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Dysregulation of DNA polymerase κ recruitment to replication forks results in genomic instability

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 09 August 2011

We have now received the reports of three referees who had assessed your manuscript on USP1 and Pol kappa. As you will see, all of them consider the study and its findings of interest and in principle suited for publication in The EMBO Journal, pending satisfactory revision to address a number of technical and interpretational/conceptual issues detailed in the reports. Safe for the further-reaching point 6 of referee 2 (extension of the study to analyze ELG1 roles), all of these concern appear to be well-taken and directly relating to the data shown, so I would like to invite you to respond to them in the form of a revised version of the manuscript.

In this respect, it will be important to address key technical points (such as the siRNA off-target problem raised by referees 1 and 2) and criticisms regarding experimental conclusiveness (e.g. the concern on slow fork compensation indicated by referees 2 and 3). Referee 2 comments on some more general conceptual issues regarding relative effects of USP1 loss on FA and Pol kappa pathways, and referee 3 recommends to overall shift some more emphasis towards the translesion polymerase aspects. Finally, please carefully check the insertion of Greek letters and the completeness of references and in-text citations, and amend the revised manuscript with a brief author contribution statement and conflict of interest declaration.

Pending adequate revision, we should be happy to consider the study further for publication. Please note however that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. When preparing your letter of response to the referees' comments, please also bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:

http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Please do not hesitate to contact me in case you should have any additional question regarding this decision. I look forward to your revision.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript by Jones et al. describes the impact of the Deubiquitinase Usp1 on genome integrity. They showed that Usp1 removes PCNA, FANCD2 and FANCI monoubiquitination throughout the cell cycle and that Usp1 protects against genome instability independent of FA-pathway. The major mechanism by which Usp1 promotes genome stability is by inhibiting the PolKappa-recruitment without damage. The experiments are well designed and conducted. The resulting data is conclusive. Especially the rescue experiments give detailed insight into the direct impact of Usp1 on genome stability.

Major points:
1) Due to the known problem of specificity of the RNAi-methodology the usage of a single siRNA targeting Usp1 for the whole analysis should be further addressed. Ideally, rescue experiments using siRNA-insensitive Usp1 overexpression are conducted.

Minor points:
1) This reference is not complete: Lin JR, Zeman MK, Chen JY, Yee MC, Cimprich KA (2011) SHPRH and HLTF Act in a Damage-
Specific Manner to Coordinate Different Forms of Postreplication Repair and Prevent Mutagenesis. Mol Cell

Referee #2 (Remarks to the Author):

In this study, Jones et al attempt to understand why intrinsic genomic instability is observed in cells lacking USP1 function. The authors confirm previous findings that depletion of USP1 results in an elevated level of PCNA, FANCI and FANCD2 ubiquitination. They concentrate on the increased PCNA ubiquitination as a driving factor in the increase of genome instability. They find that cells depleted of USP1 have increased number of micronuclei, which they use as a marker for genomic instability throughout the paper. USP1-depleted cells also have increased foci of Pol and Pol when these are overexpressed, but only the depletion of Pol decreases the micronuclei formation in the setting of USP1 depletion. The authors also found that overexpression of Pol or forced localization of it with PCNA leads to genomic instability. Based on their finding that USP1-depleted cells have slow replication forks, a phenotype that is rescued by Pol depletion, the authors propose a model whereby inappropriate engagement of Pol and PCNA, as a result of loss of USP1 function, interferes with DNA synthesis that ultimately results in replication stress and genomic instability.

I have a conceptual issue with the paper and it stems from the USP1 participating in both the

Fanconi anemia pathway and the PCNA-Ub pathway and the difficulty in separating these two functions. The authors show in Figure 3C that the FANCI depletion as well as FANCA depletion leads to increase in micronucleation. Depletion of USP1 alone gives rise to a similar level of micronucleation as FANCI or FANCA depletions. Co-depletion of FANCI and USP1 causes a further increase in micronucleation. We know from previous work that at least some of the USP1 depletion phenotype is due to USP1's effect on the Fanconi anemia pathway. The further increase in micronucleation seen in the double depletion of FANCI and USP1 is presumably dependent on USP1-PCNA functional interaction (or incomplete depletion with siRNAs as I point out below). However, as shown in figure 3A, depletion of Pol causes a full rescue of the micronucleation pehonotype in the USP1 depleted cells, as if the Fanconi anemia branch of the USP1 function had no role in generating the instability. This is unlikely based on the known cellular and mouse KO phenotypes where lack of USP1 renders the Fanconi pathway not functional. With Pol gone, USP1 depletion should still impact the Fanconi anemia pathway and cause instability. Given the data in this paper, it is hard to explain why there is a full rescue of the micronucleation phenotype with Pol and other assays besides the micronucleation are needed to really figure out what is going on. If the authors result is correct, they should entertain the possibility that somehow the Fanconi anemia pathway inhibition (by USP1 depletion) is also suppressed by Pol depletion.

