Two-stage dynamic DNA quality check by xeroderma pigmentosum group C protein

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

1st Editorial Decision 05 March 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees, whose comments are enclosed. As you will see, all three reviewers recognise the high quality of your work, and appreciate that it adds significantly to the current model of XPC function. However, there are some concerns as to the conceptual advance made (stated more explicitly in the confidential remarks to the editor), particularly in the light of the model proposed by Min and Pavletich, which you extend. We feel that your analysis does provide interesting and important new insight, and therefore would like to invite you to submit a revised version of your manuscript. I would, however, like to stress that - given these limitations in terms of conceptual novelty - it is essential that you fully address all the concerns of the reviewers, in order to ensure that the mechanistic advance is absolutely convincing.

I will not go through all the points raised in detail, but in particular, referees 1 and 3 find that the DNA binding analyses need to be extended in order to better characterise the binding preferences of different XPC constructs. In addition, both referees 1 and 2 comment upon the apparent discrepancy between your work and that of Yasuda et al. in terms of the role of DDB; it would be important to resolve these issues. Finally, I would like to draw your attention to the concerns of the referees regarding appropriate citation of previous publications.

I should add that it is EMBO Journal policy to allow only a single round of revision. Therefore, acceptance of your paper will depend upon the next, final version, which will have to be re-evaluated by at least some of the referees.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.
Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Deletion and site-directed mutagenesis analysis of the DNA damage sensor XPC revealed that a surprising short part (15%) of this protein, comprising two beta-hairpin domains (BHD1,2), is responsible for damage sensing (i.e. hetero-duplex DNA). The authors nicely show that the dynamic properties of this sensor are determined by a small peptide sequence (N-EXT) adjacent to the BHD1,2 that antagonizes DNA binding. The authors show that this DNA-repulsive action enhances the mobility of the protein in nuclei. They suggest that this property provides sufficient dynamics to XPC to efficiently detect lesions and that a further extension of this domain by a third beta-hairpin (BHD3) stabilizes the transient sensor to single-stranded regions.

General Comments:
This is a nice and solid piece of work, providing evidence for a two-stage DNA damage sensing/binding mode of the global genome NER DNA damage initiating protein XPC, and is further detailing of their previous work on dissecting the XPC DNA damage sensing mode. Although this analysis might be considered as a very detailed analysis of binding domains and residues of XPC that further substantiates earlier structural work (of a functional S. cerevisiae homolog Rad4), the most intriguing finding is however the identification of a DNA-repulsive (as dubbed by the authors) property of a small domain adjacent to the DNA binding domain that may provide sufficient dynamics to the protein to efficiently scan the genome for helix-distortions. The combination of both in vitro DNA binding studies with live cell analysis is particular attractive and provides a further solid base on their claims.

Specific comments:
1. Within the introduction and throughout the paper the authors do not mention nor discuss the role of the other components of the XPC complex (RAD23B and Cen2). This omission should be corrected, particularly the established role of RAD23B in DNA damage sensing. Are the different DNA binding properties of the truncation mutants due to loss or gain of RAD23B binding?
2. Along the same line the role of the other GG-NER initiator is also completely ignored.
3. Particularly the first part could be significantly reduced and toned down. It is not new to use GFP-tagging to monitor XPC translocation to DNA damage in living cells. A reference to this work (Hoegstraten, 2008) should be included in the result session when describing this procedure. As such the entire Fig.1 is a bit superfluous. Do not see the need of showing both a BW and color image, the notion that non-tagged GFP is expressed everywhere in the cell (Fig 1 a) has been shown million times. Assembly kinetics have been shown previously (Politti, 2005; Hoogstraten, 2008). One image would be sufficient to show that their applied method to introduce local DNA damage would be sufficient.
4. Idem for Figure 2A. It was known before that W690S has a poor binding to damaged DNA (Yasuda, 2007; Maillard, 2007; Hoegstraten, 2008).
5. The authors should discuss the discrepancy with Yasuda, that they claim is still able to bind to DNA damage but that this requires DDB2.
6. Page 8, Fig 4: The authors discuss that the binding of the truncated mutant XPC 1-766 does not depend on DDB2 by analyzing it also in CHO cells (data not shown). Although CHO have a reduced amount of DDB2 they are entirely devoid of it. A similar siRNA k.d. as in Yasuda (perhaps using even the same siRNA target sequences) should be performed (see comment above).
7. Figure 4 and 5: The authors should discuss the apparent discrepancy between the mutants 1-766 and 1-831, as the latter shows in the FRAP exp. a UV-sensitive response (transient immobilization) absent in the former, however both mutants exhibit a similar partial correction of the UV-sensitivity of XP (Fig 4) in the reactivation assay.
8. Typo on page 9, last paragraph first sentence: "The fragments......... and XPC1-831...." . I guess
this should be: XPC607-831.

