THE EMBO JOURNAL

Manuscript EMBO-2009-73211

Rad8Rad5/Mms2-Ubc13 Ubiquitin Ligase Complex Controls Translesion Synthesis in Fission Yeast

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Review timeline:

Submission date:	20 November 2009
Editorial Decision:	18 December 2009
Additional correspondence (Appeal):	12 January 2010
Editorial Decision:	19 January 2010
Revision received:	02 March 2010
Editorial Decision:	25 March 2010
Revision received:	07 April 2010
Acceptance letter:	12 April 2010

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 18 December 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are enclosed below. As you will see, these reviewers unfortunately do not offer strong support for publication of the study in The EMBO Journal. Although the referees consider, to varying extents, your current findings and conclusions potentially interesting, none of them appears to be convinced that your present set of experiments has provided sufficiently decisive evidence to strongly support the proposed interpretations. I will not repeat the individual and often overlapping points of criticism in this letter, however a recurring concern is that alternative interpretations do not appear to be conclusively ruled out. In light of these unanimous opinions, I am afraid we will not be able to proceed with publication of the study, and I hope you will understand that we also do not see ourselves in the position to invite - and thus to some degree commit to - a revised version of the manuscript, given that it is currently not at all clear whether further experimentation to address the referees' concerns would in fact be able to substantiate the main conclusions, or rather confound them. I am sorry we cannot be more positive on this occasion, but would in any case like to thank you for the opportunity to consider this manuscript, and hope that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

Referee #1 (Remarks to the Author):

Using a plasmid-based in vivo assay for translesion synthesis in S. pombe, Coulon et al report in this manuscript that effective TLS requires RAD8, the fission yeast homologue of S. cerevisiae RAD5. Most current thinking sees RAD8/RAD5 as being involved in promoting an error-free, recombinational mode of DNA damage avoidance, through a combination of its helicase activity (demonstrated for RAD5) and ability to polyubiquitinate PCNA. However, the notion that the division in the RAD6 pathway between translesion synthesis mediated by PCNA monoubiquitination and damage avoidance mediated by PCNA polyubiquitination may not be quite so straightforward has been around for a long time.

SIGNIFICANCE
RAD5 was initially identified in a screen for mutants defective in UV-induced mutagenesis and was initially dubbed REV2 (REV for reversionless) [Lemontt 1971, Genetics 68, 21-33]. However, subsequently this phenotype was only seen for some rad5 alleles and at certain reporters. Using assays in which individual lesions and the outcome of their bypass can be more clearly linked, recent data has demonstrated a clear defect in translesion synthesis in RAD5 mutants both for abasic sites [Pages et al., 2008, Genetics 180, 73-82) and for T-T [6-4] photoproducts [Zhang & Lawrence 2005, PNAS 102, 15954-15959], although in the former work, from the authors of the present manuscript, this did not involve the helicase or ubiquitin ligase activity of the protein. It must be said that these observations somewhat limit the immediate impact of the present paper and I was rather surprised that some of the historical work was not discussed in more detail.

TECHNICAL ISSUES
The authors configure their very neat plasmid assay to monitor in-frame TLS events and, by placing the DNA lesion opposite a region of heterology, have a nice in-built control for replication of the undamaged plasmid strand, which they monitor primarily by expression of the lacZ' gene. They convincingly demonstrate the kinetics of replication by assaying the proportion of DpnI-resistant plasmid with time. Elegant though this system is, I feel there are a number of issues with this assay that require addressing.

Although the authors state that the assay is configured only to monitor TLS and not damage avoidance of the lesion, I am not so sure that this is the case. I cannot see anything really to prevent a template switch event. Such an event would, as far as I can tell, result in a product indistinguishable from that created by replication of the undamaged strand. [To my knowledge little is known about how a short stretch of heterology opposite a lesion would affect recombinational damage bypass but I would very much doubt that the crossover to the newly synthesised daughter strand would occur at the lesion, rather than shortly before. I would therefore expect that there is sufficient homology here for recombination to occur]. Can the authors provide any firm evidence that this can or cannot happen?

This may go some way to explain the rather surprisingly low frequencies of TLS, even in comparison to S. cerevisiae. If the Lac- colonies comprise both the replicated undamaged strand and the damaged strand replicated by damage avoidance then the frequency of TLS may be underestimated. It is therefore not currently possible, from the data presented, to tell whether a decreased number of Lac+ (i.e. TLS) products is due to failure to replicate the lesion containing strand and hence loss of its progeny or whether it has been replicated by a damage avoidance pathway. It is thus essential that the absolute numbers of recovered replicated plasmids are shown. An estimation of replication of the undamaged strand vs. those generated by DA pathways could be obtained by co-transfecting an identical undamaged plasmid carrying a separate bacterial resistance marker.

It is potentially also important that the assay can only detect in-frame events and not those associated with frameshifts.

INTERPRETATION
The authors put forward the perfectly plausible model in which polyubiquitination acts in effect as a glue to promotes polymerase switching during TLS. However, I do not think that the model

illustrated in Figure 4 is fully consistent with the data shown in Figure 3. If one just takes the case of TLS of the CPD lesion, the data clearly suggest that RAD8 and RAD30 do not work in the same pathway: the rad8/rad30 mutant does much less TLS than either single mutant and actually has a level of TLS comparable to the pcnK164R mutant. It is possible that the interpretation of these results is complicated by the issue of Lac- colonies arising by damage avoidance on the lesion strand, discussed above. In any event I feel that this data needs careful reinterpretation.