Other major points:

1. The central theme of the paper is intrinsic genomic instability resulting from USP1 depletion. However, the authors rely on micronucleation assay as the sole marker for this phenomenon. This assay needs to be correlated with a more widely used indicators of the instability. The best of them would be analysis of the chromosomal abnormalities on metaphase spreads. The authors mention fragile site abnormalities as a possible source of the instability and they would be able to see those on metaphase spreads. The other assay might be differential cellular sensitivity to different damaging agents- for example a crosslinker to look at the Fanconi pathway and aphidicolin to look at the DNA replication pathway. Some of the findings should also be replicated in a different cell line than the U2OS cells.

2. The authors use protein depletion using just one siRNA for most siRNAs in this study without rescuing the phenotypes with siRNA-resistant cDNAs. This is not ideal given high rates of off target effects of siRNAs. The authors could have used the available USP1 KO mouse cells and Fanconi anemia patient cell lines for many of the experiments. Because these would be nulls, any additional depletions would be much easier to interpret. Any kind of epistasis analysis in the setting of possibly incomplete depletions (hypomorphic alleles) is not possible. For example, it is possible that the increase in the micronucleation that was seen with co-depletion of FANCI and USP1 (Figure 3C) was due to incomplete depletion with single siRNA and more complete inhibition of the Fanconi anemia pathway when the two were depleted together. Using the null cell line for one of the involved genes and depleting the other component would have been a proper experiment to do.

3. There is another conceptual problem with Figure 3C. USP1 depletion inhibits the Fanconi anemia pathway so inhibition of the same pathway would not be expected to suppress the phenotype of micronucleation. To test if some of the micronucleation in the USP1-depleted cells was due to the Fanconi anemia pathway inhibition, one would have to engineer a cell line in which FANCD2 and FANCI were appropriately regulated even without USP1. I am not suggesting that the authors do that since that would be extremely difficult, but I am just pointing out that they got an expected result and that they cannot conclude that "the FA pathway does not promote genomic instability caused by USP1 depletion" (page 9)

4. An important connection between genomic instability and loss of USP1 function is via increased interaction between Pol and PCNA. The conclusion that genomic instability associated with overexpression of Pol is through the engagement of the UBD domain with ubiquitinated PCNA needs further support. An IP that shows loss of interaction between

PCNA and Pol would provide this and complete the experiment in Figure 4A. The only IP performed in the study is in Figure 4D that examines an artificial constitutive interaction of Pol p21 PIP WT and PCNA.

5. It is surprising that USP1 depletion is associated with slower fork speed but not slower progression through S-phase. Although it is discussed in Page 17 that is reduced fork speed is compensated for by an overall increase in origin firing/initiation, the evidence is absent. The DNA fiber analysis is incomplete and the Pol mutants from Figure 4 should also be included to show that the fork sppeds are also decreased in those settings. Cell cycle analysis and/or growth rates of those cells should also be included to make sure that the decrease in micronucleation is not due to poor growth.

6. Human ELG1 has been identified to play a role in regulating the level of ubiquitinated PCNA through its interactions with PCNA and USP1 but is not involved in regulating FANCD2 monoubiquitination. Examination of the effect of depleting ELG1 on genomic instability would be helpful in dissecting the USP1's function of genome maintenance independent of FANCD2 and FANCI de-ubiquitination.

Minor points:

1. The 53BP1 foci shown in Figure 2D are not commonly used as markers for elevated levels of replication stress but rather double strand break formation.

2. In some places the Greek letters do not come through (including the abstract and some figures)

Referee #3 (Remarks to the Author):

In this paper Tony Huang and colleagues show that depletion of the DUB USP1 in U2OS cells results in increased micronuclei formation, which they subsequently show is dependent on pol kappa. In further elegant studies they show that dysregulated recruitment of pol kappa to replication forks generates micronuclei, whereas similar dysregulation of the related pol eta does not have this effect. This work highlights an important difference between the role of pol eta, which clearly protects from genome instability, and pol kappa, which causes genome instability. Some issues need clarifying, but overall, this paper provides important insights into this Y-family polymerase.

Specific comments:
1. Title: I don't feel that the title gives sufficient emphasis to the most important aspects of the paper. The authors started off with USP1 depletion, but the paper is more about pol kappa. I would suggest something along the lines of "Dysregulation of pol kappa recruitment to replication forks results in genome instability." Likewise, the abstract should put more emphasis on pol kappa rather than on USP1.

2. P. 7 2nd para: Ogi et al (2005) showed that the ability of pol kappa to accumulate at foci was much less than that of pol eta. Although this paper is cited, this observation is not mentioned and it's glossed over both in the text and in Fig 2C. In Fig 2C the fold change in foci formation is plotted. The actual % of cells with foci should be shown rather than the fold change. The implication of the text is that pol eta and pol kappa foci formation is similar. They show in Fig 2B, like Ogi et al, that co-localisation of pol kappa and PCNA is actually quite poor. They don't show pol eta data but there is a lot of evidence in the literature that pol eta colocalisation with PCNA is very good. Since the work in the current manuscript provides a good explanation for this difference, there is no reason to conceal it.

