63-linkage is mimicked by a diubiquitin vinyl amide, which conjugates to USP1 when USP1/UAF1 attempts to cleave the Ub-Ub bond. These assays are interesting and shed new light on the deubiquitination mechanim. The studies in Figure 4 designed to support the notion that UAF1 obstructs USP1's exo activity appear to reveal that mutations in UAF1 limit the deubiquitination to exo- and endo mechanism and restrict the base cleavage of mono-ubiquitinated PCNA. I find this section in the Results rather confusing.

The authors conclude the study with a bold and speculative model that takes into account other published data but overstates their findings. For example, the claim that USP1/UAF1 controls the onset of DDT by "stabilizing" mono-ubiquitinated PCNA is far-fetched and not addressed in the paper.

Specific concerns:

Figure 1: I agree with the interpretation of panels B-D, but not with that of panel 1E. The time course very clearly shows that monoubiquitinated PCNA is a transient product in the reaction, and that there is quite a significant amount of base-cleavage, as unmodified PCNA is the end product of the reaction. The same is true for 1F.

> Indeed, we did not intend to communicate that PCNA-Ub is not cleaved and we realize that our writing was not clear. The enzyme is able to do this, and this was already well established in the literature. We intended the word 'base-cleavage' with a narrower meaning, only referring to the removal of a chain of ubiquitins from PCNA. We have now defined this usage explicitly, and introduced the term 'mono-cleavage' for removal of the last ubiquitin from a PCNA monomer. We have added a panel to Fig. 1A (now 2A) and text to Fig. 1A (now Fig. 2A) legend and the results section to explain this.

Figure 2: I am concerned here that the kinetic modeling is not in agreement with the kinetics shown in Figures 1E and F. The simulation in panel C is poorly described. If this simulation were correct, one would expect to see a much more stable accumulation of mono-ubiquitinated PCNA in Figures 1E and F.

> We understand that this was confusing. The simulations change dependent on the amounts of enzyme and substrate. The simulations shown in Fig. S6AB mirror the conditions in the FP assay, not the one in DUB assays of Fig. 1EF (now Fig. 2EF) (which have higher concentration of USP1/UAF1). We have improved the description of conditions for the simulation and also added a second simulation to the supplementary figures (new panel Fig. S6C) that has conditions that resemble the concentrations used in figure 2E-F.

Figure 3: Structures of USP1/UAF1 have been reported previously. What's new here is the conjugation with di-ubiquitin. For the reader to appreciate the differences, it would have been nice to contrast the structures of free vs. di-ubiquitin bound USP1/UAF1.

Indeed! Previous structures were already mentioned, but we have extended the comparison in the results, and added Fig. S8CDE to show and highlight the comparison. We have added RMSD values to the figure to reflect the differences.

Figure 4: The UAF1 SLD/Anc mutant appears to inhibit base cleavage, as mono-ubiquitinated PCNA accumulates significantly in this mutant. In other words, these domains facilitate the cleavage of mono-ubiquitin from PCNA. There is indeed a slight delay in the erosion of polyubiquitin, but the most compelling difference is the stark accumulation of mono-ubiquinated PCNA in the double mutant. The gels have different degrees of exposure and if you follow the accumulation of free ubiquitin, there is a slight delay in the mutant, but it is not clear to me that a slightly longer exposure of the gels in the middle and right panels wouldn't show a fairly similar signal for mono-ubiquitin to the WT reaction.

There is indeed more accumulation of PCNA-Ub in the Anc/SLD mutant, but we think that this is a result of the mutant slowing down overall. We have tried to clarify our description of this mutant and explain that we see two things: relatively more endo-cleavage and overall slowing down, resulting in increased temporal enrichment of PCNA-Ub. The contrast of the gels was indeed a bit off, so we have adjusted the contrast to make the gels more similar.

Figure 6: The model is solely based on the kinetic modeling and models proposed in other studies that have not been proven. In yeast, template switching is the preferred DDT pathway and TLS is restricted to G2 because Rev1 is only expressed in G2 phase of the cell cycle. While it is possible that cells deal differently with smaller and bulky lesions, the authors do not address this question in their paper and simply entertain it to provide a biological context to their claims that USP1/UAF1 has a strong preference for exo-cleavage of polyubiquitinated PCNA.

