THE EMBO JOURNAL

Manuscript EMBO-2011-79408

Generation of DNA single strand displacement by compromised nucleotide excision repair.

Camille Godon, Sophie Mourgues, Julie Nonnekens, Amandine Mourcet, FrÈderic Coin, Wim Vermeulen, Pierre-Olivier Mari, Giuseppina Giglia-Mari

Corresponding author: Giuseppina Giglia-Mari, CNRS

Review timeline:

Submission date:	08 September 2011
Editorial decision:	13 October 2011
Revision received:	17 January 2012
Editorial Decision:	07 March 2012
Revision received:	22 June 2012
Accepted:	27 June 2012

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.)

Editorial decision 13 October 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received the comments of three expert referees, which are copied below. As you will see, all three reviewers consider your new findings on the UV repair defect in XP-D/CS cells potentially very interesting and important, and therefore in principle suitable for eventual publication in our journal. Nevertheless, referee 1 raises one major issue with regard to the current interpretation in the paper, namely that your present set of data does not necessarily refute the previously proposed model, but might on the other hand also be reconciled with it. In this respect, the referee offers a number of well-considered points as well as experimental suggestions to further explore the possible consistency between old and new models. During revision of the study, please take these points carefully into consideration and try to address them as diligently as possible both in the revised manuscript as well as in the point-by-point response letter.

Should you be able to adequately respond to this main conceptual issue, as well as to address the various other more specific points raised in the reports, we should be able to consider the manuscript further for publication. Please let me stress that eventual acceptance or rejection will not depend on your ability to definitely disproving the previous model, but rather on carefully deliberating, presenting and integrating the various - and sometimes complementary - pieces of previously reported and newly generated evidence to achieve as balanced and rational as possible a final interpretation. I should add that it is our policy to allow for only one round of major revision, and that it will therefore be essential that you attempt to fully clarify all of the referees' concerns during its course.

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.

When preparing your letter of response to the referees' comments, please 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

From an editorial point of view, there are a few additional issues I would like you to address at this point:
- please alter the numbered citation format to adhere to the EMBO J reference style as specified in our Guide to Authors
- we will need brief Author Contribution and Conflict of Interest statements at the end of the manuscript (before the reference section)
- please use straight lines to connect data points in the curves in Fig 3D, as appropriately done in all other cases

Should you have any additional question regarding this decision or your revision, please do not hesitate to contact me. I look forward to your revision.

Yours sincerely,
Editor
The EMBO Journal

_____

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This manuscript seeks to further understand the molecular basis for the severe XP/CS patient phenotype when caused by a subset of inherited mutations in the human xpd gene, and for the highly cancer-prone phenotype of a knockin mouse model of the human disorder carrying one of these, a G602D mutation in the XPD protein that was causative in human patient XPCS2. Since the extent of UV-induced repair synthesis is surprisingly high in XP-D/CS cells, the nature of the defect that gives rise to the severe XP/CS phenotype, in contrast to XP alone, has been a puzzle.

The present study by Godon et al addresses a novel model for XP-D/CS that was previously proposed by A.R. Lehmann and his collaborators. The Lehmann model is based on results published in two papers (refs. 25 and 27) which showed that XP-D/CS cells introduce damage-induced, NER-dependent breaks that are NOT at sites of damage, and further showed that these breaks are dependent on transcription. It proposes that the mutant TFIIH in XP-D/CS cells is defective in switching from its transcription initiation function to its NER function and hence incorrectly recruits NER factors including incision proteins to undamaged promoters, resulting in breaks associated with transcription rather than UV lesions. In contrast, the Godon et al manuscript presents results that are interpreted as providing evidence that argues strongly against this model. The authors interpret their new results to suggest instead a quite different model that involves a defect in the second NER incision at damage sites due to inability of the mutant TFIIH to stably retain XPG, resulting in strand displacement synthesis and recruitment of Exo I. These are striking results based on cutting-edge imaging technologies that have important implications which demand careful consideration. However, while some aspects of the new model are clearly supported by the presented data, other aspects of the study fail to directly address the findings of the previous Lehmann papers and in fact misinterpret them to some extent, as well as failing to take into account the known biochemical properties of the XP-D/CS mutant TFIIH. It is thus by no means clear that the new findings refute the previous model, although they do add substantial, significant new information. Overall, the data presented are strong and likely to be of considerable importance, but the interpretation of the data is

one-sided and does not consider alternative possibilities.

In summary, elucidating the peculiar repair defect in XP-D/CS is of major import for better understanding the multiple roles of TFIIH in NER, transcription-coupled repair, and transcription initiation. The results presented in this manuscript, especially the novel findings that Exo1 is recruited to damaged regions in the XP-D/CS cells and that XPG is less stably immobilized, will thus be of broad general interest to the DNA repair and transcription communities. However, both major revision and addition of further data will be necessary to support the conclusions (or to suggest different conclusions). Specific comments follow.

MAJOR SPECIFIC COMMENTS:

1. A major premise underlying the authors' interpretation of their data is that ".. within this 'off-site NER' model, the UV-processing dependent gH2AX signal should occur throughout the whole nucleus upon local UV irradiation and not be restricted to the UV exposed area ..." (p. 15), in contrast to what is observed. However, nothing about the "off-site NER" model demands that the whole nucleus must be involved in the effect. The model suggests that incision is uncoupled from damage, but it doesn't require that every TFIIH complex in the cell is participating. It is entirely plausible that only TFIIH engaged in transcription initiation at promoters within a nuclear domain containing the local damage might be affected. In fact, it was convincingly established some ten years ago that local UV damage impacts transcription only in the locally damaged area (Mone et al, EMBO Reports, 2: 1013-1017, 2001). Once the initial premise is discarded, nothing in the presented data distinguishes between lesions and transcription sites in the locally damaged regions.

2. The experiments of Fig. 1B,C take full advantage of crossing two powerful mouse models and the authors' expertise in live imaging. The results are very interesting in showing prolonged immobilization of the XpdG602D mutant TFIIH at damage and are fully consistent with the inability of this mutant TFIIH to allow NER (Dubaele et al, Mol Cell 11:1635-1646, 2003) but near-normal DNA binding capability of an archael XPD homolog with the corresponding mutation (ref. 48). However, it is not strictly correct to say that the binding is to "DNA lesions" (p. 8, line 9); what has been shown is prolonged binding in regions containing damage.

3. The authors seem to have an incorrect recollection of the critical experiment in reference 27 (Theron et al, 2005). In that paper, breaks were analyzed after UV using the comet assay in combination with inhibition of repair synthesis by ara-C plus HU. It was this quantitative measurement that revealed a clear dependence of the breaks on transcription in XP-D/CS cells but not in wt cells, not a change in gH2AX signal as stated on p.5. In fact, the demonstration in Fig. 2A that transcription inhibition reduces the gH2AX staining in locally UV-damaged areas in XP-D/CS cells is a novel result. What is very perplexing - and unexplained by the authors - is the fact that this gH2AX staining is also reduced by transcription inhibition in wt, XP-A, and XP-G/CS cells. (The same is true of RPA accumulation in Fig. 2B.) This puzzle notwithstanding, however, the statement on p. 8, "Unexpectedly and in contrast with previous data [27]..." is misleading, as the two studies used different methods. The Theron et al paper clearly showed no effect of transcription inhibition (using a number of different inhibitors) in wt cells on strand breaks per se.

4. Interpreting the absence of staining for PAR and Ku in damaged areas at 15 minutes after UV as indicating that there are no SSBs or DSBs associated with the gH2AX staining is unwarranted, and the comparison to laser-induced damage is specious, since breaks after UV depend on cellular processing. In fact, Theron et al (ref 27) conducted a much more complete experiment with a time course. Their results showed PAR signal at 2 hr after UV in both wt and XP-D/CS cells, but its persistence only in the mutant cells - and furthermore its complete absence in XP-D cells, in marked contrast to XP-D/CS.

5. There is a serious flaw in the authors' interpretation of the RPA accumulation in locally UV damaged areas in XP-D/CS cells. As they correctly state (p. 9), "To load RPA the action of TFIIH is required, which creates single-stranded bubble around the lesions." HOWEVER, the XpdG602D mutant TFIIH is devoid of helicase activity, while remaining competent for transcription (Dubaele et al, Mol Cell 2003). It is thus very unlikely that the RPA accumulation in locally damaged areas in the XP-D/CS cells is at sites of lesions, since even though the mutant TFIIH may be recruited to the lesions (as shown by XPB staining), it will be unable to unwind. Due to the known properties of

XpdG602D TFIIH, the RPA that accumulates in damaged areas in the XP-D/CS cells is likely to be at sites of transcription, whereas in the wt and other mutant cells it is presumably at lesions as expected. Importantly, it would not be possible to distinguish such a difference by IF. (The truly confounding aspect of these results is that RPA accumulation is prevented when transcription is inhibited in ALL of the cells tested.)

6. Similarly, recruitment of ERCC1, XPG, and ubi-PCNA in the XP-D/CS cells (Fig. 3) cannot be presumed from this experiment to be occurring at sites of lesions; it could just as well represent the "off-site NER" proposed by Theron et al, who showed that gH2AX accumulation in XP-D/CS cells required NER (absent in double mutant MEFs that also had ko of XPA). A very low level of 6,4-PP removal in XP-D/CS cells as reported here has been previously published (ref. 26) but is also seen in NER-defective XP-D cells. In essence, there is no significant NER-dependent removal of UV lesions in these cells, and furthermore XpdG602D TFIIH does not allow repair synthesis in cell extracts (Dubaele et al, Mol Cell 2003), in which no transcription occurs. In this regard, it is important to recall that breakage is induced in undamaged XP-D/CS cells by transfection of a damaged plasmid, but not an undamaged one (Theron et al; ref. 27). The authors' conclusion that the events observed are occurring at lesions is simply not justified. Their own data do not rule out the alternative interpretation, and published results have established that damage in the cellular genome is not required.

7. The argument that more RPA is accumulated per lesion in the XP-D/CS cells is unsupported, given that it has by no means been shown that the recruitment is at lesions in the first place. In any case, the RPA quantification by immunofluorescence in the locally damaged spots is unconvincing, given the very high level of background staining.