3. A key conclusion from the data of Fig 3, which could be stated more explicitly, is that pol eta and the FANC proteins protect cells from genome instability, whereas pol kappa has the opposite effect.

4. The effect of the p21 PIP does indeed look dramatic in Fig 4C, but only single nuclei are shown in the panels. Some quantitation is needed.
In Fig 4D and associated text, the authors should be a little bit more circumspect in attributing the band to di-ubiquitinated PCNA. They are probably right, but as they haven't shown it directly, it would be more appropriate to refer to it as presumptive.
Finally, it would be very informative to know if the catalytically dead pol kappa showed the same increase in foci formation as the wt. Please provide this information.

5. Fig 5A shows a reduction in fork speed in USP1-depleted cells. In the Discussion, p.17, l.9 they suggest that this reduction must be compensated by increased origin firing. This is correct, but they should have been able to observe this as a reduction in inter-origin distances. Was this in fact observed?

6. Discussion, para 2 (p.14, l.17-18).The stated phenotype of pol kappa is not unique. Inactivation of pol iota also has no phenotype.
Discussion, para 4 (P.15, l.16 and following). Could a further contributory factor be a lower Km for pol kappa than other polymerases?

7. Minor points:
P.6, l.12 Utani et al, and p.15, l.11 Biertumpfel et al: dates required
Suppl Fig 1C needs more explanation, to indicate that the PI panels represent the distribution of the EdU labelled fraction of the population, rather than the distribution of the whole population.

1st Revision - authors' response 04 November 2011

Manuscript *EMBOJ-2011-78755*, "Dysregulation of translesion synthesis polymerase kappa recruitment to replication forks results in genomic instability" by M. K. Jones et al.

Here is our point-by-point response to the reviewers' comments:

Reviewer #1

The manuscript by Jones et al. describes the impact of the Deubiquitinase Usp1 on genome integrity. They showed that Usp1 removes PCNA, FANCD2 and FANCI monoubiquitination throughout the cell cycle and that Usp1 protects against genome instability independent of FA-pathway. The major mechanism by which Usp1 promotes genome stability is by inhibiting the PolKappa-recruitment without damage. The experiments are well designed and conducted. The resulting data is conclusive. Especially the rescue experiments give detailed insight into the direct impact of Usp1 on genome stability.

Major points:
1) Due to the known problem of specificity of the RNAi-methodology the usage of a single siRNA targeting Usp1 for the whole analysis should be further addressed. Ideally, rescue experiments using siRNA-insensitive Usp1 overexpression are conducted.

We have addressed the reviewers' (reviewer 1 and also reviewer 2) concerns in two ways:

1) We used a second siRNA oligo sequence against USP1 for our analysis. We found that using this second USP1 siRNA sequence, we were able to validate our results concerning increased micronuclei formation after USP1 depletion. Importantly, the increase in micronuclei formation caused by the transfection of the second USP1 siRNA sequence can also be rescued with the addition of a Polκ siRNA (similar to the study done in Figure 3A). We have included this new data in the new Supplemental Figure 3. This argues against the likelihood of a siRNA off-target effect for USP1 (sequence 1). Also, the genomic instability caused by the transfection of even the first USP1 siRNA (sequence 1) could be rescued by two different Polk siRNAs (Figure 3A). This also lessens the possibility of an off-target effect for USP1 siRNA (sequence 1).

2) We also tried rescue experiments using siRNA-insensitive USP1 overexpression (for sequence 1). While we were able to successfully design this USP1 siRNA resistant expression construct (Myc-USP1 WT siResis1) (see Reviewer-only Figure below), we found that it was technically challenging to introduce the appropriate expression levels of USP1 from an exogenous promoter with transient or stable transfection experiments. Nevertheless, we were able to rescue the ubiquitination levels of PCNA using this methodology (reduction of Ub-PCNA after USP1 knockdown using sequence 1 followed by simultaneous expression of Myc-USP1 WT siResis1). However, we discovered, unexpectedly, that overexpression of Myc-USP1 WT alone could elevate the micronuclei formation in cells both in the presence and absence of endogenous USP1 knockdown (see Reviewer-only Figure below). Unfortunately, this precludes us from interpreting the data from the siRNA rescue experiments. While it is beyond the scope of this study, we speculate that exogenous expression of USP1 (above normal endogenous levels found inside the cell) could possibly lead to the reduction of FANCD2 and FANCI monoubiquitination, which may then negatively affect genomic stability in these cells. Alternatively, overexpression of USP1 may stabilize a "yet-to-be-determined" mutagenic factor(s) through its DUB activity

(similar to how USP1 can prevent the ubiquitin-mediated degradation of ID1, ID2, and ID3 proteins in a recent elegant study by Dixit and colleagues) (Williams et al. Cell 2011). In our past studies, we have discovered several ways the cell can control the protein stability of USP1 (Cotto-Rios et al. JCB, 2011; Huang et al. NCB, 2006). Therefore, it does not come as a surprise that USP1 protein levels need to be fine-tuned throughout the cell cycle in order to prevent genomic instability in human cells.