I am not convinced that the frequencies of TLS at the TT(6-4)PP are sufficiently high to really be able to draw firm conclusions in this regard.

MINOR POINTS
The polymerases should be named with their proper Greek letters, rather than spelled out

p3, para1, 3rd line from end: 'lesion' not 'lesions'

p9, para 1, line 6: LysK63 - should be either Lys63 or K63

p9, para 2, line 4: 'evidence' not 'evidences'

Referee #2 (Remarks to the Author):

This is an interesting manuscript, as it challenges the current model of how polyubiquitylation of PCNA functions during lesion bypass. Using a plasmid system in S. pombe the authors differentiate between error-free and error-prone translesion synthesis (TLS) events at defined UV lesions and show that the enzymes involved in polyubiquitylation of PCNA are (fully or partially) required for error-free TLS across both CPDs and 6-4 photoproducts. They assign the phenotypes to different combinations of polymerases and conclude that TLS may at least partially account for the enigmatic Rad5-mediated error-free pathway in S. cerevisiae as well.

The experimental system is clean, as error-free and error-prone events are clearly distinguishable by sequencing, and TLS events cannot be confused with other means of bypass due to the non-matching sequence on the undamaged strand. The results therefore appear to be meaningful. However, I do have some concerns regarding (a) events that are not measured by the assay described here and (b) the need for additional control experiments to support some of the statements. My major comments are as follows:

1. TLS events are given as a percentage of total replicated plasmids, but I did not find any information about how efficient replication overall was in the absence of PCNA modification or TLS polymerases. Does the overall recovery of plasmid DNA show any decrease or delay in some of the mutants? I suppose this is difficult to assess due to the late time points at which the DNA is recovered (i.e. after the culture has started to grow, which will level out any possible differences in the efficiency of replication), but the assay might as a result cause a somewhat disproportionate "blowing up" of minor events that are measured because a large number of plasmids are simply not replicated at all. Therefore, we never know whether a drop in TLS as measured here is compensated by TLS-independent events or whether replication of the corresponding plasmid molecules is never completed.

2. Along a similar line, it remains unclear what the relative importance of the TLS events described here is for rad8/ubc13/mms2 function overall. The assay measures the contribution of these genes to TLS, but this does not give any information about the relevance of TLS-independent rad8-dependent events, which are not picked up by the assay. Some orthogonal assay would be important to determine this.

3. According to previous work from the Lehmann lab, rad8 and mms2 mutants are equally sensitive to UV, which would indicate that there is no ATPase-dependent, polyubiquitin-independent aspect of rad8 function, as there is for Rad5 in S. cerevisiae. Based on this, I am not sure whether the results obtained here in S. pombe can be extrapolated to S. cerevisiae, as the authors do at the end of the discussion.

4. Another event that the assay does not show is frameshift errors during TLS, as these would show up as white colonies. What is noted here as a reduction in error-free TLS might simply be a shift towards this type of mutagenic event, possibly because a polymerase (Pol zeta?) that normally wouldn't operate on this lesion is forced to act in a suboptimal way. It would be important to gain some insight on how likely and how frequent this scenario is.

5. Fig. 3A: The notion that rev1 and rev3 do not show a phenotype by themselves does not necessarily mean that they are not involved in error-free TLS across CPDs - they may simply be redundant with another polymerase. In particular, rad30 dinB double mutants still show higher levels of TLS than pcna-K164R mutants. Additional mutant combinations should therefore be tested, both as double and/or triple polymerase mutants and in combination with rad8. The difference between the mutants that can still monoubiquitylate PCNA and those that cannot implies that polyubiquitylation is only partially responsible for TLS, and the additivity of the phenotypes of rad8 and rad30 mutants implies that the polyubiquitin-dependence is not due to Pol eta. These relationships should be worked out in more detail.

6. Fig. 3C: As above, the notion that none of the polymerase single mutants shows a drop in mutagenic TLS does not imply that they are not involved - again they could be redundant. It would therefore be helpful to see mutant combinations. If the authors wish to conclude that a different polymerase is responsible, they should show this by making appropriate mutants, because it is a far-reaching statement to conclude that monoubiquitylation affects polymerases other than those for which ubiquitin binding has been demonstrated.

7. Finally, I am not sure whether these results should be extrapolated to humans or S. cerevisiae without any experimental evidence.

Referee #3 (Remarks to the Author):

This manuscript addresses the role of the Rad8(Rad5)/Mms2-Ubc13 genes in TLS of TT-CPD and TT(6-4) in S. pombe, using a plasmid system. The authors conclude that TLS is regulated not only by the rhp18(Rad18) and monoubiquitination, but also by the Rad8(rad5)/Mms2-Ubc13 system via poly-ubiquitination. The questions addressed are of interest and importance. However, there are considerable concerns about the experimental system, some critical controls are missing, and the results are too preliminary to justify the author's conclusions.