8. The above criticisms of interpretation notwithstanding, it should be noted that much of the data per se is very compelling and important. On the one hand, the demonstration of lack of recruitment of ubi-PCNA in XP-G/CS cells is a nice confirmation of the current hypothesis that the presence of XPG is required to initiate repair synthesis. On the other hand, the demonstrated recruitment of ERCC1 and XPG to locally damaged areas in the XP-D/CS cells could (by an interpretation that differs from that of the authors) be considered to be an important confirmation of the hypothesis that aberrant NER occurs at transcription initiation sites, presumably dependent on XPB activity to create the transcription bubble. This could be directly tested by the simple expedient of asking whether the recruitment is prevented by inhibition of transcription initiation in the XP-D/CS cells but not wt. Furthermore, the dramatic results using a very clever BrdU labeling technique to show that long stretches of ssDNA are present in damaged areas only in the XP-D/CS cells, that XPG is less stably bound, and that Exo1 is recruited are very clean, very exciting, and certain to be of major significance for understanding the aberrant damage-induced events in XP-D/CS cells. The authors' model for strand displacement synthesis is consistent with these results - but may apply to the hypothesized "off-site NER". If, as Theron et al propose, aberrant NER, including repair synthesis, occurs not at lesions but at transcription initiation sites, then the creation of ss DNA and recruitment of Exo1 should also be prevented by transcription inhibitors.

MINOR POINTS:

1. Introduction, p. 3: Reference 8 is not the correct citation for "forms a complex with TFIIH to prevent the dissociation of the CAK and XPD". Reference 40 should be cited here instead. In addition, in the second paragraph, reference to a more current review of TCR would be appreciated.

2. Introduction, p. 4: The statement "Mutations in one of the NER factors ..." is incorrect as written, since only mutations in XPB or XPD can cause all three of these disorders.

3. Introduction: Potential use of the XpdG602D mouse model in drug testing is not the most compelling rationale for this study, which is of fundamental importance in its own right. In any case, all experiments except for those of Fig. 1B,C were conducted with normal or mutant human cells.

4. Introduction, p. 6, line 11: missing) after "UV damage".

5. Results, p. 7: insert "one" into "which allows [one] to follow the dynamic behavior...". Delete mention of MDFs as irrelevant to this study, since only keratinocytes seem to have been used.

6. Results, p. 9: "...and obtained similar results (Figure S3B)..." XPB staining in an XP12Ro XPA primary cell looks quite different from the transformed cells as accumulation in the damaged spot is very hard to see and the staining looks much more diffuse. Perhaps this cell is not representative?

7. Results, p. 10: "...we found that ERCC1 is recruited to locally UV-exposed areas in WT and XP-G cells...". The ERCC1 foci are very difficult to see in the WT cells in Fig. 3A. Can this image be improved?

8. Fig. 3D&E: These are poorly prepared, with axis labels, symbols, error bars, and symbol key all too small and faint for good visibility. In addition, the XP-G and XP-A cell data are plotted using the same color, which leads to some confusion. Different colors should be used. The figure legend should state how lesions were detected and quantified.

9. Fig. 4B: What does the * represent? No explanation is given.

10. Methods, p. 21: No source is given for the anti-ERCC1 antibody. Typographical errors: Should be "prior to UVC"; please insert "treated" after "mock" (mock-treated coverslips).

Referee #2 (Remarks to the Author):

Mutation in the XPD subunit of the NER/transcription factor TFIIH are associated with at least three disorders: XP, CS and TTD. A subset of mutations in XPD cause a combined XPD/CS phenotype and cells from XPD/CS patients have been studied for the their unusual phenotype, believed to include the formation of DNA breaks away from sites where NER takes place. In the present paper, the consequences of an XP/CS causing allele (XPD-G602D) are investigated using a mouse model in which TFIIH contains a YFP-labeled XPB enabling the authors to follow TFIIH with a XP/CS allele in cells. The authors show that rather forming breaks at distal sites, XPD-G602 results in a weakened association of TFIIH and XPG, leading to inefficient incision by XPG, strand-displacement synthesis and ExoI-mediated degradation of the lesion-containing strand. The beauty of this work lies in the link of this proposed model with the recently elucidated mechanism of NER-dependent UV-induced DNA damage signaling. Accordingly, XP/CS cells have an inability to complete NER, due to inefficient incision by XPG and resulting in the over-activation of the signaling pathway that ultimately leads to genomic instability. I believe that the findings presented here are novel, important and of general interest and that the work is sound. I believe that there are a few important issues that should be addressed prior to publication.

- The recruitment of RPA to sites of local UV damage has previously believed to a part of NER independent of transcription as is also shown in Figure 2A. The fact that inhibition of transcription would lead to reduced levels of RPA is surprising and is in contrast with the current literature. This discrepancy should be reconciled.

- The previous study of PCNA ubiquitination in the context of UV damage indicated that this occurs independently of NER activity, even in XPC- or XPA-deficient cells. Can the authors explain why PCNA ubiquitination would be inhibited in XPCS2 or XPCS1RO cells?

- The manuscript would benefit from a thorough editing of the text. Many sentences are grammatically incorrect and/or poorly structured.

Minor points:

- Ref 8 is not appropriate. Ito et al, Mol Cell 2007 (Ref 40) should be cited here instead.

- p3. 3rd line from bottom: format reference

- complete Reference 15

- The XPD/CS mouse model is in various contexts referred to as: XpdG206D (p4), XP-D/CS (p7) or TFIIH XP/CS (p.7). Please decide on one nomenclature to avoid confusion.

- p13. 7th line: Reference a recent paper showing the involvement of human ExoI removing flap structures: Sertic et al PNAS 2011, 108, 13647

Referee #3 (Remarks to the Author):

Nucleotide excision repair (NER) is a highly orchestrated process required to remove helix-distorting DNA lesions that promote cancer and aging. Over 30 proteins are required to complete the reaction and return DNA to its pristine state. NER occurs in multiple steps of protein recruitment and attrition from the site of DNA damage. This manuscript reports a remarkable and novel finding-that a mild perturbation of this highly ordered process leads to the generation of unexpected repair intermediates that promote genomic instability.
The authors use creative genetic tools to perturb the mobility of TFIIH and stability of it's binding partners, while at the same time monitoring the complex in cells.
The data is made more exciting by the fact that the TFIIH mutation studied is linked to human disease and a cancer-prone mouse model.
The manuscript is extremely well-written and should be accessible to a broad audience.
The data is clearly presented and justifies the conclusions.
A very complete battery of controls are provided in the supplemental material.

The manuscript counters a previous interpretation of how this particular mutation in the XPD subunit of TFIIH promotes genomic instability. The authors here do an excellent job of countering this with solid data that conflicts with that interpretation, yet a diplomatic presentation. Their new interpretation is provocative, well-justiified and truly paradigm shifting for aficionados of NER. The work will also have implications for those considering SNPs in DNA repair genes and cancer risk.

The lesson that gH2AX signal does not always equate to SSBs or DSBs is also deserving of reaching a broad audience.

Very minor editorial comments:
ERCC1/XPF should be ERCC1-XPF. "/" is used to indicate 2 names for 1 protein.
It is preferable to use -in 'the" presence- rather than -in presence- (throughout the paper)
Double-strand and single-strand is preferable to no hyphen.
There is a close parenthesis missing on page 6
pg. 7 XpbY/Y is not italicized

The increased RPA recruitment is likely to activate ATR to a greater extent in XP-D/CS cells than other NER mutants. One wonders if an ATR inhibitor would diminish genomic instability or cytotoxicity? - as a therapeutic angle for XP/CS patients.

1st Revision - authors' response 17 January 2012

Answers to referee #1

We would like to thank Referee #1 for his careful and thorough reviewing of our work. We acknowledge this referee's point of view and have tried to answer in the most unbiased way to all his points, also trying to find (when possible) a common denominator that could bring together our results with those of earlier studies.

First of all we want to stress that our work is not meant to discard the previous model, but more to understand the molecular mechanisms that are implicated in the $TFIIH^{XP/CS}$ faulty behavior, at least in the early to very early time window we have investigated. Although our observations are not meant to go against a particular model, we have proposed an alternative (or complementary) way of explaining how the (previously) very well described DNA breaks could be formed in the XP-D/CS genome after UV-exposure. We had thought our approach clear but, since referee #1's attention has been mainly focused on discarding our starting point (which impacts how the rest of

our data can be interpreted), probably it is not clear enough.

Therefore, to improve the focus of this study on its main idea, i.e. disclosing a molecular mechanism that produces breaks after UV exposure in XP-D/CS cells, we have trimmed down our sentences dealing with the on-site/off-site debate throughout the article, but without taking out any scientific data. In this way, we let the readers make their own opinion about "on-site/off-site" considerations.

Nonetheless, we wish to respond to the referee's claims of "serious flaws", "specious arguments" etc, through this point-by-point letter, explaining with greater clarity the evidences that led us to think that an alternative or complementary model could exist.

MAJOR SPECIFIC COMMENTS

POINT 1.

A major premise underlying the authors' interpretation of their data is that " within this 'off-site NER' model, the UV-processing dependent g*H2AX signal should occur throughout the whole nucleus upon local UV irradiation and not be restricted to the UV exposed area ..." (p. 15), in contrast to what is observed. However, nothing about the "off-site NER" model demands that the whole nucleus must be involved in the effect.*

Since the very first mobility measurements (via FRAP experiments) of GFP-tagged proteins in the nucleus of living cells, it has been shown and generally accepted that NER proteins are highly mobile and can diffuse throughout the nucleus in mere seconds (typically less than 10 sec.). It was later shown (via XPB in cultured cells) that TFIIH binds to promoters during transcription elongation for only a few seconds at a time before unbinding and returning to the pool of "freely" diffusing proteins. This very dynamic picture of protein interactions is also seen during UV-induced NER, although to a lesser extent: the core NER proteins constantly bind and unbind their substrate during DNA-lesion processing with characteristic half-times ranging from 1 to 3 minutes.

Such protein dynamics (mostly governed by diffusion and protein binding affinities) imply that a given TFIIH complex will diffuse most of the time, sometimes randomly encounter a substrate to bind to, unbind rapidly, diffuse some more, bind more strongly to a specific substrate (e.g. a DNA lesion with XPC already bound), unbind again (more or less promptly depending on the presence of downstream NER factors), diffuse, etc. Within this framework, one would then assume that local UV exposure would trigger distal aberrant NER processing throughout the nucleus (at any active promoter) simply because TFIIH molecules will inevitably diffuse around the nucleus whether or not they are engaged in transcription or NER.