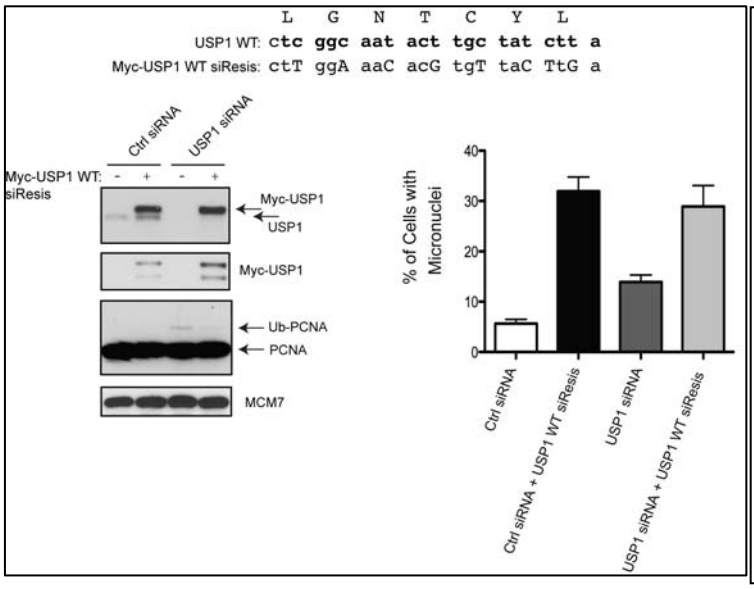

Figure 1. Generation of siRNA Resistant USP1 expression construct. (Top) Schematic of silent mutations introduced in the Myc-USP1 WT siResis construct to prevent being targeted by the USP1 siRNA (sequence 1). Letters on top denote amino acids encoded by the USP1 cDNA sequence. DNA sequence in bold represent the USP1 oligo siRNA (sequence 1). U2OS cells were transfected with USP1 siRNA (sequence 1) for 24 hours, then subsequently transfected with empty vector or Myc-USP1 WT siResis construct for another 24 hours. Cells were then replated for Western blot analysis (Left) or for micronucleation assay (Right) and terminated 24 hours later.

Minor points:
1) This reference is not complete: Lin JR, Zeman MK, Chen JY, Yee MC, Cimprich KA (2011) SHPRH and HLTF Act in a Damage-
Specific Manner to Coordinate Different Forms of Postreplication Repair and Prevent Mutagenesis. Mol Cell

Corrections have been made in the referencing of this paper.

Reviewer #2

In this study, Jones et al attempt to understand why intrinsic genomic instability is observed in cells lacking USP1 function. The authors confirm previous findings that depletion of USP1 results in an elevated level of PCNA, FANCI and FANCD2 ubiquitination. They concentrate on the increased PCNA ubiquitination as a driving factor in the increase of genome instability. They find that cells depleted of USP1 have increased number of micronuclei, which they use as a marker for genomic instability throughout the paper. USP1-depleted cells also have increased foci of Pol κ and Pol η when these are overexpressed, but only the depletion of Pol κ decreases the micronuclei formation in the setting of USP1 depletion. The authors also found that overexpression of Pol κ or forced localization of it with PCNA leads to genomic instability. Based on their finding that USP1-depleted cells have slow replication forks, a phenotype that is rescued by Pol &#x03BA depletion, the authors propose a model whereby inappropriate engagement of Pol κ and PCNA, as a result of loss of USP1 function, interferes with DNA synthesis that ultimately results in replication stress and

genomic instability.

I have a conceptual issue with the paper and it stems from the USP1 participating in both the Fanconi anemia pathway and the PCNA-Ub pathway and the difficulty in separating these two functions. The authors show in Figure 3C that the FANCI depletion as well as FANCA depletion leads to increase in micronucleation. Depletion of USP1 alone gives rise to a similar level of micronucleation as FANCI or FANCA depletions. Co-depletion of FANCI and USP1 causes a further increase in micronucleation. We know from previous work that at least some of the USP1 depletion phenotype is due to USP1's effect on the Fanconi anemia pathway. The further increase in micronucleation seen in the double depletion of FANCI and USP1 is presumably dependent on USP1-PCNA functional interaction (or incomplete depletion with siRNAs as I point out below). However, as shown in figure 3A, depletion of Pol κ causes a full rescue of the micronucleation pehonotype in the USP1 depleted cells, as if the Fanconi anemia branch of the USP1 function had no role in generating the instability. This is unlikely based on the known cellular and mouse KO phenotypes where lack of USP1 renders the Fanconi pathway not functional. With Pol κ gone, USP1 depletion should still impact the Fanconi anemia pathway and cause instability. Given the data in this paper, it is hard to explain why there is a full rescue of the micronucleation phenotype with Pol κ and other assays besides the micronucleation are needed to really figure out what is going on. If the authors result is correct, they should entertain the possibility that somehow the Fanconi anemia pathway inhibition (by USP1 depletion) is also suppressed by Pol κ depletion.