The discussion is strongly focused on DDT pathway choice, which the authors do not address experimentally. To make a strong case for pathway choice, cellular assays are needed that assess the frequency of TLS and TS in USP1 KO mutants.

We agree that cellular experiments will be necessary to fully address how USP1 regulates pathway choice, and perhaps we were too enthusiastic with our interpretations. We are trying to generate the necessary tools, but have realized in our previous studies (Keijzer et al, LSA 2024) that knockout USP1 cells are very variable in their behaviour. As USP1 has multiple functions, both preventing premature ubiquitination and then shifting the equilibrium during damage, it will be important to do a very carefully controlled study in cells, and we feel this is beyond the scope of our current manuscript.
For this manuscript we have therefore simplified Figure 7 and toned down the discussion, merely pointing out the possibility that the observed kinetic mechanism could potentially affect pathway distribution.

Reviewer #2 (Remarks to the Author):

This manuscript describes a kinetic comparison of PCNA poly and mono deubiquitination in detail. The authors used chemically modified VA to capture Endo Ub and the fluorescent tag TMR on the distal Ub to follow Exo deUb. By employing these two different techniques they argue USP1/UAF1 prefers exo-cleavage, removing the distal ubiquitin preferentially. Then they took this further with carefully designed kinetic modelling to quantify kinetics at different steps of the deUbiquitination reaction. A CryoEM study was also carried out with USP1/UAF1 in complex with K63 diUb and this structure gives some new insight of how USP1/UAF1 cleaves the Exo bond. The validation report looks fine apart from a couple of minor points. Interestingly the authors noticed a conserved negative patch for the proximal ubiquitin and mutants on this patch will actually give higher deubiquitin activity. These lead the authors to conclude the different kinetics of the deUb allow enrichment of monoUb PCNA to have a regulatory role in DDR.

This is a well written manuscript with clear figures. I have 3 major points and some minor points to mention here:

1. The study doesn't demonstrate how USP1-UAF1 specifically deubiquitinates polyUb PCNA. There are some issues with the data presented in figure 1 - figure 1D the right hand panel doesn't show an increase in the amount of USP1-PCNA-Ub2 despite the legend and text asserting that it does. It shows a decrease in the amount of USP1, which lends some support, but where has the USP1 gone if it is not visible in the conjugate lane? It is clear in figure 1B, so why not in this panel? Figure 1 D left panel and the data in figure 5 are not consistent - the 1D left panel shows almost no fluorescence signal after 20s, while figure 5 shows the same experimental reagents (third panel) retains signal after 1000 seconds. Thus figure 5 doesn't support the first turnover conclusion put forward in the interpretation of the data in figure 1. It would be good to compare PolyUb PCNA deubiquitination with another polyubiquitinated protein or even the PolyUb in Fig1 without PCNA in the gel based assay as a negative control.

> We can understand the confusion of the reviewer, which is primarily caused by a difference in enzyme concentration between Fig. 1 and Fig. 5 (Now Fig. 2 and Fig. 6). To address this we mention concentrations used in the text and performed the simulations with the kinetic model at multiple enzyme concentrations. We have added an experiment with only the most distal ubiquitin fluorescent to show the first turnover in a different manner (new Fig. S2).
> To address the concerns about Fig. 1D (now Fig. 2D), we have replaced the experiment of Fig. 2D with an improved experiment. We have also changed the contrast of Fig. 1E (now Fig. 2E) left panel to make it more consistent with the rest. Finally we added a new experiment on free chains (Fig. S2B), to show that PCNA is not important for the exo-cleavage.

Also it's nice to see how USP1/UAF1 cleave diUb on it's own, the Cryo-EM structure of USP1/UAF1 in complex with polyUb PCNA will help us to understand how PCNA binding will change from cleavage of Exo and Base Ub. A recent paper demonstrates ATAD5 plays a scaffold role in PCNA deUb may help explain where the specificity comes from and probably needs to be considered in the experiment setup (https://www.pnas.org/doi/10.1073/pnas.2315759121), or at least discussed as something

that may impact the interpretations of the data.