Detailed points:
1. The corresponding author has already published in collaboration with the Prakash lab that in S. cerevisiae Rad5 regulates TLS. The present study shows a similar effect in another yeast, and therefore novelty is low. In the Discussion the authors mention the S. cervisiae paper, but argue that there was no requirement for either the helicase or the ubiquitin ligase functions of Rad5 in S. cerevisiae. However, they did not examine whether the Ub ligase activity of Rad8 and/or its helicase activities are required in S. pombe - which they should do.
Their finding that in S. pombe also UBC13 and MMS2 were required is not sufficient evidence, since UBC13/MMS2 is an E2, and E2s have usually multiple E3s.
2. It appears that the authors consider TLS and damage avoidance as the only mechanisms generating Lac+ and Lac- phenotypes. Another possibility is that neither TLS nor damage avoidance occur: the damaged strand is lost, and the intact strand is selectively replicated. The authors should address this issue and estimate the magnitude of such a mechanism. It might cause a significant error in their estimates of the extents of TLS. In fact it might explain why TLS across the TT(6-4) lesion was 0.8%, which to me looks like background level (see below).
3. The extent of the apparent TLS of the TT(6-4) lesion is extremely low (0.8%), which is essentially at background level. This raises the concern that whatever effects were observed, they were not due to the TT(6-4) lesion. It is not uncommon that preparations of plasmids with a site-specific lesion have 1% or even a larger fraction of with no lesion, or with another 'contaminating' type of damage. The authors should show what percentage of their plasmids indeed carried the TT(6-4) lesion, and describe how they determined it. Same for the TT-CPD plasmid.
4. The authors use a mmr-deficient mutant, which may cause a background of mutations due to low fidelity of replication. What is the frequency of these mutations on each of the strands? This can be estimated by analyzing the frequency and sequence of both Lac+ and Lac- colonies obtained from

plasmids grown in wild-type S. pombe versus their tester strain.
5. If I understood the authors' arguments, they assume that a Lac+ phenotype represents TLS, whereas the Lac- phenotype represents the undamaged strand. The authors argue that all base substitution mutations formed by TLS will lead to a Lac+ phenotype, whereas Lac- colonies are the result of replication of the undamaged strand or tolerance by damage avoidance. However, the UV lesions may produce also frameshift mutations, which should cause a Lac- phenotype by TLS, and minus 1 frameshift mutations on the undamaged strand will cause a Lac+ phenotype independent of TLS. Do the authors have an estimate on whether these possibilities could have biased the interpretation of the results? This is especially important since the strains are mmr-defective, as discussed in point 3 above.
6. The authors have not examined the effect of UV irradiation on TLS in their system. This should be examined.
7. The authors should examine the effect of Rad8(Rad5)/Mms2-Ubc3 mutations on in vivo UV mutagenesis in S. pombe. One would predict a significant effect if Rad8/MMS2-Ubc13 regulate TLS.
8. This method does not really measure extents of TLS (see point 2 above). Therefore the term 'extent of TLS' is misleading. Similarly, in Fig 4 the authors wrote that error-free TLS across TT(6-4) is 70%. This is again misleading. Their estimate of total TLS was 0.8% which is more or less background levels, and one cannot deduce that it is 70% accurate TLS. This "accurate TLS" might in fact be simple replication of background unmodified sequences in the TT(6-4) plasmid.

Additional correspondence (Appeal) 12 January 2010

I wanted to get back to you concerning the above mentioned manuscript. I understand, by reading the comments of the three referees, that we need to explain more thoroughly our assay. However, for having developed and worked with that kind of in vivo TLS assay since 1989, I can assure that the results are highly reliable. Our experience with this assay has mainly been gained working in E. coli, more recently in S.cerevisiae and in S pombe in the present paper.
I feel that all three referees have recognized that our paper offers a completely new perspective on the poorly defined branch of the RAD6/18 pathway that depends upon the Rad8/Mms2-Ubc13 poly-ubiquitin complex. Most of their comments question the way our assay is functioning and in retrospect I feel that this is fully justified. I have written a short document describing how the TLS assay works, it also explains that the assay does not monitor DA.
Could you please reconsider your decision on the basis of this document. I feel that it would be unfair to reject the paper on the basis of what I consider to be a mere misunderstanding of the methodology that we implemented. We would of course need to introduce this additional information into the revised paper and respond to all other concerns the referees have expressed.

Best regards,
Robert

Background information describing the significance of the plasmid-based TLS assay.

When a (short) plasmid that contains a replication-blocking lesion in one strand is being replicated, there is functional uncoupling of the replication machinery at the lesion site: ie, the polymerase that replicates the lesion containing strand is transiently blocked at the lesion site while replication of the undamaged strand proceeds unperturbed (in vivo: [1]; in vitro: [2, 3]). Indeed, the undamaged strand replicates with the same kinetics as either of the strands in an undamaged plasmid [1]. This scenario applies whether the lesion is in the leading or in the lagging strand. Such plasmids are ideal tools to monitor TLS in vivo as they deliver the lesion in the context of a genuine replication fork. In contrast, such probes are not suited for the study of Damage Avoidance since the replication fork does not come to a real stop in view of the relatively short size of the plasmid that allows complete unwinding of the two parental strands. In S.cerevisiae, the replication pattern of plasmids is not affected in strains ubc13 and mms2 deficient in the RAD6/18 error-free branch[4] compared to the wt strain, thus indicating that the system does not monitor DA. In order to monitor DA events one needs to implement plasmid constructs carrying two replication-blocking lesions, one in each strand