The model suggests that incision is uncoupled from damage, but it doesn't require that every TFIIH complex in the cell is participating. It is entirely plausible that only TFIIH engaged in transcription initiation at promoters within a nuclear domain containing the local damage might be affected.

Indeed the "Off-site model" does not require that <u>every</u> TFIIH complex in the cell participates, but because diffusion will "mix" the unbound complexes of the entire nucleus in seconds, <u>any</u> randomly chosen (and hence randomly positioned) TFIIH complex could participate i.e. there is no evidence of any process capable of countering diffusion and restraining TFIIH to within a "nuclear domain"

In fact, it was convincingly established some ten years ago that local UV damage impacts transcription only in the locally damaged area (Mone et al, EMBO Reports, 2: 1013-1017, 2001).

In the context of the Mone et al. paper, the reason for transcription to be hindered only within the locally damaged area is the very large number of UV-induced DNA lesions present on active genes that will repeatedly stall RNA Polymerase II processing and therefore trigger Transcription Coupled NER events.

Once the initial premise is discarded, nothing in the presented data distinguishes between lesions and transcription sites in the locally damaged regions.

Referee #1 has successfully drawn our attention to the fact that much of our subsequent data interpretations rely on this initial premise. Closer scrutiny has revealed a gap in our reasoning: we

have assumed that TFIIH binding to promoters is unchanged by the XPD^{G602D} mutation. Some of our results would be compatible with the "Off-site model" if mutant TFIIH complexes were abnormally retained at their promoter binding sites.

To resolve this issue, we have conducted Chromatin Immunoprecipitation (ChIP) experiments on the promoters of 4 different expressed genes in both MRC5 (Wild type) and XP-D/CS cells, prior to and one hour after exposure to UV-C light. Our results indicate that TFIIH binding to active promoters decreases considerably and equally in both cell types after exposure to UV light: the XPD^{G602D} mutation clearly does not lead to abnormally strong or prolonged binding of $TFIIH^{XPCS}$ to active promoters.

Text added/modified/deleted:
The results of this experiments are presented in Fig 1 panel D and described at p. 8, section: "*Mobility of hybrid TFIIH complex (XPB-YFP with XPD^{G602D}) after DNA damage induction and transcription inhibition*"

POINT 2.
The experiments of Fig. 1B,C take full advantage of crossing two powerful mouse models and the authors' expertise in live imaging. The results are very interesting in showing prolonged immobilization of the XpdG602D mutant TFIIH at damage and are fully consistent with the inability of this mutant TFIIH to allow NER (Dubaele et al, Mol Cell 11:1635-1646, 2003) but near-normal DNA binding capability of an archael XPD homolog with the corresponding mutation (ref. 48). However, it is not strictly correct to say that the binding is to "DNA lesions" (p. 8, line 9); what has been shown is prolonged binding in regions containing damage.

The number of UV-induced DNA lesions (at the doses used throughout our study) is an order of magnitude greater than the number of active promoters (*circa* 100 000 *versus* 10 000 respectively). When performing a localized UV exposure, this difference becomes even larger because only about 1/10 to 1/5 of the nucleus is exposed with an increased UV dose to maintain approximately the same number of DNA lesions as in a global irradiation. In short, within the locally damaged area, the number of UV-lesions (Global Genome NER substrate) far exceeds the number of lesions on active genes (Transcription Coupled NER substrate), which also exceeds the number of active promoters. The resulting low concentration of transcribed genes with UV-lesions (within the locally exposed area) is best illustrated by the quasi-inability to observe, *in cellulo,* the recruitment of Transcription Coupled NER specific proteins such as CSB and CSA, or other NER factors in cells where Global genome NER is abrogated such as XPC deficient cells. Consequently, the overwhelming majority of events probed *via* NER related factors, within locally UV-irradiated areas must *statistically* be associated to Global genome NER processing.

POINT 3.
The authors seem to have an incorrect recollection of the critical experiment in reference 27 (Theron et al, 2005). In that paper, breaks were analyzed after UV using the comet assay in combination with inhibition of repair synthesis by ara-C plus HU. It was this quantitative measurement that revealed a clear dependence of the breaks on transcription in XP-D/CS cells but not in wt cells, not a change in g*H2AX signal as stated on p.5. In fact, the demonstration in Fig. 2A that transcription inhibition reduces the* g*H2AX staining in locally UV-damaged areas in XP-D/CS cells is a novel result.*

We thank referee #1 for having pointed out this incongruence in the text. We now have modified the text at p. 5 accordingly.

What is very perplexing - and unexplained by the authors - is the fact that this g*H2AX staining is also reduced by transcription inhibition in wt, XP-A, and XP-G/CS cells. (The same is true of RPA accumulation in Fig. 2B.) This puzzle notwithstanding, however, the statement on p. 8, "Unexpectedly and in contrast with previous data [27]..." is misleading, as the two studies used different methods. The Theron et al paper clearly showed no effect of transcription inhibition (using a number of different inhibitors) in wt cells on strand breaks per se.*

It is indeed very perplexing and still unexplained; however it is a highly reproducible result.

Each cell-type we tested responded in the same way to the transcription inhibition treatment. While XPB accumulation remains unchanged, RPA accumulation and the gH2AX signal are abolished after alpha-amanitin treatment.

We believe that transcription inhibition is not directly impeding RPA accumulation on the local damage, but that this phenomenon is a secondary effect due to the fact that many genes will be shut down during the alpha-amanitin treatment (which is 16 h long), some of them producing also proteins that could be needed for RPA accumulation or upstream of this accumulation on the local damage. In fact, if any protein upstream of this accumulation will have a short lived m-RNA, the protein will not be produced any more and the cell will be deficient for one of the NER reaction steps. The nature of this protein remains unknown and it was not our purpose to investigate into this phenomenon in this article. We just used this staining to show that transcription inhibition blocks the gH2Ax signaling because the DNA opening is not made for one reason we still do not know hence RPA is not loaded (RPA being the signal needed to induce the ATR signaling). However, here we use RPA accumulation just as a sensor for this helix opening.

Moreover and most importantly, we used RPA staining (as well as the gH2Ax staining) to show that transcription inhibition does not just specifically affect XPCS2 cells but all the cells tested (WT, XPA and XPG) demonstrating that there is no specific transcription-related XPCS2 response.

Text added/modified/deleted:

In order to make this point clear in the text, we included a more unambiguous explanation of our data and our purpose at p. 10 (end of section "Effect of transcription inhibition on UV-induced DNA damage response"). We also corrected the statement at p. 8 (now p. 9), as suggested by the referee #1, by deleting the sentence " and in contrast with previous data".

POINT 4.

Interpreting the absence of staining for PAR and Ku in damaged areas at 15 minutes after UV as indicating that there are no SSBs or DSBs associated with the g*H2AX staining is unwarranted, and the comparison to laser-induced damage is specious, since breaks after UV depend on cellular processing. In fact, Theron et al (ref 27) conducted a much more complete experiment with a time course. Their results showed PAR signal at 2 hr after UV in both wt and XP-D/CS cells, but its persistence only in the mutant cells - and furthermore its complete absence in XP-D cells, in marked contrast to XP-D/CS.*

We have used Ku and PAR to complement the use of gH2AX because of their specificity as indicators of DSBs and SSBs. Since throughout this study we have mostly looked at early events after UV exposure (1 hour or less), we wanted to verify if the gH2AX signals we observe at those early time points are correlated to DNA breaks or not. We favored 15 minutes post UV irradiation because we feared that at post 1 hour, (a) not enough DSB would remain (DSB repair by NHEJ is fast) to be detectable using Ku as a direct probe and (b) all SSBs would also have been repaired by the *very* fast SSB repair pathways. For completeness we have added to the supplementary figure S2A, the 1 hour time point for both Ku and PAR.

Concerning the laser induced DNA damage, we used this method as a positive control for Ku and PAR immunofluorescence since figure S2A shows only negative Ku and PAR accumulation. We also used the laser micro-irradiation to show that the levels of gH2AX signal we observe after exposure to UV would normally correlate with detectable levels of Ku (DSBs) and PAR (SSBs) accumulation.

We deeply regret that referee #1 suggests we are trying to mislead the reader, and believe "specious arguments" are best left to other arenas. Our intention was to interpret the absence of Ku and PAR as indicating that there are no DNA breaks *at these early time points only*. This result does not in any way indicate that (cellular processing dependent) DNA breaks will not be produced later on, as has been found by Theron et al.

Text added/modified/deleted:

The text in the introduction, results and discussion sections has been carefully modified to clarify this. We also added to the supplementary figure number 2A the result of Ku and PAR IF at 1 hour post UV in addition to the 15 min experiment. Figure S2B is unchanged.

POINT 5.

There is a serious flaw in the authors' interpretation of the RPA accumulation in locally UV damaged areas in XP-D/CS cells. As they correctly state (p. 9), "To load RPA the action of TFIIH is required, which creates single-stranded bubble around the lesions." HOWEVER, the XpdG602D mutant TFIIH is devoid of helicase activity, while remaining competent for transcription (Dubaele et al, Mol Cell 2003). It is thus very unlikely that the RPA accumulation in locally damaged areas in the XP-D/CS cells is at sites of lesions, since even though the mutant TFIIH may be recruited to the lesions (as shown by XPB staining), it will be unable to unwind. Due to the known properties of XpdG602D TFIIH, the RPA that accumulates in damaged areas in the XP-D/CS cells is likely to be at sites of transcription, whereas in the wt and other mutant cells it is presumably at lesions as expected. Importantly, it would not be possible to distinguish such a difference by IF. (The truly confounding aspect of these results is that RPA accumulation is prevented when transcription is inhibited in ALL of the cells tested.)