> We agree and it is useful to mention this, but also the role of the various E3 ligases and other factors. We have added a paragraph to the discussion on factors affecting the levels of PCNA-Ub and the USP1-dependent cleavage.

2. The kinetic analysis is interesting but requires more work to improve robustness of the conclusions:
a. The authors seem to imply that a model with only exo-cleavage fits better than a general model with option for exo-, endo- and base cleavage. A more general model should provide better agreement with experimental data even if it is overfit and so the authors description suggests that the fitting process for the general model did not converge. The authors should clarify or correct this. In particular the fact that the errors are symmetric suggests the error analysis may not be robust enough for the conclusions drawn in such a multi-parameter model.

> We have improved the description of the generation of the model, explaining that a more general model had multiples constants, that either did not converge, indicating that they did not influence the fit, or went to zero value, indicating that they are not contributing.
> For simplicity we chose to show the standard errors, which are symmetric by definition. We have now added the 95% confidence interval (supplementary table 1) and they are indeed not symmetric.

b. Following a, Given that the kinetic models have many parameters a thorough parameter uncertainty analysis should be performed and reported to identify if there are any correlations between the fitted parameters (I believe this is Fitspace in the Kintec software).

> We have now shown the Fitspace data explicitly in new Figure S12.

c. Several different models have been compared throughout the kinetic analysis and so a more robust description of the comparisons would be suitable, for example using the Bayesian Information Criterion (BIC).

> Since our procedure only involved elimination of unnecessary steps, we did not obtain competing models that fit the results, therefore this type of comparison is not applicable.

3. The idea of different deUb kinetics regulate mono or poly Ub PCNA therefore fine tune different DDR pathway is too speculative and needs cell biology to back it up. Does the DE/KK mutant lead to less monoUb in cells, for example? It may also be useful to test whether the DE/KK mutation has the same increase in deubiquitination of a different USP1 substrate before reaching the conclusion that the difference in the impact of mutations on chains is to preserve or enrich monoubiquitinated PCNA. It's certainly a possibility (although the data in figure 1 suggest that PCNA is fully deubiquitinated very quickly), but there are other possible interpretations of this experiment. Again ATAD5 might be important here.

As already mentioned in the response to reviewer 1, we agree that cellular experiments will be necessary to fully address how USP1 regulates pathway choice. We are trying to generate the necessary tools, but have realized in our previous studies (Keijzer et al, LSA 2024) that knockout USP1 cells are very variable in their behaviour. As USP1 has multiple functions, both preventing premature ubiquitination and then shifting the equilibrium during damage, it will be important to do a very carefully controlled study in cells, and we feel this is beyond the scope of our current manuscript.
For this manuscript we have therefore simplified Figure 7 and toned down the discussion, merely pointing out the possibility that the observed kinetic mechanism could potentially affect pathway distribution.

Minor points:
1. In Fig 1 E and F why not only aim one Ub per PCNA trimer as in B to D to have a more direct comparison?

Our kinetics do not show any effect of multiple PCNA monomers being modified, these reactions are independent. In practice, it is technically useful to start from fully occupied PCNA-Ub to be able to make 100% PCNA-Ub_2.

Result 1 paragraph 3 last sentence “USP1 does not conjugate to K63-PCNA-UbVAUb-UbTMR,indicating that USP1 exclusively uses exo-cleavage in the first turnover and thus has a strong preferencefor trimming ubiquitin chains on PCNA. “ but in Fig1C and 1D we can see USP1+PCNA-Ub2 at 0.5 min so not much difference. Also it will be good to compare UbVAUb vs UbUbTMR to show if VA slows down the deUb reaction.

These assays were not intended to report rates, but merely to provide a qualitative, visual aid about the cleavage preference. To aid this, higher enzyme concentrations were used to aid visualization, and as a result the reaction is faster and not much difference can be observe between Fig. 1C and Fig. 1D (now Fig. 2C and Fig. 2D). As USP1/UAF1 is highly efficient, all distal Ub-TMR is rapidly cleaved from PCNA-Ub_3 in Fig. 2D after which USP1/UAF1 proceeds with cleavage of the remaining PCNA-Ub_2 within 30 seconds.