([5], Fuchs, RP unpublished results).
In conclusion, the plasmid system with a single replication-blocking lesion in one strand accurately measures the efficiency of replication across a given lesion (TLS) compared to the efficiency of replication of a non-damaged strand. Indeed, in the first replication cycle, the damage-containing strand will suffer from a delay that reflects the intrinsic difficulty to bypass the lesion under investigation, while the undamaged strand replicates with unmodified kinetics. Indeed, we observe no decrease in colony forming ability between unmodified and lesion-containing plasmid an observation that also reflects the fact that a single lesion does not block plasmid amplification as a result of replication uncoupling of the two strands. In the following cycles, the daughter strand that results from the TLS event will now undergo cycles of amplification with the same kinetics as the undamaged strand. After many cycles of replication (here in S. pombe), the plasmid mixture is extracted and the ratio of lac+ plasmids (ie blue colonies in E. coli, originating from the damaged strand) to lac- plasmids that stems from the initially undamaged strand (ie white colonies in E coli) quantitatively reflects the efficiency of the TLS event compared to the efficiency of replication of the undamaged strand that acts as an internal standard. In the absence of lesion the ratio between lac+/lac- is close to 1 as expected. For example, TT_CPD is bypassed much more easily than TT_64. On the other hand, for a given lesion, the assay monitors (quantitatively) the extent at which a given mutant allele affects the TLS pathway in vivo: for ex for TT-CPD, in a pcna-K164R strain TLS is reduced to 5% of its value in a wt strain. As noted by the referees, the assay does not detect frameshift mutations, but this is not a major issue considering the very low frequency of frameshift mutations induced by UV lesions.

1. Pages V, Fuchs RP. Uncoupling of leading- and lagging-strand DNA replication during lesion bypass in vivo. Science 2003,300:1300-1303.
2. Higuchi K, Katayama T, Iwai S, Hidaka M, Horiuchi T, Maki H. Fate of DNA replication fork encountering a single DNA lesion during oriC plasmid DNA replication in vitro. Genes Cells 2003,8:437-449.
3. McInerney P, O'Donnell M. Functional uncoupling of twin polymerases: mechanism of polymerase dissociation from a lagging-strand block. J Biol Chem 2004,279:21543-21551.
4. Pages V, Johnson RE, Prakash L, Prakash S. Mutational specificity and genetic control of replicative bypass of an abasic site in yeast. Proc Natl Acad Sci U S A 2008,105:1170-1175.
5. Zhang H, Lawrence CW. The error-free component of the RAD6/RAD18 DNA damage tolerance pathway of budding yeast employs sister-strand recombination. Proc Natl Acad Sci U S A 2005,102:15954-15959.

2nd Editorial Decision 19 January 2010

Thanks for your message regarding your request that we reconsider our decision on your manuscript EMBOJ-2009-73211, and apologies for the delay in getting back to you on this. As I told you, I sent your rebuttal letter to one of the original referees (#2) to ask whether he/she felt that your explanation allayed the concerns raised as to the validity of your assay. I have now heard back from the referee, who accepts that there had been some confusion about your assay, and who recognises that the points you make do at least partially deal with the criticisms highlighted by the reviewers. Consequently, he/she now feels that it would be appropriate to invite you to submit a revised version of your manuscript.

We are happy to go along with this recommendation, but I would like to make a number of points. Firstly, it will be essential that you adequately explain and clarify your assay system so that it can be understood not only by experts in the field (who already had some difficulties with the system, as revealed in the review) but also by the non-specialist reader. Secondly, the reviewers all raised a number of points not directly related to the system used that will have to be dealt with in your revision. Thirdly, referee 2 - having read your appeal letter and looked again in detail at the manuscript - has concerns as to whether the system, developed in S. cerevisiae, can be directly transferred to S. pombe, and argues that its specificity needs to be convincingly demonstrated in the S. pombe system. His/her comments on this point are appended below.

Should you feel that you can address all these concerns, I would therefore like to invite you to submit a revised version of your manuscript. Clearly, this will have to be subjected to a further round of stringent re-review (including not only referee 2 but also the other referees, who have not been involved with this re-considered decision), and there is of course no guarantee as to the outcome of this.

Please let me know if you have any further questions, and I look forward to receiving your revision in due course.

Referee 2 comments:

There is, however, one general concern that has arisen from the rebuttal: the author writes that their assay does not measure DA, and he bases this statement on the observation that in S. cerevisiae mutants in ubc13 or the E3 activity of Rad5 are not defective in this assay. Their current study is based on S. pombe, and here ubc13 mutants now DO show a considerable defect, comparable to that of the Rad5 homolog Rad8. To an unbiased observer, this result could of course mean that Rad8 and Ubc13 are needed for TLS in S. pombe as opposed to S. cerevisiae; alternatively, however, one could conclude that in S. pombe ≠ in contrast to S. cerevisiae ≠ the assays DOES pick up alternative bypass events. I do not see why the first option should be more likely than the second. For example, the kinetics of replication fork stalling or unwinding of the strands could be different in the two organisms. I therefore think that in their revision the authors would have to convincingly demonstrate that they do not monitor anything else than TLS, not simply by extrapolation of results from from S. cerevisiae. I think the differences between the two studies (this one and the one by Pages, 2008) make it obvious that this should be done.

1st Revision - authors' response 02 March 2010

Referee #1 (Remarks to the Author):

Using a plasmid-based in vivo assay for translesion synthesis in S. pombe, Coulon et al report in this manuscript that effective TLS requires RAD8, the fission yeast homologue of S. cerevisiae RAD5. Most current thinking sees RAD8/RAD5 as being involved in promoting an error-free, recombinational mode of DNA damage avoidance, through a combination of its helicase activity (demonstrated for RAD5) and ability to polyubiquitinate PCNA. However, the notion that the division in the RAD6 pathway between translesion synthesis mediated by PCNA monoubiquitination and damage avoidance mediated by PCNA polyubiquitination may not be quite so straightforward has been around for a long time.