9. Fig 6G, The authors nicely show that BHD3 confers a preference for ss DNA, rather than for heteroduplex DNA. This is a surprising observation, since it is expected that for its damage-sensing function in cells, it should have preference for small hetero-duplex regions in a ds background and was shown previously for the full length protein (Kusumoto, 2001). How does the full length protein behave in this assay?

Referee #2 (Remarks to the Author):

In this manuscript, Camenisch and co-workers try to determine a minimal structural unit of XPC protein required for recognition of DNA damage and initiation of nucleotide excision repair (NER). XPC is a critical DNA damage sensor in the global genome NER subpathway in higher eukaryotes, so that it must be of biological relevance to understand the precise molecular mechanism underlying in vivo damage search conducted by it.

Based on the published domain structure of yeast XPC homolog, RAD4, the authors tested a series of XPC deletion mutants for in vivo accumulation to local UV damage, ability to support in vivo NER reactions, and in vitro DNA binding activities. They first show that the C-terminal part of XPC (607-940) containing three consecutive beta-hairpin domains (called BHD1, BHD2, and BHD3, respectively) is sufficient to accumulate in local UV damage foci, whereas the more N-terminal part involving the transglutaminase-homology domain (TGD) seems still necessary for removal of UV damage. Most surprisingly, the BHD3 domain, which was shown to interact directly with damaged sites and thus supposed to be most important for its damage recognition function, seems to be dispensable for in vivo targeting of XPC to local UV damage and even for damage removal itself. They further show that the N-EXT subdomain, located between BHD2 and BHD3, has some positive effects to enhance in vivo mobility of XPC, whereas BHD3 may contribute to stabilize the ‘ultimate damage recognition complex’ after the ‘dynamic damage sensor’ composed of BHD1 and BHD2 once finds a DNA site that has disrupted base pairs. Although this two-step damage recognition mechanism sounds quite attractive, this reviewer has several concerns that should be clarified before publication.

1. In this work, the authors quantify and express accumulation of GFP-fused XPC (Wt or mutants) as ‘increase in fluorescence (%)’. These values are directly influenced by initial levels of GFP fluorescence within the regions of interest. Because they use transient expression of GFP-XPC, it must be critical to show how they could control the expression levels and choose appropriate cells for comparison among different mutants.

2. Most striking is that some mutant XPCs lacking its C-terminus (1-766 and 1-831) appear to be partially active in removing UV-induced photolesions in vivo (Fig. 4B). Are the corresponding mutant proteins also active in in vitro NER reactions? According to the data by Uchida et al. (DNA Repair, 1, 449-461, 2002), XPC (1-734) and (1-815) seem totally inactive in vitro. How could this apparent inconsistency be explained?

3. There has been accumulating evidence that DDB promotes recruitment of XPC to UV-damaged sites. Although the authors state that XPC (1-766) could accumulate to local UV damage in Chinese hamster ovary cells, which lack the p48 subunit of DDB, this does not necessarily mean that observed accumulation of each XPC deletion mutant was not affected by the presence of DDB. What they could do is, for instance, suppressing expression of endogenous p48 with siRNA to see if accumulation of each XPC mutant is affected.

4. In Fig. 2, the authors show that the XPC W690S mutant fails to bind DNA and to accumulate to local UV damage. In contrast, Fig. 7C indicates that the same mutant may be partially active in removing UV photolesions (similar assays in Fig. 3B and Fig. 4B show much lower background activities with GFP controls). Again how can these results be reconciled?

5. The authors claim that the evolutionarily conserved glutamate residue (E755 in human XPC) in the N-EXT domain may have some repulsive effects against DNA and thus enhance in vivo mobility of XPC. In the yeast RAD4, however, this glutamic acid is not conserved (T555 at the corresponding position) and, they say, one at a nearby position (E550) may compensate for the
missing negative charge. If this is the case, it is conceivable that the mutant XPC containing two amino acid substitutions (K750E and E755K) should behave like Wt XPC. Such data would strongly support their model.

6. 'TDG' in Fig. 3A should be 'TGD'.