The referee #1 will certainly agree with us that on many occasions already, in vitro studies have led to some results that could not be confirmed in vivo. Purified complexes acting on naked DNA damaged by a Cys-platinum lesion in a test tube is far from being the ideal situation for a protein to fully show what its function or malfunction is. In vitro studies are of course useful but cannot always give us the real picture of what happens in the cell. A remarkable example of this is the TTDA protein. Although TTDA is dispensable in the in vitro NER-reaction, its absence causes a complete NER deficiency in a knock-out TTDA mouse (results presented at several occasions by Dr Vermeulen and paper in preparation). Many XPD mutations (not just XP-D/CS, but also XP and TTD) hinder in vitro the helicase activity (Fan et al, Cell 2008), but only a few of those mutations have been tested for their helicase activity in vivo (Nishiwaki et al, DNA Repair, 2008). Comparing these two works, one can clearly remark that an almost null activity in vitro corresponds to at least 20 to 40 % RPA accumulation levels, demonstrating that zero helicase activity does not seem to exist in vivo.

Undeniably, in XP-D/CS cells at least 15% of lesions are repaired on average (20% of 64PP and 10% of CPD, cf figure 3D and E). This “residual” repair cannot be just a random response of the NER system (or experimental noise), but shows that $TFIIH^{XP/CS}$ still has a certain “residual” activity in opening the helix and most importantly at the right place i.e. on the lesions. In this case $TFIIH^{XP/CS}$ is in the right environment, surrounded by the whole NER machinery (including components we still do not know of), on a chromatinised substrate and on UV-induced lesions.

Concerning RPA accumulation at locally UV-exposed areas, Referee #1 is correct to state that it is not possible via IF to directly distinguish DNA-lesions from other substrates such as promoters in a locally damaged area. However, we insist on the fact (cf. responses to point 1 and 2) that there simply are not enough active genes and promoters in these areas to recruit NER factors to detectable levels by IF (TFIIH and most other NER factors are essentially at a 1 to 1 stoichiometry with their substrates). Only the UV-lesion substrates are numerous enough to be probed via NER factors in the local damage areas.

POINT 6.

Similarly, recruitment of ERCC1, XPG, and ubi-PCNA in the XP-D/CS cells (Fig. 3) cannot be presumed from this experiment to be occurring at sites of lesions; it could just as well represent the "off-site NER" proposed by Theron et al, who showed that g*H2AX accumulation in XP-D/CS cells required NER (absent in double mutant MEFs that also had ko of XPA).*

Here we invoke the same arguments as for Points 2 and 5.

A very low level of 6,4-PP removal in XP-D/CS cells as reported here has been previously published (ref. 26) but is also seen in NER-defective XP-D cells. In essence, there is no significant NER-dependent removal of UV lesions in these cells, and furthermore XpdG602D TFIIH does not allow repair synthesis in cell extracts (Dubaele et al, Mol Cell 2003), in which no transcription occurs.

In the paper cited (Dubaele et al, Mol Cell, 2003), no cellular extracts were used, but just recombinant proteins produced in baculovirus. As stated before in point 5, the null activity observed in vitro could well be partially restored when the complexes are in their in vivo context.

Once again, we think that the level of lesion removal in XP-D/CS cells is too high to be a background level of repair or noisy data (cf. point 5) because it is not the lowest repair level

observed: XPG cells show no removal of lesions and the XPG data points are statistically different from the XP-D/CS points (cf. figure 3D,3E).

In this regard, it is important to recall that breakage is induced in undamaged XP-D/CS cells by transfection of a damaged plasmid, but not an undamaged one (Theron et al; ref. 27). The authors' conclusion that the events observed are occurring at lesions is simply not justified. Their own data do not rule out the alternative interpretation, and published results have established that damage in the cellular genome is not required.

This piece of evidence remains an unexplained observation. From this experiment the pan-nuclear signal theory has been drawn, i.e. that the presence of lesions on a plasmid could induce an *undefined* signal that would indicate *only* to the $TFIIH^{XP/CS}$ complexes engaged in transcription that they should trigger the NER reaction on promoters. For a decade the nature of this signal has not been uncovered and has eluded many research groups dedicated to the study of NER.

POINT 7.
The argument that more RPA is accumulated per lesion in the XP-D/CS cells is unsupported, given that it has by no means been shown that the recruitment is at lesions in the first place

The remark concerning the nature of the recruiting substrate in the local damage area has been addressed (cf. responses to Points 1, 2, 5 and 6).

In any case, the RPA quantification by immunofluorescence in the locally damaged spots is unconvincing, given the very high level of background staining.

It seems that Referee #1 refers to RPA nuclear staining (figure 4A) as background staining. This is not the case as RPA is present throughout the nucleoplasm, hence is detected within the nucleus because our IF conditions essentially preserve "most" of its nuclear localization/signal. Background staining (outside of the cells) is on the contrary, satisfyingly low.

Quantification of the RPA recruitment signal in the locally damaged (LD) area was achieved using a state of the art confocal microscope designed for quantitative imaging. Images were then analysed with the ImageJ software by using a straightforward approach that provides remarkably robust estimates of LD accumulation signals:

(a) The average fluorescence within the LD area was measured.
(b) The average fluorescence of the nucleus was measured (excluding the LD area and all nucleoli so as not to introduce a bias), then subtracted to the one measured in (a).
(c) The resulting value corresponds to the average net fluorescence due to the accumulation of RPA.

Providing *average* fluorescence values (in arbitrary units) rather than *total* (or integrated) fluorescence values for the LD accumulation of RPA was necessary because of the size variations of the irradiated LD regions from one cell to the next *i.e.* average (per pixel) fluorescence does not depend on the LD surface area.

Text added/modified/deleted:
Text in the results section ("*RPA accumulation on UV-induced damages containing a* $TFIIH^{XP/CS}$ *complex***"**, end of page 12) was reformulated. Figure legend and a separate Methods section for the quantification of RPA were also reformulated.

POINT 8.
The above criticisms of interpretation notwithstanding, it should be noted that much of the data per se is very compelling and important. On the one hand, the demonstration of lack of recruitment of ubi-PCNA in XP-G/CS cells is a nice confirmation of the current hypothesis that the presence of XPG is required to initiate repair synthesis. On the other hand, the demonstrated recruitment of ERCC1 and XPG to locally damaged areas in the XP-D/CS cells could (by an interpretation that differs from that of the authors) be considered to be an important confirmation of the hypothesis that aberrant NER occurs at transcription initiation sites, presumably dependent on XPB activity to create the transcription bubble. This could be directly tested by the simple expedient of asking

whether the recruitment is prevented by inhibition of transcription initiation in the XP-D/CS cells but not wt.
Furthermore, the dramatic results using a very clever BrdU labeling technique to show that long stretches of ssDNA are present in damaged areas only in the XP-D/CS cells, that XPG is less stably bound, and that Exo1 is recruited are very clean, very exciting, and certain to be of major significance for understanding the aberrant damage-induced events in XP-D/CS cells. The authors' model for strand displacement synthesis is consistent with these results - but may apply to the hypothesized "off-site NER".If, as Theron et al propose, aberrant NER, including repair synthesis, occurs not at lesions but at transcription initiation sites, then the creation of ss DNA and recruitment of Exo1 should also be prevented by transcription inhibitors.

Transcription inhibition alters the possibility of RPA to be recruited on the local damage by a mechanism that is probably secondary to transcription inhibition *per se* as already discussed above. Hence, when RPA is not recruited to the damage, factors depending on the presence of RPA will not be recruited either, this includes ERCC1-XPF complex. Because this factor will not be recruited, neither will be Exo1. The effect of transcription inhibition has been observed in all cell type tested, transformed and primary. There is no reason to think that without a crucial factor as RPA on the lesions, the other factors would be recruited. As stated at the beginning, this article is not meant to go into the effects of transcription inhibition on NER factors accumulation but just to elucidate the mechanisms that control faulty protein behaviors.

MINOR POINTS:

1. *Introduction, p. 3: Reference 8 is not the correct citation for "forms a complex with TFIIH to prevent the dissociation of the CAK and XPD". Reference 40 should be cited here instead. In addition, in the second paragraph, reference to a more current review of TCR would be appreciated.*

   Corrected

2. *Introduction, p. 4: The statement "Mutations in one of the NER factors ..." is incorrect as written, since only mutations in XPB or XPD can cause all three of these disorders.*

   Corrected, we deleted “one of the”

3. *Introduction: Potential use of the XpdG602D mouse model in drug testing is not the most compelling rationale for this study, which is of fundamental importance in its own right. In any case, all experiments except for those of Fig. 1B, C were conducted with normal or mutant human cells.*

   Corrected, we deleted the sentence “Because of the potential use of the *Xpd*^{G602D} mouse model in drug testing” and sentences related.

4. *Introduction, p. 6, line 11: missing) after "UV damage".*

   Corrected

5. *Results, p. 7: insert "one" into "which allows [one] to follow the dynamic behavior...". Delete mention of MDFs as irrelevant to this study, since only keratinocytes seem to have been used.*

   Corrected.

6. *Results, p. 9: "...and obtained similar results (Figure S3B)..." XPB staining in an XP12Ro XPA primary cell looks quite different from the transformed cells as accumulation in the damaged spot is very hard to see and the staining looks much more diffuse. Perhaps this cell is not representative?*

   Indeed, we now used a cell that is more representative.

7. *Results, p. 10: "...we found that ERCC1 is recruited to locally UV-exposed areas in WT and XP-G cells...". The ERCC1 foci are very difficult to see in the WT cells in Fig. 3A. Can this image be improved?*

Although the original image is unambiguous when observed with the microscope's dedicated computer screen, its low contrast does not tolerate image enhancement well. We replaced the image with another one.

8. *Fig. 3D&E: These are poorly prepared, with axis labels, symbols, error bars, and symbol key all too small and faint for good visibility. In addition, the XP-G and XP-A cell data are plotted using the same color, which leads to some confusion. Different colors should be used. The figure legend should state how lesions were detected and quantified.*

The two graphs have been improved as required and figure legend corrected.

9. *Fig. 4B: What does the * represent? No explanation is given.*

Corrected in the figure legend: "*The * at 10 J/m^2 indicates an undetectable RPA accumulation signal in MRC5 cells.*"

10. *Methods, p. 21: No source is given for the anti-ERCC1 antibody. Typographical errors: Should be "prior to UVC"; please insert "treated" after "mock" (mock-treated coverslips).*

Corrected

Answers to referee #2

We would like to thank Referee #2 for the careful reviewing process and the nice comments on our work. We have integrated in the text and in the article most of the remarks made by referee #2 and we believe the article is now more clear.
For each point-by-point answer we will refer to the text change in the article.