SIGNIFICANCE
RAD5 was initially identified in a screen for mutants defective in UV-induced mutagenesis and was initially dubbed REV2 (REV for reversionless) [Lemontt 1971, Genetics 68, 21-33]. However, subsequently this phenotype was only seen for some rad5 alleles and at certain reporters. Using assays in which individual lesions and the outcome of their bypass can be more clearly linked, recent data has demonstrated a clear defect in translesion synthesis in RAD5 mutants both for abasic sites [Pages et al., 2008, Genetics 180, 73-82) and for T-T [6-4] photoproducts [Zhang & Lawrence 2005, PNAS 102, 15954-15959], although in the former work, from the authors of the present manuscript, this did not involve the helicase or ubiquitin ligase activity of the protein. It must be said that these observations somewhat limit the immediate impact of the present paper and I was rather surprised that some of the historical work was not discussed in more detail.

1-The S. cerevisiae data published previously have been discussed and compared to data obtained here in S. pombe.

TECHNICAL ISSUES
The authors configure their very neat plasmid assay to monitor in-frame TLS events and, by placing the DNA lesion opposite a region of heterology, have a nice in-built control for replication of the undamaged plasmid strand, which they monitor primarily by expression of the lacZ' gene. They convincingly demonstrate the kinetics of replication by assaying the proportion of DpnI-resistant plasmid with time. Elegant though this system is, I feel there are a number of issues with this assay that require addressing.

Although the authors state that the assay is configured only to monitor TLS and not damage avoidance of the lesion, I am not so sure that this is the case. I cannot see anything really to prevent a template switch event. Such an event would, as far as I can tell, result in a product indistinguishable from that created by replication of the undamaged strand. [To my knowledge little is known about how a short stretch of heterology opposite a lesion would affect recombinational damage bypass but I would very much doubt that the crossover to the newly synthesised daughter strand would occur at the lesion, rather than shortly before. I would therefore expect that there is sufficient homology here for recombination to occur]. Can the authors provide any firm evidence that this can or cannot happen?

2-We have carefully described the way our assay works by adding several ß in the beginning of the result section. While the plasmid assay allows replication across specific lesions (TLS) to be analyzed within the context of a replication fork it does not permit DA events to be recorded (see paragraph on DA events in the discussion section).

This may go some way to explain the rather surprisingly low frequencies of TLS, even in comparison to S. cerevisiae. If the Lac- colonies comprise both the replicated undamaged strand and the damaged strand replicated by damage avoidance then the frequency of TLS may be underestimated. It is therefore not currently possible, from the data presented, to tell whether a decreased number of Lac+ (i.e. TLS) products is due to failure to replicate the lesion containing strand and hence loss of its progeny or whether it has been replicated by a damage avoidance pathway. It is thus essential that the absolute numbers of recovered replicated plasmids are shown. An estimation of replication of the undamaged strand vs. those generated by DA pathways could be obtained by co-transfecting an identical undamaged plasmid carrying a separate bacterial resistance marker.

3-We have added data showing the number of primary colonies obtained following transformation into S. pombe of control and modified plasmid constructs (fig 2B). In all strains tested, the number of colonies formed by the damaged plasmid is within the same range as the number of colonies formed with the control undamaged plasmid. Our hypothesis to explain the observed results is that even if the replication of one strand is blocked by the presence of a lesion, the other undamaged strand gets fully replicated and amplified and will thus give rise to colonies with the same efficiency as the control, lesion-free, construct. This observation holds for all strains tested and again supports the notion that the present plasmid system does not record DA events. These issues have been extensively discussed in the manuscript (see paragraph "Development of a TLS assay in S. pombe" in result section).

It is potentially also important that the assay can only detect in-frame events and not those associated with frameshifts.

4-We have added a reference showing that UV-lesion induced frameshifts are rare events in both S. cerevisiae and in E. coli.

INTERPRETATION
The authors put forward the perfectly plausible model in which polyubiquitination acts in effect as a glue to promotes polymerase switching during TLS. However, I do not think that the model illustrated in Figure 4 is fully consistent with the data shown in Figure 3. If one just takes the case of TLS of the CPD lesion, the data clearly suggest that RAD8 and RAD30 do not work in the same pathway: the rad8/rad30 mutant does much less TLS than either single mutant and actually has a level of TLS comparable to the pcnK164R mutant. It is possible that the interpretation of these results is complicated by the issue of Lac- colonies arising by damage avoidance on the lesion strand, discussed above. In any event I feel that this data needs careful reinterpretation.

5-We have carefully taken into account this point and modified the text and figure 5 accordingly.

I am not convinced that the frequencies of TLS at the TT(6-4)PP are sufficiently high to really be able to draw firm conclusions in this regard.

6-For TT(6-4), we acknowledge the fact that there is a residual low level of error-free TLS that is probably caused by a small fraction of lesion-free oligonucleotide. On the other hand, the mutagenic TLS events necessarily result from the bypass of a lesion-containing oligonucleotide. Therefore, these events represent a meaningful signal that allows the genetic control of this pathway to be monitored.

MINOR POINTS
The polymerases should be named with their proper Greek letters, rather than spelled out

p3, para1, 3rd line from end: 'lesion' not 'lesions'

p9, para 1, line 6: LysK63 - should be either Lys63 or K63

p9, para 2, line 4: 'evidence' not 'evidences'

It has been fixed.

Referee #2 (Remarks to the Author):

This is an interesting manuscript, as it challenges the current model of how polyubiquitylation of PCNA functions during lesion bypass. Using a plasmid system in S. pombe the authors differentiate between error-free and error-prone translesion synthesis (TLS) events at defined UV lesions and show that the enzymes involved in polyubiquitylation of PCNA are (fully or partially) required for error-free TLS across both CPDs and 6-4 photoproducts. They assign the phenotypes to different combinations of polymerases and conclude that TLS may at least partially account for the enigmatic Rad5-mediated error-free pathway in S. cerevisiae as well.