We apologize for our poor explanation concerning our experimental results with USP1 and the FA pathway. We have modified the text to provide a better explanation of our reasoning behind the experiments and the interpretation of the results (Introduction section, pg 3; Results section, pg 8; Discussion section, pg 13). In summary, while it is true that USP1 participates in the FA pathway, the loss of USP1 does not completely abrograte FA pathway function (only partial inhibition and likely only for crosslink repair). This is an important point that is backed up by data from other groups. What has been previously reported is that loss of USP1 causes crosslinker sensitivity in a FA pathway-dependent manner. However, complete loss of the FA pathway (through knockout of either FANCL or FANCD2) has a more severe MMC sensitivity phenotype than USP1 knockout alone in both mouse and chicken DT40 model systems (Oestergaard et al., Mol Cell 2007; Kim et al., Dev Cell 2009). This suggests that in the absence of USP1, monoubiquitinated forms of FANCD2 and FANCI can still perform crosslink repair functions, albeit in a less efficient manner due to the inability to recycle back to the unmodified forms of the proteins. In our study, we are only measuring genomic instability in undamaged conditions. Thus, even an inefficient FA pathway may still be critical for function in promoting genomic stability.

We have performed various experiments in our paper to test whether this is indeed the case. First, we show that, in our human cell-based system, knockdown of either USP1 or its catalytic co-factor, UAF1, causes dramatic relocalization of both FANCI and FANCD2 to the chromatin fraction (site of DNA damage/repair) (Supplemental Figure 4A). Second, we provide new data that knockdown of USP1 causes an increase in FANCD2 paired mitotic nuclear foci, indicative of unresolved replication stress and possibly fragile site expression (Supplemental Figure 4B). This shows active recruitment of FA proteins (functional FA pathway) to deal with lingering replication stress-induced lesions (Naim and Rosselli, NCB 2009). Third, knockdown

of FANCI or FANCA in the absence of USP1 further elevates micronuclei formation in human cells (Figure 3C). Finally, we provide new data that in FANCA-deficient patient cells, USP1 knockdown can further elevate micronuclei formation (Figure 3D). Thus, this effect we observe in Figure 3C is likely not due to incomplete double siRNA knockdown. These functional data provide strong evidence to support a model whereby the FA pathway protects cells against further genomic instability in USP1-depleted cells.

Other major points:
1. The central theme of the paper is intrinsic genomic instability resulting from USP1 depletion. However, the authors rely on micronucleation assay as the sole marker for this phenomenon. This assay needs to be correlated with a more widely used indicators of the instability. The best of them would be analysis of the chromosomal abnormalities on metaphase spreads. The authors mention fragile site abnormalities as a possible source of the instability and they would be able to see those on metaphase spreads. The other assay might be differential cellular sensitivity to different damaging agents- for example a crosslinker to look at the Fanconi pathway and aphidicolin to look at the DNA replication pathway. Some of the findings should also be replicated in a different cell line than the U2OS cells.

We have now replicated our findings in a different cell line (FA patient-derived fibroblasts corrected with FANCA WT or with vector control) than the U2OS cells (Figure 3D).

Micronuclei are common in cells undergoing genotoxic or replicative stress and may contain entire chromosome pieces or fragments, making them important and highly sensitive indicators of genomic instability (Utani et al., PLoS One, 2010). The usage of the micronucleation assay has recently been effectively employed to highlight replication stress-induced genomic instability in Fanconi anemia cells (Naim and Rosselli, NCB 2009). The reviewer has a valid point in that gross chromosomal abnormalities may be better visualized on metaphase spreads. However, this has already been done previously and it was reported that the level of chromosomal aberrations were fairly low in Usp1-deficient MEFs, with less than 0.5 aberrations per cell in undamaged MEFs (Kim et al., Dev Cell 2009). Unfortunately, we feel it would be too technically challenging to quantify these infrequent events with different siRNA manipulations for non-DNA damage conditions.

We agree that it would perhaps be interesting to see whether there are specific sensitivities to DNA damaging agents, such as crosslinkers, UV, or aphidicolin, in USP1-depleted cells. We plan to investigate this question but we feel that it is beyond the scope of our current paper, since we are only trying to understand the molecular mechanism of how USP1 protects cells against genomic instability in untreated cells. We anticipate that for more error-free DNA lesion bypass, different DNA damage-induced lesions will require recruitment of selective TLS Pols to PCNA. How this can be achieved is currently an important area of study.

2. The authors use protein depletion using just one siRNA for most siRNAs in this study without rescuing the phenotypes with siRNA-resistant cDNAs. This is not ideal given high rates of off target effects of siRNAs. The authors could have used the available USP1 KO mouse cells and Fanconi anemia patient cell lines for many of the experiments. Because these would be nulls, any additional depletions would be much easier to interpret. Any kind of epistasis analysis in the setting of possibly incomplete depletions (hypomorphic alleles) is not possible. For example, it is possible that the increase in the micronucleation that was seen with co-depletion of FANCI

and USP1 (Figure 3C) was due to incomplete depletion with single siRNA and more complete inhibition of the Fanconi anemia pathway when the two were depleted together. Using the null cell line for one of the involved genes and depleting the other component would have been a proper experiment to do.