2. In my opinion Fig 1E suggest USP1/UAF1 prefer Exo Ub or cleave Exo Ub quicker as PCNA only band is constantly increasing.

USP1/UAF1 prefers to exo-cleave poly-ubiquitinated PCNA, but will still cleave some PCNA-Ub. As chains are rapidly cleaved, more and more monoubiquitinated PCNA is generated and some of that will be cleaved by USP1, resulting in appearance of the ‘PCNA only’ band. As the reaction progresses, more and more monoubiquitinated PCNA becomes available, and generation of PCNA only ‘speeds up’.

3. Fig5E suggest the negative patch mutation has greater effect on PCNA Ub bond than UbUb bond. It’s strange to see in Fig5B a nice interaction between Ub and USP1 but has very little effect with the DE mutations. Have the authors looked at the alphafold prediction on

PCNA-Ub or PCNA bind to USP1/UAF1 to identify a possible interaction region on PCNA? Possibly a negative patch?

> AlphaFold unfortunately does not predict an interaction for USP1(/UAF1) and PCNA or PCNA-Ub.
> The distance between the residues is too far to be a proper interaction, which is why we did not discuss it as such, but highlighted the presence of a possible negative pocket. However, we have added a sentence to make it more clear that this pocket is not relevant for ubiquitin binding: "This also means that a positive charge into this negative pocket does not interfere with Ub-Ub cleavage."

4. Can authors describe local resolution, especially on chain D and insert 3? Q score suggest fitting is poor, is this because of poor resolution or flexibility? It's hard to claim insert 3 contact UAF via hydrogen bonds at that resolution (last sentence of structure description section)

> Indeed, the resolution does not allow precise fitting in this region. We have added a local resolution image (new Fig. S8B). However, since the sidechains on both sides are hydrophilic, the contacts are less likely to be hydrophobic. We have rewritten the section to make clear that the suggestion is tentative and changed the way we depict insert 3 (new panel in updated Fig. 4E (previous Fig. 3).

5. There are a few issues with references - 2021a for one, when there is no b (Rennie), some duplications listed as a and b manuscripts (eg Hibbert, Hoege, Huang, Joosten), please check carefully..

> We apologize for this and have doublechecked all references.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4 (Remarks to the Author):

USP1 is an important DUB reglating the response to DNA damage, and a key substrate of USP1 is ubiquitinated PCNA. The type of ubiquitin modification on PCNA dictates pathway choice of DNA damage repair and hence it is important to understand the contribution of USP1 to this process. In this study, the authors use biochemistry, biophysical and structural approaches together with some cleverly designed chemical biology tools to analyse the recognition and cleavage mechanism of USP1/UAF1 of monoubiquitinated PCNA versus PCNA modified with K48 or K63 linked chains.

My major concern pertains to the authors' interpretation of USP1 being an exo-DUB and not examining the contribution of PCNA to substrate recognition and cleavage. Here are my

specific points and suggestions:
1. In figure 1, the cleavage of PCNA carrying K48 or K63 chains is analysed. If USP1 is an exo-DUB (as concluded by the authors), then the only product generated should be monoUb. The presence of short chains Ub2-Ub5 at the first time point suggests that endo-cleavage occurs as efficiently as exo-cleavage (monoub product) and hence USP1 is not strictly an exo-DUB. To make the claim that USP1 is an exo-DUB, the authors will have to use polyubiquitinated PCNA with a tag or fluorescent label only on the distal most ubiquitin to analyse the products formed.

> This was a very useful suggestion. The suggested experiment with fluorescent terminal ubiquitin (Fig. S2A) shows that indeed free mono-ubiquitin is the main product.
> We agree that there is a small degree of endo-cleavage and have made this more explicit in the text: "There is a small degree of endo-cleavage since minor amounts of free ubiquitin chains of different lengths appear as well. Occurrence of base-cleavage can be excluded, since PCNA-Ub accumulates at first and USP1/UAF1 does not employ base-cleavage on PCNA-Ub_2 either"

2. How does the cleavage of polyubiquitinated PCNA compare with cleavage of free ubiquitin chains? In other words, does PCNA dictate the mode of cleavage?