The experimental system is clean, as error-free and error-prone events are clearly distinguishable by sequencing, and TLS events cannot be confused with other means of bypass due to the non-matching sequence on the undamaged strand. The results therefore appear to be meaningful. However, I do have some concerns regarding (a) events that are not measured by the assay described here and (b) the need for additional control experiments to support some of the statements. My major comments are as follows:

1. TLS events are given as a percentage of total replicated plasmids, but I did not find any information about how efficient replication overall was in the absence of PCNA modification or TLS polymerases. Does the overall recovery of plasmid DNA show any decrease or delay in some of the mutants? I suppose this is difficult to assess due to the late time points at which the DNA is recovered (i.e. after the culture has started to grow, which will level out any possible differences in the efficiency of replication), but the assay might as a result cause a somewhat disproportionate "blowing up" of minor events that are measured because a large number of plasmids are simply not replicated at all. Therefore, we never know whether a drop in TLS as measured here is compensated by TLS-independent events or whether replication of the corresponding plasmid molecules is never completed.

See answer 2 and 3 to Ref#1

2. Along a similar line, it remains unclear what the relative importance of the TLS events described here is for rad8/ubc13/mms2 function overall. The assay measures the contribution of these genes to TLS, but this does not give any information about the relevance of TLS-independent rad8-dependent events, which are not picked up by the assay. Some orthogonal assay would be important to determine this.

As discussed above, our assay does not monitor DA events. Our results clearly indicate that TLS events in S. pombe depend upon the rad8/ubc13/mms2 function. Our assay does not allow to conclude whether DA events depend or not upon the rad8/ubc13/mms2 function.

3. According to previous work from the Lehmann lab, rad8 and mms2 mutants are equally sensitive to UV, which would indicate that there is no ATPase-dependent, polyubiquitin-independent aspect of rad8 function, as there is for Rad5 in S. cerevisiae. Based on this, I am not sure whether the results obtained here in S. pombe can be extrapolated to S. cerevisiae, as the authors do at the end of the discussion.

We have now discussed the above-mentioned point in the text.

4. Another event that the assay does not show is frameshift errors during TLS, as these would show up as white colonies. What is noted here as a reduction in error-free TLS might simply be a shift towards this type of mutagenic event, possibly because a polymerase (Pol zeta?) that normally wouldn't operate on this lesion is forced to act in a suboptimal way. It would be important to gain some insight on how likely and how frequent this scenario is.

See answer 4 to Ref#1

5. Fig. 3A: The notion that rev1 and rev3 do not show a phenotype by themselves does not necessarily mean that they are not involved in error-free TLS across CPDs - they may simply be redundant with another polymerase. In particular, rad30 dinB double mutants still show higher levels of TLS than pcna-K164R mutants. Additional mutant combinations should therefore be tested, both as double and/or triple polymerase mutants and in combination with rad8. The difference between the mutants that can still monoubiquitylate PCNA and those that cannot implies that polyubiquitylation is only partially responsible for TLS, and the additivity of the phenotypes of rad8 and rad30 mutants implies that the polyubiquitin-dependence is not due to Pol eta. These relationships should be worked out in more detail.

These points are discussed in the revised manuscript in result section.

6. Fig. 3C: As above, the notion that none of the polymerase single mutants shows a drop in mutagenic TLS does not imply that they are not involved - again they could be redundant. It would therefore be helpful to see mutant combinations. If the authors wish to conclude that a different polymerase is responsible, they should show this by making appropriate mutants, because it is a far-reaching statement to conclude that monoubiquitylation affects polymerases other than those for which ubiquitin binding has been demonstrated.

This point is addressed in the revised manuscript by adding data with multiple polymerase mutants (see result section).

7. Finally, I am not sure whether these results should be extrapolated to humans or S. cerevisiae without any experimental evidence.

Our paper now highlights the notion that the control of PRR pathways are clearly different in S. pombe and S. cerevisiae.

Referee #3 (Remarks to the Author):

This manuscript addresses the role of the Rad8(Rad5)/Mms2-Ubc13 genes in TLS of TT-CPD and TT(6-4) in S. pombe, using a plasmid system. The authors conclude that TLS is regulated not only by the rhp18(Rad18) and monoubiquitination, but also by the Rad8(rad5)/Mms2-Ubc13 system via poly-ubiquitination. The questions addressed are of interest and importance. However, there are considerable concerns about the experimental system, some critical controls are missing, and the results are too preliminary to justify the author's conclusions.

Detailed points:

1. The corresponding author has already published in collaboration with the Prakash lab that in S. cerevisiae Rad5 regulates TLS. The present study shows a similar effect in another yeast, and therefore novelty is low. In the Discussion the authors mention the S. cervisiae paper, but argue that there was no requirement for either the helicase or the ubiquitin ligase functions of Rad5 in S. cerevisiae. However, they did not examine whether the Ub ligase activity of Rad8 and/or its helicase activities are required in S. pombe - which they should do.

Their finding that in S. pombe also UBC13 and MMS2 were required is not sufficient evidence, since UBC13/MMS2 is an E2, and E2s have usually multiple E3s.

See answer 1 Ref#1
Many attempts to construct Rad8 Ub ligase / helicase mutants in S. pombe were unsuccessful. We agree that it would have been interesting to test such mutants. However, the fact that a similar effect on TLS is observed for Rad8, Ubc13 and Mms2 suggests that this E2/E3 poly-ubiquitin complex is required in TLS.