POINT 1.
The recruitment of RPA to sites of local UV damage has previously believed to a part of NER independent of transcription as is also shown in Figure 2A. The fact that inhibition of transcription would lead to reduced levels of RPA is surprising and is in contrast with the current literature. This discrepancy should be reconciled.

We acknowledge the misunderstanding here: we never wanted to show that RPA accumulation is transcription dependent or not and we probably did not stress this enough in the text.

Indeed, we do think that transcription inhibition is not actually impeding directly RPA accumulation on the local damage, but we think that this phenomenon is a secondary effect due to the fact that many genes will be shut down during the alpha-amanitin treatment (which is 16 h long), some of them producing also proteins that could be needed for RPA accumulation or upstream of this accumulation on the local damage. In fact, if any protein upstream of this accumulation will have an m-RNA with a very short lifetime, the protein will not be produced and the cell will be deficient in one of the steps on NER-protein accumulation on the damage (very probably the opening of the single-strand). The nature of this protein remains unknown and it was not our purpose to investigate into this phenomenon in this article. We just used this staining to show that indeed transcription inhibition blocks the gH2Ax signaling, but it is just because the DNA opening is not made for one reason we still do not know and very probably because RPA is not loaded (RPA being the signal needed to induce the ATR signaling). However, here RPA accumulation is just used as a sensor for this helix opening.

Moreover and most importantly, we used this staining (as well as the gH2Ax staining) to show that transcription inhibition does not just specifically affect XPCS2 cells but all the cells tested (WT, XPA and XPG) demonstrating that there is no specific transcription-related XPCS2 response.

In order to make this point clear in the text, we included a more unambiguous explanation of our data and our purpose at pg 10 (end of chapter "Effect of transcription inhibition on UV-induced DNA damage response")

Text added: *"It is important to stress here that RPA accumulation on local damage is in this case just used as a marker for DNA opening. We do not want to demonstrate or suggest that RPA accumulation during NER is transcription dependent, but that transcription inhibition, by a mechanism that remains to be explored, affects the opening of the double helix or most likely, by a secondary effect due to depletion of some crucial factors from the protein pool, inhibit the accumulation of an upstream protein needed for RPA accumulation"*

POINT 2.
The previous study of PCNA ubiquitination in the context of UV damage indicated that this occurs independently of NER activity, even in XPC- or XPA-deficient cells. Can the authors explain why PCNA ubiquitination would be inhibited in XPCS2 or XPCS1RO cells?

Thank you for reminding us of this work. We suppose referee #2 is referring to the work of Vrouwe and collaborators.

Indeed, PCNA-Ub in non-replicative cells can also be due to the signaling system DDR indicating to the cell that a persistent lesion remains unrepaired (which is the case in XPC and XPA cells). However, in this case PCNA-Ub happens later in time than the PCNA-Ub observed in wilt type cells, which is mainly triggered by the presence of the substrate 3' OH left by the cutting of the ERCC1/XPF endonuclease.

Of course any source of 3'OH substrate can induce PCNA-Ub but in our case, because of the timing we used to detect PCNA-Ub, we can suppose this source is primarily the NER-reaction and not the later signaling.

At early time points after UV irradiation, in XPG cells no PCNA substrate is formed, because of the absence of XPF/ERCC1 cutting, while in XPCS cells, because of a residual XPF/ERCC1 cut, PCNA-Ub can take place at a reduced level. Of course, in WT cells, because the cut is fully proficient, PCNA-Ub is stronger. It is of course not excluded that at later time points we would find a PCNA-Ub also in the XPG cells, the nature of this PCNA-Ub would be the same as the one observed for XPC or XPA cells.

We will acknowledge the article by Vrouwe and collaborators, because we believe in many points, our data on RPA accumulation and gH2Ax signaling confirm and are in line with the results presented previously in this work.
Moreover, to make this point clear in the text, we modified some sentences at pg 11.

POINT 3.
The manuscript would benefit from a thorough editing of the text. Many sentences are grammatically incorrect and/or poorly structured.

We have gone through the entire manuscript and hope to have addressed this point.

MINOR POINTS:

Ref 8 is not appropriate. Ito et al, Mol Cell 2007 (Ref 40) should be cited here instead.

Corrected.

p3. 3rd line from bottom: format reference - complete Reference 15

Corrected.

The XPD/CS mouse model is in various contexts a referred to as: XpdG206D (p4), XP-D/CS (p7) or TFIIH XP/CS (p.7). Please decide on one nomenclature to avoid confusion.

Corrected. The mouse is now Xpd^{G602D} overall in the text. The nomenclature $TFIIH^{XP/CS}$ refers to the mutated complex or cells.

p13. 7th line: Reference a recent removing flap structures: Sertic et paper showing the involvement of a human ExoI al PNAS 2011, 108, 13647

Corrected

Answers to referee #3

We would like to thank Referee #3 for the reviewing process and the nice comments on our work.
We have integrated in the text and in the article most of the remarks made by referee #3.
For each point-by-point answer we will refer to the text change in the article. We would like to thank the referee especially for his words: *"The manuscript counters a previous interpretation of how this particular mutation in the XPD subunit of TFIIH promotes genomic instability. The authors here do an excellent job of countering this with solid data that conflict with that interpretation, yet a diplomatic presentation. Their new interpretation is provocative, well justified and truly paradigm shifting for aficionados of NER*".

MINOR POINTS:

ERCC1/XPF should be ERCC1-XPF.

Corrected.

It is preferable to use -in 'the" presence- rather than -in presence- (throughout the paper)

Corrected.

Double-strand and single-strand is preferable to no hyphen.

Corrected.

There is a close parenthesis missing on page 6 pg. 7 XpbY/Y is not italicized

Corrected.

The increased RPA recruitment is likely to activate ATR to a greater extent in XP-D/CS cells than other NER mutants. One wonders if an ATR inhibitor would diminish genomic instability or cytotoxicity? - as a therapeutic angle for XP/CS patients.

We think that even if the ATR signaling cascade were to be inhibited in XP/CS cells, the mechanism involving the creation of long single-strand flaps (and ultimately the breaks that can derive from these structures) would not be hindered, because this mechanism originates directly from a faulty NER reaction and it is known that the NER reaction can proceed when ATR is inhibited. However, deleterious downstream events could be affected by ATR inhibition and it would indeed be interesting to further explore this idea in a follow-up study.

2nd Editorial Decision 07 March 2012

Thank you for submitting your revised manuscript for our consideration, and please excuse the delay in its re-evaluation, which was due to the revised article still raising a substantial number of issues, as you will see from the comments of the original referee 1 copied below. Furthermore, I noticed that several editorial points raised in my original letter were also neglected during preparation of this

revision. In conclusion, I am afraid that in our opinion, the revised manuscript is at this point still not acceptable for publication in The EMBO Journal.

As mentioned before, it is our policy to allow a single round of major revision only. In this case, given that referee 1 remains overall supportive despite their residual major concerns, I would be inclined to offer you one more opportunity to address the open issues through an exceptional second round of revision. However, I have to make it very clear that this will be the final round for this paper, and that we would have to ultimately reject if the re-revision fails to clarify the remaining problems to both the editor's and referee's satisfaction.

For the re-revision to be successful, the following will at this stage be essential:

- reformatting the paper according to EMBOJ guidelines - especially no numbered referencing

- including an Author Contribution statement as well as a Conflict of Interest declaration

- providing all supplementary text and figures combined in one single Supplementary Information PDF

- rewriting the Methods sections on 'Fluorescence Recovery After Photobleaching (FRAP)' and on 'Chromatin Immunoprecipitation', which currently read like near-verbatim copies from Giglia-Mari et al PLoS Biol 2009 and Coin et al Mol Cell 2008, respectively, and could therefore be seen as a form of self-plagiarism if published with the current wording

- addressing all the various remaining issues of referee 1 with regards to interpretation and conclusions, not only in the response letter but also in the manuscript itself

- conducting the few additional experiments requested by referee 1 (points 4 and 8)aimed at testing different predictions of the two opposing models. Given the remaining problems with decisive interpretation of the results, I am afraid this will be essential at this stage to strengthen the insights and make the manuscript eventually suitable for The EMBO Journal!

- addressing the issues raised regarding both data presentation as well as overall presentation/writing of the study. With respect to the latter, I would strongly urge you to not only carefully edit and revise the study, but to also have the manuscript proof-read and copy-edited by a native speaker of English.

Should you be willing and able to address all these points, then we would be prepared to consider a re-revised manuscript for one final round of re-assessment. I would therefore appreciate if your resubmission, in addition to the full referee rebuttal letter, also contained a response letter to the editor describing briefly how you addressed each of the points I listed above.

Please let me know if you should have any questions regarding this decision.

Yours sincerely,
Editor
The EMBO Journal

_____

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors have provided an extensive rebuttal to the reviewer comments on the original version of this manuscript, but it is disappointing that only rather minimal changes have been implemented in the manuscript itself. Interesting results of two new experiments have been added, but neither of these directly addresses the authors' model, nor do they definitively refute the opposing previous model of A.R. Lehmann and colleagues. However, as noted originally, this manuscript is significant

for the novelty and importance of the reported findings that TFIIH containing the G602D mutant form of XPD (present in the human XP-D/CS patient cells XPCS2 and in the knockin mouse model) is itself retained longer in locally UV-damaged regions but results in reduced retention of XPG, along with recruitment of Exo1 and generation of long stretches of single-stranded DNA. Although the Exo1 recruitment, ssDNA generation, and reduced XPG retention are strictly correlative observations at this stage, they do plausibly lead to the authors' model that the mutant TFIIH results in strand displacement synthesis as a result of inefficient incision by XPG. These are new and provocative results that are likely to be of considerable importance and that will surely generate a great deal of interest and additional research. The manuscript will thus be an important contribution to the DNA repair and transcription fields. However, the authors would do future readers of this study a service by modifying and extending some of the interpretations and discussion as noted below. Furthermore, also as noted in the specific comments, it is still the case that additional experiments to directly test opposing predictions of the two models would greatly strengthen the manuscript. In particular, nowhere in the currently presented results or discussion do the authors directly address the previous observation that UV-induced breaks are transcription-dependent in XP-D/CS cells but NOT in WT, and this should be remedied.