We have validated our results with a second siRNA sequence and used the FANCA-deficient patient fibroblasts in conjunction with USP1 siRNA knockdown for analysis (see above responses). Unfortunately, we were unable to obtain USP1 KO MEFs from the D'Andrea Lab in a timely manner.

3. There is another conceptual problem with Figure 3C. USP1 depletion inhibits the Fanconi anemia pathway so inhibition of the same pathway would not be expected to suppress the phenotype of micronucleation. To test if some of the micronucleation in the USP1-depleted cells was due to the Fanconi anemia pathway inhibition, one would have to engineer a cell line in which FANCD2 and FANCI were appropriately regulated even without USP1. I am not suggesting that the authors do that since that would be extremely difficult, but I am just pointing out that they got an expected result and that they cannot conclude that "the FA pathway does not promote genomic instability caused by USP1 depletion" (page 9)

We have changed our concluding sentence in the Results section (page 9). Please see above explanation for the USP1 and FA pathway connection.

4. An important connection between genomic instability and loss of USP1 function is via increased interaction between Pol κ and PCNA. The conclusion that genomic instability associated with overexpression of Pol κ is through the engagement of the UBD domain with ubiquitinated PCNA needs further support. An IP that shows loss of interaction between PCNA and Pol κ would provide this and complete the experiment in Figure 4A. The only IP performed in the study is in Figure 4D that examines an artificial constitutive interaction of Pol κ p21 PIP WT and PCNA.

We have now included new data to show that USP1 knockdown enhances the association of GFP-Polκ WT with PCNA (Figure 2D).

5. It is surprising that USP1 depletion is associated with slower fork speed but not slower progression through S-phase. Although it is discussed in Page 17 that is reduced fork speed is compensated for by an overall increase in origin firing/initiation, the evidence is absent. The DNA fiber analysis is incomplete and the Pol κ mutants from Figure 4 should also be included to show that the fork speeds are also decreased in those settings. Cell cycle analysis and/or growth rates of those cells should also be included to make sure that the decrease in micronucleation is not due to poor growth.

We have showed that USP1 knockdown in U2OS cells does not affect DNA synthesis levels and cell cycle progression compared to Ctrl knockdown cells in the EdU pulse chase experiment (Supplemental Figure 1C). It is generally accepted (not quite as novel) and has been shown by others that perturbations that cause reduced fork speed can be compensated for by an overall increase in origin firing/initiation events in order to finish S phase in a timely manner (Anglana

et al., Cell 2003). In relation to our work, a study has shown that overexpression of Polκ can result in a slower replication fork coupled to a decrease in inter-origin distances, suggesting that an overall increase in initiation events is important for maintaining the timing of S phase (Pillaire et al. Cell Cycle, 2007). Although we speculate that this may be occurring in USP1-depleted cells, more experiments are obviously necessary to prove this point (as commented by the reviewers). On a technical point, it is tremendously more challenging to generate data on inter-origin distances because it is necessary to follow (with confidence) a single DNA fiber long enough to capture at least two distinct initiation events. Unfortunately this requires fibers of a better quality and length than we were able to prepare during this time. We toned down our claim concerning slow fork compensation and provide discussion points for future analysis (Discussion section, paragraph on page 16-17).

6. Human ELG1 has been identified to play a role in regulating the level of ubiquitinated PCNA through its interactions with PCNA and USP1 but is not involved in regulating FANCD2 monoubiquitination. Examination of the effect of depleting ELG1 on genomic instability would be helpful in dissecting the USP1's function of genome maintenance independent of FANCD2 and FANCI de-ubiquitination.

We appreciate the suggestion by the reviewer. It will be interesting to test the role of ELG1 in regulating genomic stability. At this point, we think it is beyond the scope of our study.

Minor points:

1. The 53BP1 foci shown in Figure 2D are not commonly used as markers for elevated levels of replication stress but rather double strand break formation.

Unresolved replication stress due to incomplete DNA synthesis during S phase has recently been shown to transmit lingering DNA damage into G1 phase of successive cell cycles, which are marked by the 53BP1 DNA damage response protein (Lukas et al., NCB 2011; Harrigan et al., JCB 2011). These 53BP1 nuclear bodies may shield chromosomal fragile sites sequestered into these bodies against erosion and serve to prevent loss of chromosome integrity. We have modified the text for the Results section regarding this point (pg 7).

2. In some places the Greek letters do not come through (including the abstract and some figures)

We have edited the Greek letters for the manuscript and figures.

Reviewer #3

In this paper Tony Huang and colleagues show that depletion of the DUB USP1 in U2OS cells results in increased micronuclei formation, which they subsequently show is dependent on pol kappa. In further elegant studies they show that dysregulated recruitment of pol kappa to replication forks generates micronuclei, whereas similar dysregulation of the related pol eta does not have this effect. This work highlights an important difference between the role of pol eta, which clearly protects from genome instability, and pol kappa, which causes genome

instability. Some issues need clarifying, but overall, this paper provides important insights into this Y-family polymerase.