2. It appears that the authors consider TLS and damage avoidance as the only mechanisms generating Lac+ and Lac- phenotypes. Another possibility is that neither TLS nor damage avoidance occur: the damaged strand is lost, and the intact strand is selectively replicated. The authors should address this issue and estimate the magnitude of such a mechanism. It might cause a significant error in their estimates of the extents of TLS. In fact it might explain why TLS across the TT(6-4) lesion was 0.8%, which to me looks like background level (see below).

The above-mentioned concerns have been addressed in the beginning of the result section and in the discussion (paragraph on DA).

3. The extent of the apparent TLS of the TT(6-4) lesion is extremely low (0.8%), which is essentially at background level. This raises the concern that whatever effects were observed, they were not due to the TT(6-4) lesion. It is not uncommon that preparations of plasmids with a site-specific lesion have 1% or even a larger fraction of with no lesion, or with another 'contaminating' type of damage. The authors should show what percentage of their plasmids indeed carried the TT(6-4) lesion, and describe how they determined it. Same for the TT-CPD plasmid.

For TT(6-4), we acknowledge the fact that there is a residual low level of error-free TLS that is probably caused by a small fraction of lesion-free oligonucleotide. However, most of the error-free TLS events are shown to be under the control of various genes and can therefore not be the result of the replication of contaminating undamaged constructs. On the other hand, the mutagenic TLS events necessarily result from the bypass of a lesion-containing oligonucleotide. Therefore, these events represent a meaningful signal that allows the genetic control of this pathway to be monitored. Our UV-lesion containing oligonucleotides have been extensively purified by HPCL and PAGE electrophoresis.

4. The authors use a mmr-deficient mutant, which may cause a background of mutations due to low fidelity of replication. What is the frequency of these mutations on each of the strands? This can be estimated by analyzing the frequency and sequence of both Lac+ and Lac- colonies obtained from plasmids grown in wild-type S. pombe versus their tester strain.

The mutator effect of a MMR defective strain (10-5 to 10-6) is low and will not significantly affect a small locus such as the lacZ gene used here.

5. If I understood the authors' arguments, they assume that a Lac+ phenotype represents TLS, whereas the Lac- phenotype represents the undamaged strand. The authors argue that all base substitution mutations formed by TLS will lead to a Lac+ phenotype, whereas Lac- colonies are the result of replication of the undamaged strand or tolerance by damage avoidance. However, the UV lesions may produce also frameshift mutations, which should cause a Lac- phenotype by TLS, and minus 1 frameshift mutations on the undamaged strand will cause a Lac+ phenotype independent of TLS. Do the authors have an estimate on whether these possibilities could have biased the interpretation of the results? This is especially important since the strains are mmr-defective, as

discussed in point 3 above.

See answer 4 to Ref#1

6. The authors have not examined the effect of UV irradiation on TLS in their system. This should be examined.

Because of the extreme sensitivity of the parental strain (NER-, MMR- and UVR-) we cannot test the impact of UV on the TLS assay (see spot assay of figure S1). However, we did test the impact of HU with TT-CPD that lead to a similar pattern of ubiquitination of PCNA than UV. Indeed, the parental strain displays a relatively weak sensitivity to HU (data not shown). Before transformation S. pombe cells were incubated with 10 mM HU then prepared for transformation (several washes). After transformation, S. pombe cells were plated onto selective medium without HU. When cells replicate the TT-CPD plasmid, the PRR pathway is activated. We observed an increase of TLS from 17% to 25% without and with HU in WT parental strain, respectively. The whole TLS events are dependent of PCNA since in Pcn1-K164R mutant TLS rate is below 1% even in presence of HU. These results are not added to the paper and should be kept confidential.

7. The authors should examine the effect of Rad8(Rad5)/Mms2-Ubc3 mutations on in vivo UV mutagenesis in S. pombe. One would predict a significant effect if Rad8/MMS2-Ubc13 regulate TLS.

There is no in vivo UV mutagenesis assay in S. pombe yet.

8. This method does not really measure extents of TLS (see point 2 above). Therefore the term 'extent of TLS' is misleading. Similarly, in Fig 4 the authors wrote that error-free TLS across TT(6-4) is 70%. This is again misleading. Their estimate of total TLS was 0.8% which is more or less background levels, and one cannot deduce that it is 70% accurate TLS. This "accurate TLS" might in fact be simple replication of background unmodified sequences in the TT(6-4) plasmid.

We have taken into account these comments. See also answer 2 and 3 to Ref#1.

3rd Editorial Decision 25 March 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-73211R. It has now been seen again by all three referees, whose comments are enclosed below. As you will see, both reviewers 1 and 2 are now supportive of publication. Referee 3, on the other hand, still questions the validity of your assay, and consequently does not recommend publication. Given these conflicting reports, I have consulted with an external expert advisor, as well as with our Chief Editor. As a result of these discussions, we are willing to overlook the negative recommendation of referee 3, and to accept your study for publication in the EMBO Journal, pending further minor revision. Specifically, referee 1 still maintains that it would be important to show data on the actual numbers of colonies recovered under the different mutant conditions (a point also raised by the negative referee 3). I would therefore ask you to introduce a table including these data. Referee 3 also argues that your data do not conclusively demonstrate that it is the ubiquitin ligase activity of Rad5 on PCNA (as opposed to other potential activities of Rad5) that is responsible for the observed phenotypes. At this point, and given the positive recommendations of the other referees and our external advisor, I would not insist on your providing additional data along this line, but I would ask you to ensure that this point is adequately discussed.