In addition to remaining concerns about some of the presented interpretations, thorough editing is still required. The revised manuscript appears to be rather hastily prepared, and there are a great many errors throughout, both typographical and grammatical, as well as a number of very awkward phrasings. Because these problems are so numerous, they are not listed specifically. NOTE: All references throughout the manuscript after the very first paragraph are off by -1 (e.g., the text reference to [28] should actually be [29], etc.), and the supplemental figure legends S3, S4, and S7 are mixed up.

Because the authors' rebuttal best captures the remaining issues, the following specific comments relate to key points in the rebuttal and refer to points in the manuscript where appropriate.

MAJOR SPECIFIC COMMENTS:

1. The demonstration that gH2AX staining is confined to the local area of damage (p. 5; Fig. S1A) is not original to this study; in fact, it was shown originally by Theron et al. Whether the Lehmann model requires pan-nuclear transcription-dependent breaks in the XP-D/CS cells is clearly a matter of opinion. The authors have modulated their insistence on this point to some extent in the revised version, but it would be appropriate to acknowledge that this issue was considered by Theron et al.

2. The new ChIP experiment to compare the binding of TFIIH to promoters with and without UV in WT vs XP-D/CS cells (p. 8, Fig. 1D) is a worthy addition but is inadequately presented. The results (no difference between the two strains after UV) clearly do contradict the simplest prediction from the Lehmann model. They do not, however, definitively rule out the Lehmann model, since it is conceivable that the proposed switch from transcription initiation mode to NER mode (e.g. a conformational change that governs partnerships) occurs inappropriately for TFIIH complexes that are still engaged in transcription. Furthermore, while these new results are useful in lending additional support to the authors' interpretation, considerably more information must be provided in order to be acceptable for publication. There is not even any mention of what promoters were assayed, no information is provided on primers used or on control reactions, and there are no primary data. What is shown in Fig. 1D does not meet usual standards for presenting qPCR results and serves only as a summary.

3. Authors' response to previous Point 2: The numerical argument is persuasive and could profitably be incorporated into the manuscript in support of their interpretations.

4. Authors' response to previous Point 3, concerning gH2AX and RPA staining: On the face of it, the presented data argue that this staining, while UV dependent, is NOT dependent on NER, since it also occurs in cells that lack XPA or XPG. Nevertheless, the authors view it as "associated to the NER reaction" (top of p. 9) and due to "RPA engagements during NER" (p. 10) without obvious support for that conclusion. Such support was in fact provided for the case of gH2AX by Theron et al, who showed that there is NO gH2AX signal in XP-D cells, although it occurs at early times (2 hr) in WT cells and both early and persistently in XP-D/CS cells. The difference between events in XP-D vs XP-D/CS cells is a very interesting distinction that in fact could have been used to good

effect in the present study, and should at least be discussed in the context of the results presented in this manuscript. The huge surprise in the results of Godon et al is that transcription inhibition abolishes both gH2AX and RPA staining in all cells tested, not just in XP-D/CS. However, this result does not bear on refuting (or not) the Lehmann model, which was specifically based on the finding that the strand breaks depend on transcription in XP-D/CS cells but NOT in WT, and that strand breaks do not occur at all in XP-D cells. The authors do not deal with this previously reported observation, but it is necessary that they do so.
With respect to their observed puzzling universal effect of amanitin on gH2AX and RPA accumulation, the authors now propose (p. 10, and rebuttal) that this may be a secondary effect due to depletion of some hypothetical required protein factors during the 16 hr treatment that was used. While this explanation is doubtless a possibility, the prevention of RPA recruitment by alpha-amanitin treatment (which should prevent the NER reaction, as the authors point out) is inconsistent with the known lack of effect of transcription inhibition by amanitin on global NER (Christians and Hanawalt, Mutat. Res., 274:93-101, 1992). It would therefore be worth determining whether changing the inhibitor conditions used so that transcription is prevented at the time of damage but not for many hours before might give a different result in WT cells, with no effect on RPA (or gH2AX) accumulation in that case. If so, it should then be asked whether there is still an effect in XP-D/CS cells. These are experiments that really ought to be done, if only to lessen the consternation that is likely to result from publication of the current results.

5. Authors' response to previous Point 4: It is unfortunate that the authors misinterpreted the previous statement about the "specious" comparison of UV damage to laser damage. What was intended was simply to point out that the results cannot be directly compared since strand breaks are immediate in the latter case but develop over time after UV. In any case, it is not clear what point the authors wish to make with respect to the question at hand by looking at strand breaks at early times after UV (judged by Ku and PAR recruitment). The existence of strand breaks as early as 1 hr after UV in the XP-D/CS cells was well documented by Berneburg et al using the much more sensitive comet assay, and PAR staining was shown by Theron et al to be detected at 2 hr but to persist for at least 24 hrs, whereas in WT cells PAR was detected at 2 hr but gone by 4, and it was never detected in XP-D cells. What conclusions are meant to be drawn from the data shown in Fig. S2 and discussed on p. 9 is thus unclear.

6. Authors' response to previous Points 5 and 6: It is of course true that in vitro studies, particularly with purified proteins, do not always correctly reflect events in the cell. However, the lack of helicase activity in this mutant XPD has been widely reported, and furthermore the Berneburg et al paper demonstrated a complete absence not only of dual incisions but also of uncoupled incisions (of particular importance for the Godon et al model) and of repair synthesis in XP-D/CS cell extracts - which is at least closer to the situation in the cell than the results of Dubaele et al with purified recombinant TFIIH. With respect to the lesion removal presented in Fig. 3D, a low level of 6,4-PP removal in XP-D/CS cells was previously reported, but was also seen in XP-D cells which do not have strand breaks at all (van Hoffen et al, NAR 1999). These results thus cannot correctly be used as proof that there is a low level of incision at lesions in the XP-D/CS cells. (Here again, comparison to results with XP-D cells would be useful.)

7. Authors' response to previous Point 7: Addition of the technical details concerning quantification of RPA in the locally damaged areas is helpful and appreciated.

8. Authors' response to previous Point 8: Testing whether recruitment of ERCC1, the generation of "extra" ssDNA, and recruitment of Exo I in the XP-D/CS cells is prevented by transcription inhibition remains a critical requirement in order to address the authors' model vs the Lehmann model in the context of the published observation that the strand breaks observed in XP-D/CS cells require transcription. The authors argue that this would be a meaningless experiment, since they observe blockage of RPA accumulation by alpha-amanitin even in WT cells under their conditions and therefore NER could not occur. However, as pointed out in #4 above, global NER is not blocked by amanitin under previously published conditions, so likely what is necessary is to adjust conditions for the inhibitor treatment.
The authors' model also directly implies that persistent strand breaks generated by ERCC1/XPF and flap cleavage, generation of excess ssDNA, and Exo1 / FEN1 recruitment should also occur in XP-G cells, in which full-length XPG protein is present but catalytically inactive, so that the 3' incision cannot be made although TFIIH is (presumably) normal. It would be nice to see this prediction

tested, although it may arguably be beyond the scope of this paper.

9. The new experiment showing that CAK is not released normally from the mutant TFIIH in XP-D/CS cells is certainly interesting, although it is unclear how it bears on the model, and the authors offer no discussion of this finding. Given that XPG has been reported to be involved in stably maintaining both CAK and XPD in TFIIH, but that the authors conclude that XPG is less strongly associated with TFIIH in the mutant cells, the result seems somewhat counter-intuitive in view of the other conclusions.

10. Introduction, last paragraph, p. 6: The two sentences beginning with "Taken together, our observations indicate ..." are strictly inferences / interpretations, rather than firm conclusions. It is important to distinguish what is inferred from what is demonstrated. For example, it has not been directly shown that "XPG .... cannot correctly bind the TFIIH complex". A similar statement in Results, p. 15 (beginning of 2nd paragraph), should also be corrected. Yet another example is in Discussion, p. 17, where it is stated that "we were able to detect ... larger single-strand NER bubbles". In fact what was detected (convincingly so) was an increased amount of ssDNA.

MINOR POINTS:
In addition to the already noted requirement for thorough editing, a few corrections are needed that were not noted in review of the original manuscript.

1. The human cells that are mutant for XPG and XPA are incorrectly designated in the figures as deltaXPG or deltaXPA. These cells contain patient nonsense mutations that are severely truncating, but they are not gene deletions. Also, the patient cell strain/line names are correct in the Methods but incorrectly written in the figures: "RO" is correct, not "Ro" (i.e. should be XPCS1RO and XP12RO).

2. The patient cells XPCS1RO are from an XP-G/CS patient, not an XP-G patient as stated in several places in the text. (This is an important distinction.)

3. Should use RNA Pol II (or RNAPII), not RNAP2.

4. Results, p. 11 top: The ChIP western experiment does not address whether the detected proteins are "lesion-bound". This is a misstatement.

5. Discussion, p. 18: The statements concerning XPD structural data are predictions from the structures, not conclusions.

2nd Revision - authors' response 22 June 2012

Here is the point-by-point answer to referee 1, which we would like to thank for the experimental suggestions he made, which allowed us to greatly improve the interpretation of our results and understand better the results previously published on the subject. We also would like to thank him for the thorough proofreading of the manuscript, which allowed us to better phrase our propositions.

Referee #1 (Remarks to the Author):

The authors have provided an extensive rebuttal to the reviewer comments on the original version of this manuscript, but it is disappointing that only rather minimal changes have been implemented in the manuscript itself. Interesting results of two new experiments have been added, but neither of these directly addresses the authors' model, nor do they definitively refute the opposing previous model of A.R. Lehmann and colleagues. However, as noted originally, this manuscript is significant for the novelty and importance of the reported findings that TFIIH containing the G602D mutant form of XPD (present in the human XP-D/CS patient cells XPCS2 and in the knockin mouse model) is itself retained longer in locally UV-damaged regions but results in reduced retention of XPG,

along with recruitment of Exo1 and generation of long stretches of single-stranded DNA. Although the Exo1 recruitment, ssDNA generation, and reduced XPG retention are strictly correlative observations at this stage, they do plausibly lead to the authors' model that the mutant TFIIH results in strand displacement synthesis as a result of inefficient incision by XPG. These are new and provocative results that are likely to be of considerable importance and that will surely generate a great deal of interest and additional research. The manuscript will thus be an important contribution to the DNA repair and transcription fields. However, the authors would do future readers of this study a service by modifying and extending some of the interpretations and discussion as noted below. Furthermore, also as noted in the specific comments, it is still the case that additional experiments to directly test opposing predictions of the two models would greatly strengthen the manuscript. In particular, nowhere in the currently presented results or discussion do the authors directly address the previous observation that UV-induced breaks are transcription-dependent in XP-D/CS cells but NOT in WT,and this should be remedied.