> We added an experiment with the distal ubiquitin labeled on a free K63-ub chain (Fig. S2), and we still observe primarily exo-cleavage, indicating that PCNA is not important for this part of the mechanism.

3. While the authors use the minimally diUb linked substrate to infer hte endo/exo preference of cleavage, anlaysing how the off rates would vary in a PCNA modified with trimers or longer chains will strengthen the claim. I believe this would add significant insights on the selective mechanism USP1/UAF1 uses to cleave in a linkage specific manner as PCNA is not modified by dimers but rather longer chains in cells.

> Indeed, and the new experiment suggested by the reviewer (Fig. S2) confirms the exo-cleavage. We would like to examine the longer chains, but we need a highly homogeneous sample for kinetic analysis (eg. A PCNA that only has Ub_4-chains) and that is not something we are able to produce. For a purely theoretical analysis, the complexity of our approach becomes too large: the number of options expands too much.

4. In figure 3, can the authors test if an additional S2' or S3' is present for Ub binding, say using AlphaFold and sequence conservation? This would help explain the endo-cleavage observed.

> AlphaFold does not predict such a ubiquitin-binding site nor does it predict our cryo-EM structure.

5. In relation to point 2, can the authors comment on where PCNA interacts with

USP1/UAF1 and show this on the structure in figure 3. This will help establish why PCNA-Ub is a poorer substrate compared to PCNA-polyUB.

> We do not know where PCNA interacts and AlphaFold also does not predict it. We are trying to get the structure experimentally, but would rather not speculate now.

6. If PCNA has no role, then a kinetic model would show Kcut to be the same for both PCNA-Ub and free ubiquitin chains.

> We do understand the confusion about k_{cut} . In our model we use k_{cut} exclusively for the actual cleavage of the isopeptide bond and is therefore not the same as k_{cat}. Since this means that k_{cut} is always between a Ub C-term and a lysine side chain, there is no difference between substrates. All binding aspects are taken up in the other rates.
> To explain this better we have added a paragraph to the results: "To deconvolute the model, we have separated dissociation constants (off-rates, the numbered constants in the model) from cleavage of the isopeptide bond (k_{cut}) itself. As a result, k_{cut} could be shared in all cleavage steps, as USP1 is always cleaving an isopeptide bond, regardless of substrate. On the other hand, the numbered constants (k) are unique, as they are product-dependent. In classical Michaelis-Menten kinetics, these two values together appear as k_{cat}."

7. Since there are 3 ubiquitination sites on PCNA, does USP1 cleave in cis or trans?

> Our data do not address this. All we can say is that our data do not show any sign of cooperativity and hence the cleavages are independent.

8. the modifications seen in figure 1 are difficult to see as they are very faint on the gel, could the authors please quantify the band intensities of the products.

> True, the gel of Fig. 1D (now Fig. 2D) could have been better. We have repeated the experiment and replaced the gel in new Fig. 2D.

The following comments are intended to help the authors clarify their message or provide additional information for the reader:

1. To characterize the cleavage reaction, the authors generate a global kinetic model. However, it is not always clear how this model was generated. The conceptual difference between the distributive and processive reaction described in Figure 2B and Supplementary Table 1 is clear, but it lacks the description on how they affect the model. In addition, they state the results did not fit either model but rather a mixture of both, while not showing any fitting. I would suggest including a supplementary figure with fittings to each model so the reader can understand better and a reference or detailed methods section explaining the global model, perhaps highlighting the overall mathematical differences between the processive and distributive reaction.

Thanks. We have now added a new supplementary figure to show the large initial model and explain what steps were taken in order to come to the final kinetic model (Fig. S4).We essentially went through hundreds of variations of the model, improving the fit until we could do so no longer. There are simply too many intermediates to show this in a readable format.
We've also changed the text in the results section to highlight the main choices. Finally we added a figure with the statistics of the final fitting (Fig xx).