Please can you address these last remaining concerns in a final revision of your manuscript? Once we receive this, we should then be able to accept your study without further delay.

With best wishes,
Editor

The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

I think that by and large the authors have put forward a strong, but not watertight, case that their assay does not pick up damage avoidance events. The assertion that the two strand are completely unwound before replication is very strong, although the evidence for this is not really direct.

The authors now show the number of colonies produced per µg of DNA for wild type cells (Figure 2B). They assert in the rebuttal letter that 'This observation holds for all strains tested...'. Does this mean that all the mutants tested revealed similar colony recovery figures to wild type? Even in situations where the leading strand would be predicted to be lost due to complete failure of TLS and unavailability of DA mechanisms (e.g. pcnK164R)? I would have expected an overall reduction of c. 50% in the efficiency of plasmid recover in such cases. It would still be helpful to see this data for the other mutants in a supplementary table.

However, overall the results remain interesting and provocative, especially in the differences highlighted with respect to what has been shown in S. cerevisiae.

Referee #2 (Remarks to the Author):

The authors have now provided a very detailed and clear explanation of the experimental system and have addressed my concerns adequately. Hence, I am now supportive of publication because I think the assay is validated adequately, and the results are novel and interesting and provide a challenge to currently accepted models. I am not sure whether this study will be the last that there is to say about the mechanism of TLS, and it is unclear how much of the results may be a S. pombe-specific phenomenon, but they will certainly spark further investigations.

Referee #3 (Remarks to the Author):

The revised manuscript does not meet the two main critiques of the original manuscript, namely the concerns about the experimental system used, and the lack of evidence that it is indeed the polyubiquitination of PCNA by Rad8(Rad5)/Mms2-Ubc13 Ubiquitin ligase complex that controls TLS in S. pombe.
Detailed critique:
1. It appears that the authors greatly underestimate the complexity of the events occurring during replication of a damaged plasmid in S. pombe, and as a result do not take into account potential complications in their experimental system. Their entire case rests on calculation of relative TLS efficiency, which is done by diving blue colonies (which they consider to be TLS events) and white colonies, which they consider to be replication of the undamaged strand. This is problematic, because white colonies can also be the result of (a) damage avoidance and (b) TLS via a frameshift mechanism, as I and other reviewers pointed out. These are critical points, because when assayed in cells in which particular TLS genes were knocked out, the relative fractions of these events may be different from wt cells, and cause errors in the calculations. For example, the factor of 2, by which they multiply the frequency of blue/while colonies may vary considerably among mutants, and cause misinterpretation of results and effects.
The authors have not presented convincing data to indicate that damage avoidance does not occur in their system; In fact it is my impression that it does occur. They do not relate to the possibility of at least partial loss of the damaged strand, and to variations in the preferential replication of the undamaged strand. They also do not relate to the possibility that in mutant cells there will be a much higher fraction of frameshift TLS events, which will be scored as replication, not TLS.
2. The authors and others have previously reported that Rad5 is involved in TLS. The novel claim here is that it is the polyubiquitination of PCNA by Rad8(Rad5)/Mms2-Ubc13 which is required for TLS. That would have been a very nice result indeed, however, the author do not provide any convincing evidence for it. The fact is that the Rad5 involvement in TLS in S. cerevisiae does NOT

involve polyubiquitination of PCNA. In addition, in mammals the HTLF protein, which is a Rad5 homolog, and has ubiquitin ligase activity on PCNA, is also a transcription factor. So the authors have to provide much more experimental evidence to prove that in S. pobme it is the polyubiquitination activity of Rad8(Rad5)Mms2-Ubc13 on PCNA that is required for TLS.
3. The author's did not provide actual colony counts of several experiments to illustrate the performance of their assay, although they were asked to do so. Instead they present 'Numbers of colonies/ug DNA', which does not reveal much about the actual numbers of colonies. It is imperative that the actual absolute colony counts in several experiments be provided. This is especially true for the 6-4 PP experiments, where the relative extents of TLS are at the limit of detection, and their significance questionable.
4. The authors claim that they have not UV irradiated their cells due to their extreme UV sensitivity. I do not understand this argument. Why couldn't they use low UV doses? This has been done before with hypersensitive yeast cells.

2nd Revision - authors' response 07 April 2010

Please find the final version of the manuscript Stephane Coulon et.al. entitled "Rad8Rad5/Mms2-Ubc13 Ubiquitin Ligase Complex Controls Translesion Synthesis in Fission Yeast."

We have modified our manuscript taking into account your final concerns:

1. A new table, showing the number of colonies recovered after transformation of the different S. pombe strains, has been added to the manuscript in supplementary material as recommended by referees #1 and #3 (Table S1). This table has been correctly referenced in the paragraph "Development of a TLS assay" of the manuscript.

2. The fact that we did not conclusively demonstrated that it is the ubiquitin ligase activity of Rad8 that is responsible for TLS has been discussed. The following sentence has been added to the discussion paragraph: "In contrast, in S. pombe, error-free TLS across TT-CPD or TT(6-4) is equally affected in all three mutants (rad8 or ubc13 mms2) that inactivate the E2-E3 complex (Fig 3 and 4), although we have not formally proven that it is the ubiquitin ligase activity of Rad8Rad5 on PCNA that is required for TLS".

Acceptance letter 12 April 2010

Many thanks for submitting the final version of your manuscript, and apologies for the slight delay in dealing with this - I was on holiday last week. However, I have now had the chance to look through the manuscript, and am pleased to tell you that we can now accept it for publication without further modification. You should receive the formal acceptance e-mail shortly.