Specific comments:
1. Title: I don't feel that the title gives sufficient emphasis to the most important aspects of the paper. The authors started off with USP1 depletion, but the paper is more about pol kappa. I would suggest something along the lines of "Dysregulation of pol kappa recruitment to replication forks results in genome instability." Likewise, the abstract should put more emphasis on pol kappa rather than on USP1.

We have followed the recommendations of the reviewer and have changed the title and abstract to put more emphasis on Pol kappa rather than on USP1.

2. P. 7 2nd para: Ogi et al (2005) showed that the ability of pol kappa to accumulate at foci was much less than that of pol eta. Although this paper is cited, this observation is not mentioned and it's glossed over both in the text and in Fig 2C. In Fig 2C the fold change in foci formation is plotted. The actual % of cells with foci should be shown rather than the fold change. The implication of the text is that pol eta and pol kappa foci formation is similar. They show in Fig 2B, like Ogi et al, that co-localisation of pol kappa and PCNA is actually quite poor. They don't show pol eta data but there is a lot of evidence in the literature that pol eta colocalisation with PCNA is very good. Since the work in the current manuscript provides a good explanation for this difference, there is no reason to conceal it.

We have added the representative images that show Pol eta foci formation and co-localization with PCNA in Ctrl and USP1 knockdown conditions (new Supplemental Figure 2). We have also modified the text in the Result section to state the fact that Pol kappa foci formation and co-localization with PCNA in the absence of DNA damage is quite poor, which we also observed in our experimental conditions (pg 6). The rationale behind using fold difference/change in assessing polymerase foci formation is that different polymerases may have different baseline percentage of cells with foci in undamaged conditions. Using fold change will equilibrate the baseline differences between each TLS polymerase when analyzing the effects of USP1 knockdown.

3. A key conclusion from the data of Fig 3, which could be stated more explicitly, is that pol eta and the FANC proteins protect cells from genome instability, whereas pol kappa has the opposite effect.

We have followed the recommendation of the reviewer and incorporated this in the text (pg 8-9).

4. The effect of the p21 PIP does indeed look dramatic in Fig 4C, but only single nuclei are shown in the panels. Some quantitation is needed.
In Fig 4D and associated text, the authors should be a little bit more circumspect in attributing the band to di-ubiquitinated PCNA. They are probably right, but as they haven't shown it directly, it would be more appropriate to refer to it as presumptive.
Finally, it would be very informative to know if the catalytically dead pol kappa showed the same increase in foci formation as the wt. Please provide this information.

We now explained the localization of the Polκ p21 PIP chimera more precisely and included new data to show that it is exclusively in nuclear foci during S phase (Supplemental Figure 5A, pg 11). We have also removed the arrow pointing to a di-ubiquitinated PCNA band in Figure 4D-F. However, we included a new data showing that the slower migrating PCNA bands generated from an anti-GFP co-IP of extracts expressing Polκ p21 PIP chimera protein are likely ubiquitinated forms of PCNA. Incubating the IP sample with a recombinant USP2 catalytic domain enzyme (can cleave non-specific ubiquitin chains from proteins, Boston Biochem) can remove the slower migrating PCNA bands (Figure 4F). We also included new data to show that the catalytically dead pol kappa (Polκ p21 PIP CAT) showed the same increase in foci formation as the wildtype version (Supplemental Figure 5C).

5. Fig 5A shows a reduction in fork speed in USP1-depleted cells. In the Discussion, p.17, l.9 they suggest that this reduction must be compensated by increased origin firing. This is correct, but they should have been able to observe this as a reduction in inter-origin distances. Was this in fact observed?

We commented on this issue (see above).

6. Discussion, para 2 (p.14, l.17-18).The stated phenotype of pol kappa is not unique. Inactivation of pol iota also has no phenotype.
Discussion, para 4 (P.15, l.16 and following). Could a further contributory factor be a lower Km for pol kappa than other polymerases?

We have removed the statement and modified the text accordingly (Discussion section, pg 14). We included the point that pol kappa may have a lower Km for ubiquitinated PCNA than other polymerases due to its two UBZ domains (Discussion section, pg 15).

7. Minor points:
P.6, l.12 Utani et al, and p.15, l.11 Biertumpfel et al: dates required
Suppl Fig 1C needs more explanation, to indicate that the PI panels represent the distribution of the EdU labelled fraction of the population, rather than the distribution of the whole population.

We thank the reviewer for the comments and have edited the references and expanded on the explanation for Supplemental Figure 1C in the Supplemental Figure Legend section.

2nd Editorial Decision 16 November 2011

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original reviewers, and I am pleased to let you know that the referee considers the study substantially improved and now in principle suited for publication, pending clarification of a few remaining specific issues (see below). I am therefore returning the study to you for one final round of minor modification, kindly asking you to revise the study according to these suggestions and comments. When re-revising the study, please also carefully check for the manuscript and figures for correct presentation of Greek letters (e.g. I noted that polymerase labels in the model Figure 7 where not accurately reproduced in my PDF version).