2. When explaining the model, authors highlight the assumptions they make. Although most of them are well defined, I find it hard to comprehen that the Kcut going from PCNA_Ub_Ub_USP1 to PCNA_Ub_USP1_Ub and the one going from PCNA_Ub_USP1 to PCNA_USP1_Ub being same. Looking at the fluorescence polarization assays of USP1/UAF1 activity comparing PCNA-Ub and PCNA-Ub2 (Figure 2A) and the description of the results from the authors throughout the manuscript, it is clear the USP1 cleaves much more efficiently the distal Ub from PCNA-Ub2 than the Ub of a monoubiquitylated PCNA. Could the authors clarify how they calculated the Kcut and why it is the same for different substrates?

As discussed in the answer to reviewer 2, we intend to use k_{cut} solely for the isopeptide bond cleavage. We have clarified this in the text as follows: "To deconvolute the model, we have separated dissociation constants (off-rates, the numbered constants in the model) from cleavage of the isopeptide bond (k_{cut}) itself. As a result, k_{cut} could be shared in all cleavage steps, as USP1 is always cleaving an isopeptide bond, regardless of substrate. On the other hand, the numbered constants (k) are unique, as they are product-dependent. In classical Michaelis-Menten kinetics, these two values together appear as k_{cat}."

3. There are other DUBs that have been shown to have exo-DUB and chain length preferences. Could the authors discuss the activity observed here with those reported previously?

Thanks for the suggestion. We have added a paragraph to the discussion to address this. Many DUBs use a flexible mixture between exo- and endo- cleavage, and the chain length, or the chain type usually plays a role in deciding which of the two is used. As far as we are aware, it is mostly USP14 and UCHL5, (proteasome DUBs), that have a strong exo-cleavage preference driven by a structural component that allows binding of chains to only promote exo-cleavage.

4. The authors generate mutations based on the cryo-EM structure that confer higher USP1 DUB activity. Could the authors comment if these residues (or this region) are mutated or have SNPs that may indicate a role in disease.

We have not been able to identify this. USP1 is generally speaking relatively rarely mutated in cancer (in e.g. cbioportal, cosmic), and we did not find any mutations in these residues. We are not aware of other diseases where USP1 is mutated.

5. The authors suggest a model for USP1/UAF1's role in directing the DNA damage tolerance

pathway choice. In the absence of cellular data supporting their model, I would advise that this be toned down. Alternatively, they could perform cellular experiments to support this hypothesis.

We have followed this advice.

Overall, the results provided in this manuscript are interesting and of good quality but hard to follow in some areas. I would encourage the authors to revise the manuscript to make it more accessible to the non-specialist reader.

Thanks for the constructive review

Rebuttal

We thank the reviewers for their useful comments and vigilance. This allowed us to correct two errors and clarify our arguments, leading to an improved version of the manuscript, where experimental results and speculation are clearly distinguished.

Reviewer #1 (Remarks to the Author):

In general, the manuscript has been improved. There is still a strong focus on "pathway choice" (Figure 7) and this is decidedly not directly addressed in the manuscript. Although the legend to Figure 7 has more hypothetical language than in the original version, it is essentially the same.

1)The speculation about pathway choice as it is presented in the discussion is relatively pointless. I would much rather see the authors discussing their findings with those by Nusawhardana et al (which the authors cite), who showed that USP1 inhibition suppresses gap formation at replication forks in a manner that is at least partially dependent on REV1, and thus on the mono-ubiquitination of PCNA. These findings are seemingly at odds with the notion that USP1 has a strong preference for exo-cleavage in vivo, and would much more support a model in which USP1 does both, deubiquitinating PCNA and the polyubiquitination chains on PCNA. I think the reader will understand that in vitro findings and in vivo regulation are two different issues, but a discussion in the context of the above-mentioned in vivo findings is more valuable than a speculative model on pathway choice that is inconsistent with the current literature.

To further address the perception of overinterpretation, we now removed all speculation from the introduction entirely, so that it only addresses out results. We have also further toned down the abstract: “This unusual mechanism can cause temporal enrichment of monoubiquitinated PCNA during polyubiquitination. It will be interesting to see how this affects DDT pathway balance."