In addition to remaining concerns about some of the presented interpretations, thorough editing is still required. The revised manuscript appears to be rather hastily prepared, and there are a great many errors throughout, both typographical and grammatical, as well as a number of very awkward phrasings. Because these problems are so numerous, they are not listed specifically. NOTE: All references throughout the manuscript after the very first paragraph are off by -1 (e.g., the text reference to [28] should actually be [29], etc.), and the supplemental figure legends S3, S4, and S7 are mixed up.

Figure legends and references have been corrected.

Because the authors' rebuttal best captures the remaining issues, the following specific comments relate to key points in the rebuttal and refer to points in the manuscript where appropriate.

MAJOR SPECIFIC COMMENTS:

1. The demonstration that gH2AX staining is confined to the local area of damage (p. 5; Fig. S1A) is not original to this study; in fact, it was shown originally by Theron et al. Whether the Lehmann model requires pan-nuclear transcription-dependent breaks in the XP-D/CS cells is clearly a matter of opinion. The authors have modulated their insistence on this point to some extent in the revised version, but it would be appropriate to acknowledge that this issue was considered by Theron et al.

We acknowledged the work of Theron et al. and further modulated the requirements of a pan-nuclear signal in XP-D/CS cells in the original Lehmann model, by adding at the end of page 5 the following text:

”The original off-site incision model could imply the existence of a pan-nuclear transcription-dependent UV-induced DNA break formation in XP-D/CS cells. However, similarly to what has been previously shown (Theron et al, 2005), at early time points after local UV irradiation H2AX phosphorylation (YH2AX, a marker for DNA breaks) is massively present at the UV exposed areas (Supplementary Figure S1A and S1B for H2A ubiquitination). Although this would argue against a pan-nuclear DNA break formation as previously noted (Theron et al, 2005), a localized YH2AX signal in XP-D/CS cells is not a sufficient proof to refute or validate the off-site incision model at promoter sites.”

2. The new ChIP experiment to compare the binding of TFIIH to promoters with and without UV in WT vs XP-D/CS cells (p. 8, Fig. 1D) is a worthy addition but is inadequately presented. The results (no difference between the two strains after UV) clearly do contradict the simplest prediction from the Lehmann model. They do not, however, definitively rule out the Lehmann model, since it is conceivable that the proposed switch from transcription initiation mode to NER mode (e.g. a conformational change that governs partnerships) occurs inappropriately for TFIIH complexes that are still engaged in transcription.
Furthermore, while these new results are useful in lending additional support to the authors' interpretation, considerably more information must be provided in order to be acceptable for publication. There is not even any mention of what promoters were assayed, no information is provided on primers used or on control reactions, and there are no primary data. What is shown in Fig. 1D does not meet usual standards for presenting qPCR results and serves only as a summary.

To clarify this point, we added the names of the genes used in this experiments in the M&M section as well as the procedure used to quantify the qPCR results. The qPCR results of each gene are now presented in the supplementary figure S2. We hope this responds to referee 1's request for "primary data", however we believe that showing actual qPCR raw data curves would not at all be expected by future readers. We refer to this extra information in the main text at page 9. Since the qPCR experiment was not over interpreted and serves only to show that the mutant TFIIH complex is not abnormally retained at promoter sites, it did not seem necessary to modify the text any further.

3. Authors' response to previous Point 2: The numerical argument is persuasive and could profitably be incorporated into the manuscript in support of their interpretations.

As previously suggested by referee 1, we changed our phrasing at page 9 to: "in regions containing high concentrations of DNA lesions". The numerical argument has been introduced in the discussion at page 24.

4. Authors' response to previous Point 3, concerning gH2AX and RPA staining: On the face of it, the presented data argue that this staining, while UV dependent, is NOT dependent on NER, since it also occurs in cells that lack XPA or XPG. Nevertheless, the authors view it as "associated to the NER reaction" (top of p. 9) and due to "RPA engagements during NER" (p. 10) without obvious support for that conclusion.

The text page 10 "associated to the NER reaction" was changed to "associated to the early NER processing" i.e. at least up to TFIIH recruitment. The text page 12 "and confirm that the observed signals were indeed due to RPA engagements during NER" was removed.

Such support was in fact provided for the case of gH2AX by Theron et al, who showed that there is NO gH2AX signal in XP-D cells, although it occurs at early times (2 hr) in WT cells and both early and persistently in XP-D/CS cells. The difference between events in XP-D vs XP-D/CS cells is a very interesting distinction that in fact could have been used to good effect in the present study, and should at least be discussed in the context of the results presented in this manuscript.

We thank referee 1 for suggesting the use of XP-D cells to complement this work. We added to our major experiments the cell line XP6BE that bears the same XP-D causative mutation (R683W) as the cell line used by Theron et al. (XP1BR). However, in contrast to the data cited by referee 1 (Theron et al, Fig 2a), we clearly observed a localized YH2AX signal in these cells 1 hour after local UV exposure (Figure 2A row 5). Although Theron et al. globally exposed cells to 5 J/m2 of UVC and looked for YH2AX at longer times after UV (2,4 and 6 hours), it is not very clear to us why 1 hour after local UVC exposure (60 J/m2), we observe such a different result i.e. YH2AX in XP-D cells. Both YH2AX and RPA recruitment seen in the XP-D cells were suppressed by the 16 hour-long transcription inhibition (as seen for all other cell types we tested, see following point) but not by the 6-hour treatment.

The huge surprise in the results of Godon et al is that transcription inhibition abolishes both gH2AX and RPA staining in all cells tested, not just in XP-D/CS. However, this result does not bear on refuting (or not) the Lehmann model, which was specifically based on the finding that the strand breaks depend on transcription in XP-D/CS cells but NOT in WT, and that strand breaks do not occur at all in XP-D cells. The authors do not deal with this previously reported observation, but it is necessary that they do so.
With respect to their observed puzzling universal effect of amanitin on gH2AX and RPA accumulation, the authors now propose (p. 10, and rebuttal) that this may be a secondary effect due to depletion of some hypothetical required protein factors during the 16 hr treatment that was used. While this explanation is doubtless a possibility, the prevention of RPA recruitment by alpha-amanitin treatment (which should prevent the NER reaction, as the authors point out) is inconsistent with the known lack of effect of transcription inhibition by amanitin on global NER (Christians and Hanawalt, Mutat. Res., 274:93-101, 1992). It would therefore be worth determining whether changing the inhibitor conditions used so that transcription is prevented at the time of damage but not for many hours before might give a different result in WT cells, with no effect on RPA (or gH2AX) accumulation in that case. If so, it should then be asked whether there is still an effect in XP-D/CS cells. These are experiments that really ought to be done, if only to lessen the consternation that is likely to result from publication of the current results.

In order to clarify the issues raised here by referee 1, we assessed which alpha-amanitin concentration is needed to block transcription in our WT, XP-D/CS, XP-G, XP-A and XP-D cells, while keeping the treatment duration as short as possible as suggested by referee 1 (measured by Eu incorporation into newly synthetized RNAs, cf suppl Figure S2 and data not shown). Interestingly we observed that for the same concentration of alpha-amanitin, not all cell types reacted in the same way, namely XP-D/CS cells were remarkably resistant to transcription inhibition by alpha-amanitin. The "optimum" alpha-amanitin treatment we determined was 20µg/ml for 6 hours. We would like to stress the fact that if one would treat the cells we used in this study with the concentration and time used in the paper of Christians and Hanawalt (Mut. Res 1992) as suggested by referee 1 (i.e. 10µg/ml for 5.5 hours of treatment), one would not have full transcription inhibition in WT, XP-D/CS and XP-A cells (the cells used in Christians and Hanawalt's study were CHO cells). This result is very important because it shows that different cell types can show different sensitivities to certain molecules.
We repeated all the transcription inhibition experiments under this "short-time treatment" condition (20 µg/ml for 6 hours) and compared it to the results we (mostly) already had for the "long-time treatment" (25 µg/ml for 16 hours). We found that, regardless of the cell line, transcription inhibition by the short-time treatment had no effect on the YH2AX signal, RPA, XPF and XPG recruitment after local UV exposure (updated figures 2 and 3). Similarly, in XP-D/CS cells, formation of ssDNA and recruitment of Exo1 were not affected by the short-time alpha-amanitin treatment (figure 5C and 5D). However, we have observed that the 16 hour long treatment hinders RPA recruitment and YH2AX signaling (figure 2) but not XPF and XPG recruitment (figure 3). Additionally in XP-D/CS cells the 16 hours alpha-amanitin treatment abolishes ssDNA formation (updated figure 5C) and Exo1 recruitment (updated figure 5D) after local UV exposure.
The striking differences observed between the short and long treatments would support the idea that lengthy transcription inhibition by alpha-amanitin can lead to unwanted secondary effects such as depletion of short-lived factors. Regardless of the exact nature of the secondary effects of lengthy transcription inhibition by alpha-amanitin, our data clearly show that ssDNA formation in XP-D/CS cells is not transcription dependent *per se*.

One possible explanation for the differences observed between this study and the previous ones is based on the fact that any observed event that disappears after a lengthy transcription inhibition (as with alpha-amanitin) cannot be systematically regarded to be directly dependent on ongoing transcription. As for the use of other "transcription inhibitors" such as DRB, H8 or Actinomycin D, although they require much shorter incubation times (typically 2 hours), they are also considered less reliable because of their lack of specificity (DRB and H8 are kinase inhibitors and Actinomycin D is a DNA intercalator)

The new data is now included in the results section and corresponding figures. The new results also appear in the discussion section, where we have stressed the fact that we have not studied the production of DNA breaks at later time points but only the XP-D/CS specific mechanism that leads to the early formation of ssDNA stretches.

The key (quasi full page) text changes are at pages 10 and 12.