I hope you will be able to return a re-revised version as early as possible; after that, we should hopefully be able to swiftly proceed with acceptance and production of the study.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #2 (Remarks to the Author):

The authors took some of the suggestions to heart and improved the paper by performing additional experiments including using a second siRNA against USP1 to confirm their results and using a FANCA mutant cell line to show that USP1 depletion leads to further increase in micronucleation phenotype independent of FA pathway.

I still have a couple of issues.

In figure 2D, which has been added, the increase of the GFP-Polkappa and PCNA interaction upon USP1 depletion is not very convincing. There is clearly more GFP-Pol kappa in the input, so it is hard to judge if the increase that is seen in the IP is meaningful.

In Figure 3D, it would be important to show the extent of rescue of the micronucleation phenotype in USP1 depleted FA-A cells (-/+ FANCA), after depletion of pol kappa. This would be an experiment to complement that shown in 3A and would answer my original conceptual issue (first point in my original review) about how much of the micronucleation phenotype in USP1 cells is due to the FA pathway dysfunction.

Discussion: The authors state "While it is known that the FA pathway can also be regulated by USP1, the functional intersection between FA proteins and USP1 is likely only critical during DNA damage conditions, such as in crosslink repair, but not in the maintenance of genomic stability in undamaged cells." I have to object to this statement since there is really no data in the paper that speaks to that. The fact that for most of the experiments the authors did not use DNA damage conditions does not mean that the USP1-FANCI/D2 crosstalk is not important under those conditions. The FA cell lines have abnormalities even without damage (as shown by others and in this paper). The above statement is unnecessary for the interpretation of the USP1-PCNA-polkappa axis, which is the focus of the paper, and will create confusion in the Fanconi anemia field.

2nd Revision - authors' response 16 November 2011

Manuscript *EMBOJ-2011-78755R*, "Dysregulation of DNA polymerase kappa recruitment to replication forks results in genomic instability" by M. K. Jones et al.

Here is our point-by-point response to the comments of Reviewer #2:

Reviewer #2

The authors took some of the suggestions to heart and improved the paper by performing additional experiments including using a second siRNA against USP1 to confirm their results and using a FANCA mutant cell line to show that USP1 depletion leads to further increase in micronucleation phenotype independent of FA pathway.

I still have a couple of issues.

In figure 2D, which has been added, the increase of the GFP-Polkappa and PCNA interaction upon USP1 depletion is not very convincing. There is clearly more GFP-Pol kappa in the input, so it is hard to judge if the increase that is seen in the IP is meaningful.

The reviewer is correct in observing a slight increase in GFP-Pol kappa protein in the input lanes after USP1 knockdown. We frequently see this protein stability effect on TLS polymerases after USP1 knockdown or UV damage (Huang et al,. Nat Cell Biol 2006). We think this is not simply due to some technical problems like pipeting errors, but actually is reflected by the condition or state of ubiquitinated PCNA. Conditions that increase or elevate PCNA ubiquitination encourages TLS polymerase association with the modified PCNA. This interaction may enhance the stability of the TLS polymerase itself. This effect has been observed in other papers specifically for Pol kappa when doing the crosslink IP experiments (after UV or BPDE damage) (Guo et al., JBC 2008; Bi et al., MCB 2006), and also for other TLS polymerases. We are currently looking into how TLS polymerase stability can be controlled in a UBD-dependent manner.

In Figure 3D, it would be important to show the extent of rescue of the micronucleation phenotype in USP1 depleted FA-A cells (-/+ FANCA), after depletion of pol kappa. This would be an experiment to complement that shown in 3A and would answer my original conceptual issue (first point in my original review) about how much of the micronucleation phenotype in USP1 cells is due to the FA pathway dysfunction.

Since USP1 knockdown in FA-A cells dramatically enhanced genomic instability, this strongly suggests that these two pathways (USP1 vs FA pathway) mediating genomic integrity are not epistatic to each other. Another way to show this would be to knockdown Pol kappa and show that it will have no effect on preventing genomic instability in FA-A cells. This is testable, but is beyond the scope of this study, since we are not trying to understand how the FA pathway protects against genomic instability in this study.

Discussion: The authors state "While it is known that the FA pathway can also be regulated by USP1, the functional intersection between FA proteins and USP1 is likely only critical during DNA damage conditions, such as in crosslink repair, but not in the maintenance of genomic stability in undamaged cells." I have to object to this statement since there is really no data in

the paper that speaks to that. The fact that for most of the experiments the authors did not use DNA damage conditions does not mean that the USP1-FANCI/D2 crosstalk is not important under those conditions. The FA cell lines have abnormalities even without damage (as shown by others and in this paper). The above statement is unnecessary for the interpretation of the USP1-PCNA-polkappa axis, which is the focus of the paper, and will create confusion in the Fanconi anemia field.

We understand the concerns of the reviewer and have modified the text accordingly (see Discussion section).