We also realize that we made an unfortunate, and unintentional mistake in the sentence addressing the Nusawardhana paper, where we wrote “in a PCNA ubiquitination independent manner”, which definitely should have been “in a PCNA ubiquitination dependent manner”. Apologies for that, we have corrected this.

Apart from that, we did not intend to say that USP1 does not cleave monoubiquitinated PCNA. USP1 does indeed do both. We admit that our writing in the discussion may have been confusing. We altered the first paragraph of the discussion to summarize what we have found in our paper. Then we adjusted the second paragraph to clarify that this is our interpretation: “With this kinetic mechanism in mind, we can speculate about a potential role of USP1/UAF1 in directing DNA damage tolerance (Fig. 7), in anticipation of *in vivo* experiments that are required to verify this model.”

In that paragraph we now further clarify that USP1 also cleaves PCNA-Ub: “when PCNA is monoubiquitinated and translesion synthesis takes place, USP1/UAF1 will deubiquitinate PCNA-Ub after the lesion has been bypassed. However, when both mono- and polyubiquitination take place, or when there is only polyubiquitination, the kinetic data suggests that USP1/UAF1 will primarily trim chains, which could temporarily enrich for monoubiquitinated PCNA.“

With this addition, our discussion builds on the foundations about DNA damage tolerance that we introduced in the introduction (or Fig. 7 legend). It should now be more clear that the exo-cleavage preference only comes into play when there is only polyubiquitinated PCNA, or when both mono- and polyubiquitinated PCNA are present. In that case our data suggest that USP1 will prefer to cleave the latter.

2)On page 11, lines 491, the authors claim that "The findings of this study reveal how the intrinsic.....a role in regulating DNA damage tolerance". I do not agree with this statement. The authors present biochemical and biophysical data that describe a strong preference for exo-cleavage in vitro, and only in vitro. We don't know whether this intrinsic biochemical property is modulated in vivo to work differently.

We agree that our data do not show how this works in vivo, but we think biochemical analysis could lay the ground work for future, in vivo studies. In order to reflect this better, we have changed the sentence to: ”The findings in this study reveal detailed, mechanistic insights into how USP1/UAF1 operates on polyubiquitinated PCNA in vitro. It is possible that these kinetic mechanisms play a role in in regulation of PCNA in DNA damage tolerance. “

Reviewer #2 (Remarks to the Author):

For the most part the authors have addressed our previous concerns.

However, there are still a few points that I am a little confused about, and would appreciate some clarification.

1. the authors did not respond to the suggestion of comparing UbVAUb with UbUbTAMRA to see if VA slows down the deUb reaction - did they just miss the comment, or have I missed something in the revised manuscript?

We may not have been sufficiently clear in our previous rebuttal: The UbVaUb substrate makes a covalent complex that inactivates USP1 after the first turnover. Therefore we can not compare the speed on this substrate with a normal/UbUbTMR substrate. With progressively less USP1, the reaction with UbvaUb will slow down over time, which is not the case for UbUbTMR, making a comparison meaningless.

2. There is no legend for figures 4D and 4E.

Thanks for pointing that out, apologies for missing that. We have added the legend to the figures.

3. The authors state: "As USP1/UAF1 is highly efficient, all distal Ub-TMR is rapidly cleaved from PCNA-Ub3 in Fig. 2D after which USP1/UAF1 proceeds with cleavage of the remaining PCNA-Ub2 within 30 seconds." Perhaps a shorter time point would show exclusive exo-cleavage, but with the current data it's hard to draw that conclusion.

We come to the exclusive exo-cleavage conclusion by a process of elimination. We adjusted the results to explain this thought process and make it explicit what bands could be expected for each mechanisms and which are visible on the gel: We do not see bands that would indicate endo-or base-cleavage.

We have changed the results as follows: ”We do not observe a band for fluorescent USP1+PCNA-Ub3, which would indicate endo-cleavage, or a band expected for base- cleavage (either a free fluorescent Ub3 band, or a USP1+UbVaUb (as detected in 2B)). All labeled, cleaved ubiquitin appears as a free UbTMR, and USP1 conjugates to PCNA-Ub2, thus indicating exo-cleavage.”