5. Authors' response to previous Point 4: It is unfortunate that the authors misinterpreted the previous statement about the "specious" comparison of UV damage to laser damage. What was intended was simply to point out that the results cannot be directly compared since strand breaks are immediate in the latter case but develop over time after UV.

Laser induced DNA damage was primarily used as a positive control for our Ku80 and PAR antibodies. We have gone through the text of the manuscript and made sure that it is not stated otherwise.
Text added page 11: "We verified that the antibody staining for Ku80 and PAR functioned in our assays by staining laser-induced SSBs and DSBs in XP-D/CS cells (supplementary figure S4B)."

In any case, it is not clear what point the authors wish to make with respect to the question at hand by looking at strand breaks at early times after UV (judged by Ku and PAR recruitment). The existence of strand breaks as early as 1 hr after UV in the XP-D/CS cells was well documented by Berneburg et al using the much more sensitive comet assay, and PAR staining was shown by Theron

et al to be detected at 2 hr but to persist for at least 24 hrs, whereas in WT cells PAR was detected at 2 hr but gone by 4, and it was never detected in XP-D cells. What conclusions are meant to be drawn from the data shown in Fig. S2 and discussed on p.9 is thus unclear.

The Comet assay can apparently detect as few as 50 DNA breaks. When DNA damage is present in a localized area of the nucleus, Ku80-GFP can be used as a probe for DSBs in live cells with a detection threshold of approximately 100 to 150 DSBs ("Kinetics of endogenous mouse FEN1 in base excision repair." Kleppa et al. In press, NAR.). Using Ku80 anti-bodies should increase detection contrast by reducing the amount of Ku80 present in the nucleus that is not bound to DSBs, thus may lower the threshold to around 100 DSBs.
Since the YH2AX signals we observe after local UV exposure are very strong, we wanted to test if in the same time frame (1 hour or less), SSBs or DSBs could already be detected in XP-D/CS cells. None were detected but we would expect DNA breaks to appear at later time points. The only conclusion we draw from this is stated at page 11:
"Both of these markers were not detected (above threshold levels) at the local UV damage in XP-D/CS cells (supplementary Figure S4A) at both early time points (15 min and 1 hour) after UV irradiation, indicating that the strong localized YH2AX signal we have observed in these cells is not primarily due to the presence of large concentrations of SSBs or DSBs in these areas. It is however expected that DNA breaks will appear in greater numbers at later time points."

6. Authors' response to previous Points 5 and 6: It is of course true that in vitro studies, particularly with purified proteins, do not always correctly reflect events in the cell. However, the lack of helicase activity in this mutant XPD has been widely reported, and furthermore the Berneburg et al paper demonstrated a complete absence not only of dual incisions but also of uncoupled incisions (of particular importance for the Godon et al model) and of repair synthesis in XP-D/CS cell extracts - which is at least closer to the situation in the cell than the results of Dubaele et al with purified recombinant TFIIH. With respect to the lesion removal presented in Fig. 3D, a low level of 6,4-PP removal in XP-D/CS cells was previously reported, but was also seen in XP-D cells which do not have strand breaks at all (van Hoffen et al, NAR 1999). These results thus cannot correctly be used as proof that there is a low level of incision at lesions in the XP-D/CS cells. (Here again, comparison to results with XP-D cells would be useful.)

Here we do not quite understand the comment of referee 1. He indicates that low levels of 6,4-PP removal in XP-D/CS (and XP-D) cells have previously been reported (van Hoffen et al, NAR 1999) then concludes that our own similar observation "cannot correctly be used as proof that there is a low level of incision at lesions in the XP-D/CS cells". Since we can compare the low level of lesion removal of XP-D/CS cells to the "very" low levels in XP-A (for CPDs) or XP-G/CS cells (statistically not different from zero lesion removal), we see no reason not to state that there is a low level of incision in XP-D/CS cells. As for comparing to XP-D cells, since it has been reported that they also present a low level of 6,4-PP removal, we do not see how this experiment would give any new insights into this issue.

Here is the new text we have added pages 14 and 15:
"Although seemingly in disagreement with previous data, indicating that there is no quantifiable incision activity in XP-D/CS cell extracts on a naked DNA bearing a single cisplatin lesion (Berneburg et al, EMBO 2000), our results show that in living cells, in the proper chromatin context, a small proportion of UV-lesions are in fact removed by the NER machinery. This would imply that at least some UV-induced lesions will lead to the proper positioning of the NER machinery and the excision of the damaged strand."

7. Authors' response to previous Point 7: Addition of the technical details concerning quantification of RPA in the locally damaged areas is helpful and appreciated.

We thank referee 1 for his help in clarifying this part of the manuscript.

8. Authors' response to previous Point 8: Testing whether recruitment of ERCC1, the generation of "extra" ssDNA, and recruitment of Exo I in the XP-D/CS cells is prevented by transcription inhibition remains a critical requirement in order to address the authors' model vs the Lehmann model in the context of the published observation that the strand breaks observed in XP-D/CS cells require transcription. The authors argue that this would be a meaningless experiment, since they

observe blockage of RPA accumulation by alpha-amanitin even in WT cells under their conditions and therefore NER could not occur. However, as pointed out in #4 above, global NER is not blocked by amanitin under previously published conditions, so likely what is necessary is to adjust conditions for the inhibitor treatment.

In light of the additional experiments we conducted following the remarks of referee 1 in point 4, we repeated the ssDNA and Exo1 experiments under the short and long alpha-amanitin treatment durations. As noted in our answer to point 4, the 6h alpha-amanitin treatment prior to local UV exposure in XP-D/CS cells did not affect ssDNA formation and recruitment of Exo1 (figures 5C and 5D columns 3 and 4), while the 16 hour long treatment abolished both (figures 5C and 5D, columns 5 and 6).
Text added page 16:
"Interestingly, while short-time transcription inhibition does not affect ssDNA production (Figure 5C, columns 3 and 4), 16 hours treatment with alpha-amanitin inhibit the formation of ssDNA in XP-D/CS cells (Figure 5C, columns 5 and 6). These results are in accordance with the inhibition of RPA recruitment under the same transcription inhibition conditions observed in all cell types studied (Figure 2B, columns 5 and 6)."

The authors' model also directly implies that persistent strand breaks generated by ERCC1/XPF and flap cleavage, generation of excess ssDNA, and Exo1 / FEN1 recruitment should also occur in XP-G cells, in which full-length XPG protein is present but catalytically inactive, so that the 3' incision cannot be made although TFIIH is (presumably) normal. It would be nice to see this prediction tested, although it may arguably be beyond the scope of this paper.

At this stage of our study, although the suggested experiments are of strong interest, we agree with referee 1 that they are beyond the scope of this paper.
On more scientific grounds, we could argue that inefficient binding of XPG in XP-D/CS cells is mechanistically different from the (presumably) proper binding of a catalytically inactive XPG protein. The model we currently propose does not include detailed enough information on the actual processes that leads to the formation of excess ssDNA, hence does not provide, in our opinion, any prediction on what would occur with a catalytically inactive XPG protein. Nonetheless, we agree with the referee 1 that this issue could be a future road to explore.

9. The new experiment showing that CAK is not released normally from the mutant TFIIH in XP-D/CS cells is certainly interesting, although it is unclear how it bears on the model, and the authors offer no discussion of this finding. Given that XPG has been reported to be involved in stably maintaining both CAK and XPD in TFIIH, but that the authors conclude that XPG is less strongly associated with TFIIH in the mutant cells, the result seems somewhat counter-intuitive in view of the other conclusions.

In order to clarify this point, text page 23 was reformulated to:
"These results are in agreement with XPD structural data (Fan et al, 2008; Liu et al, 2008; Wolski et al, 2008) which predict that the XP-D/CS mutation confers a certain rigidity to XPD, influencing mainly the dynamic protein-protein interactions within TFIIH, such as CDK7, and possibly with closely interacting proteins such as XPG (Ito et al, 2007). It is possible to imagine that the XPDG602D would change the conformation of TFIIH in such a way that CDK7 (and probably the CAK) would be more strongly retained to the core, while XPG would on the contrary be less bound to TFIIH."

10. Introduction, last paragraph, p. 6: The two sentences beginning with "Taken together, our observations indicate ..." are strictly inferences / interpretations, rather than firm conclusions. It is important to distinguish what is inferred from what is demonstrated.

Page 6, "indicate" was changed to "suggest"

For example, it has not been directly shown that "XPG .... cannot correctly bind the TFIIH complex".

Page 7, the text was modified to “the XPG endonuclease, which, in the presence of the XPDG602D mutation, is less efficiently bound to its protein partners (in the absence of UV) and chromatin (in presence of UV damage)”

A similar statement in Results, p. 15 (beginning of 2nd paragraph), should also be corrected.

Pages 18 and 19 were corrected for similar statements.

Yet another example is in Discussion, p. 17, where it is stated that "we were able to detect ... larger single-strand NER bubbles". In fact what was detected (convincingly so) was an increased amount of ssDNA.

References to “single-strand NER bubbles” were corrected to “single-strand DNA stretches” or “single-strand DNA substrate”

MINOR POINTS:
In addition to the already noted requirement for thorough editing, a few corrections are needed that were not noted in review of the original manuscript.

1. The human cells that are mutant for XPG and XPA are incorrectly designated in the figures as deltaXPG or deltaXPA. These cells contain patient nonsense mutations that are severely truncating, but they are not gene deletions. Also, the patient cell strain/line names are correct in the Methods but incorrectly written in the figures: "RO" is correct, not "Ro" (i.e. should be XPCS1RO and XP12RO).

This has been corrected throughout the manuscript.

2. The patient cells XPCS1RO are from an XP-G/CS patient, not an XP-G patient as stated in several places in the text. (This is an important distinction.)

This has been corrected throughout the manuscript.

3. Should use RNA Pol II (or RNAPII), not RNAP2.

This has been corrected throughout the manuscript.

4. Results, p. 11 top: The ChIP western experiment does not address whether the detected proteins are "lesion-bound". This is a misstatement.

This has been corrected to “Recruitment of XPF to NER complexes …” page 13.

5. Discussion, p. 18: The statements concerning XPD structural data are predictions from the structures, not conclusions.

This has been corrected at page 23.

Acceptance 27 June 2012

Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised in the previous round of review, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal. You shall receive a formal letter of acceptance shortly.

Yours sincerely,
Editor
The EMBO Journal