4. Our comment about concentrations - comparing figure 2D to figure 6E seems to show a different outcome - explicitly in fig 2D there is complete loss of fluorescent signal before 30s, while in 6E, there is still signal way longer than that. There is no concentration listed in the legend for figure 2D of the substrate (despite the authors stating in their response that the concentrations are in the text - I couldn't find it in the text or legend, and I can't tell from the methods section what the concentration of PCNA-Ub-VA-Ub-Ub-TMR is - is it 0.67, 1.33, or 2.67uM?), whereas in figure 6E, the substrate concentration is on the y axis, and I am still struggling to be able to compare the two experiments, can the authors please tell me how to do so? Would it be easier to interpret a ratio of enzyme:substrate for example? If the conc is 2.67uM that is comparable to the 4uM starting conc in fig 6E, in which case the differences in remaining signal after 30 secs is quite stark.

As discussed above, the crosslinking experiments were designed to make sure that bands were visible on a gel after a single turnover reaction. Therefore conditions are very different. As stated in the methods, the concentration of PCNA-Ub_{va}Ub-Ub^{TMR} trimers was 2 uM. To make this more explicit we now specify the concentrations in the legend of Figure 1, and in results we mention: ”In these assays, we used a relatively high concentration of USP1/UAF1 (200 nM), to make sure the bands are clearly visible on gel”

The enzyme concentrations are important here. In the UbvaUb conjugation experiments, the concentration of USP1/UAF1 (200 nM) is 20-fold higher than the concentration of USP1/UAF1 in the figure 6 experiments (10 nM). The initial reaction rate therefore with the same concentration of substrate, is 20-fold faster. With this 20-fold increase in mind, we think both experiments show similar results and there are still minimal amounts of PCNA-Ub_{va}Ub-Ub^{TMR} are still visible on the fluorescent image from 0.5 to 4 minutes.

However, we did not intend for Fig. 1D to be compared to the FP assays, as Fig. 1D is intended as a qualitative assay and the FP assays are quantitative.

5. Our original review we raised the following concern: "figure 1D the right hand panel doesn't show an increase in the amount of USP1-PCNA-Ub2 despite the legend and text asserting that it does. It shows a decrease in the amount of USP1, which lends some support, but where has the USP1 gone if it is not visible in the conjugate lane? It is clear in figure 1B, so why not in this panel?" In the new 'improved' experiment in Fig 2D, there is still a loss of the USP1-PCNA-Ub2 signal, which is inconsistent with the earlier experiments (now Fig2C). We are not disputing the data, data are data! But we are still confused by the assertion in the text that this complex is being formed when it looks in the gel as though it is decreasing. The USP1 decreases but the complex does not increase, how do the authors interpret this in light of their preferred explanation?

It is indeed an occasional issue that in our Coomassie stained gels USP1 disappears prematurely (see e.g. 2F). We have not been able to address this issue satisfactory. However, we have tested activity, and the proteins retains full activity even after 30 minutes compared to a fresh aliquot.

6. Given how much of it has been toned down, due to the overstating in the original manuscript, I'm not sure that the use of 'enrichment' in the title and abstract is reasonable.

The enrichment reflects the biochemical results in this study. It is observed in our kinetic analysis (Figure 3) and in the assays of Fig. 2EF. In the text we highlight that we can observe this enrichment/accumulation in the Fig. 2EF assays (in line with figure 3 assays), and that the enrichment depends on the presence of poly-ubiquitin chains on PCNA.

To even better reflect our data we changed the title to: "USP1/UAF1 targets polyubiquitinated PCNA with an exo-cleavage mechanism that can temporarily enrich for monoubiquitinated PCNA" to also reflect that this enrichment is temporal and not absolute.

This review was prepared by both reviewers 2 and 3.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4 (Remarks to the Author):

The authors have addressed all the comments and concerns of the reviewers and the manuscript is significantly improved. I am happy for it to accepted for publication.