Referee #3 (Remarks to the Author):

One of the fundamental unanswered questions in DNA repair is how relatively low numbers of DNA repair proteins can monitor billions of base pairs within the context of chromatin in the mammalian cell nucleus to find damaged sites. In human cells, global nucleotide excision repair is initiated by XPC binding to the damage site. Previously Vermeulen and coworkers have used XPC-GFP constructs to follow the movement of XPC into and out of the nucleus, as well as, diffusion to UV photoproducts in living cells. In this present study the authors have studied WT and mutants of XPC protein. Building on in vitro studies with purified proteins the authors in this present study use XPC-GFP constructs to examine the redistribution of XPC to damaged sites in living cells. They show that this redistribution is severely reduced by a W690A or F733A mutations and to a lesser extent by W531A and W542A mutants. The kinetics and extent of redistribution were well correlated with binding to 135 base oligonucleotide in this study and other binding experiments in previous studies. These studies are a significant advance to the field. The manuscript could be improved if the authors address the following points:

1. Introduction. The authors should point out that the bacterial NER repair protein, UvrB also contains a beta-hairpin which directly interacts with DNA in a melted or open conformation. This beta-hairpin and more specifically Tyr96 are essential for damage recognition. This important functional homology between eukaryotic and prokaryotic NER proteins was not discussed in the original crystal structure paper of the Rad4/Rad23B heterodimer bound to a UV photoproduct by Min & Pavletich (2007). Furthermore, since the manuscript discusses many of the structural implications of the mutations in XPC, it would greatly benefit from adding a figure showing the structure of the Rad4 protein and indicate the specific residues and regions that were modified in the present study.

2. Please indicate in the legend of Figure 2, that the 135 oligonucleotide used in binding experiments is ssDNA not a DNA duplex. What effect is observed for the binding of these mutants to double-strand DNA or a heteroduplex as shown in Figure 6, panel E.

3. What is the level expression of the XPC-GFP construct as compared to the normal level of the protein in human cells? Is there sufficient hRad23B in the cells to make the heterodimer, the active form of the searching complex? A Western blot of XPC levels from a normal cells versus XPC-GFP constructs would be useful. Larger than normal amounts of the protein would ultimately lead to more activity of mutant proteins and differences in overall repair kinetics.

4. The results section discussing data displayed in Figure 4, the authors are encouraged to give actual percentages rather than qualitative descriptors such as "completely lost" and "a significant level".

5. Other than being a clever name, the N-EXT domain gives little meaning to the reader, based on the crystal structure of Rad4, the domain contains a $\beta$ turn/$\beta$ sheet structure. Since this domain is in the middle of the protein and not near the N-terminus of the protein. Thus for clarity to the readers the authors are strongly encouraged to use the structural motif rather than inserting a potentially confusing name into the literature.
5. Top of page 9, the authors are urged to develop a table of the diffusion rates, and avoid descriptions like, "... is significantly diminished..." or "... strikingly reduced nuclear mobility ..." (on page 10) with regard to the FRAP experiments. A discussion of how these, what appear to be, modest changes in diffusion would mean to dynamic 3D searching and damage location would be helpful. Knowing the concentration of proteins and the volume of the nucleus and the rate of diffusion, it would be possible to calculate how long a complete search of the genome would take.

6. Top of page 10, please clarify what is meant by the sentence: "A side-by-side comparison of XPC607-741 and XPC607-766 shows that the N-EXT subdomain leads to a > 50% drop in nucleic acid binding (Figure 6F), suggesting that the enhanced nuclear mobility conferred by amino acids 742-766 (Figure 5B) results from a partially antagonistic DNA-repulsive effect." What seems to be occurring in experiments presented in Figure 6 Panels EFG using different substrates is that the βturn/βsheet fold just proximal to BHD confers increased binding to single-strand DNA, as compared to double-strand DNA, but is not antagonistic to DNA binding in general, and thus seems to allow specificity of single-strand DNA over duplex DNA. These data indicate that part of the role of this domain may be to view the lesion in the context of strand opening, analogous to UvrB's beta-hairpin. Previous studies by the Wood lab and also Egly and coworkers have show localized melting of the DNA by XPC. It would be of interest to see if the E755K mutant lacks the ability to open the DNA at a site of the lesion. Finally, for comparison, it would be good to show the behavior of full length WT XPC binding to these three different substrates. Does full length XPC have the preference for single-strand DNA over duplex DNA?

7. In vitro DNA binding analysis of the E755K mutant to the three different substrates would be informative as it is not clear if this mutation would increase binding to all three types of DNA or only single-strand DNA. This experiment is important as binding to DNA duplex substrate containing a defined lesion was not used in any of this study. A comparison of the three types of substrates also has implications on the model discussed in Figure 8.

8. The authors are encouraged to include in the discussion a summary and citation of work by Vermeulen and co-workers who have pioneered the use of FRAP for the analysis the dynamics of DNA repair proteins, including XPC-GFP constructs in living cells. The present studies in this manuscript represent an important advance, but placing this work in the context of the field is essential. Several of Vermeulen's articles are referenced, but the work is not well described in the introduction or discussion.

1st Revision - authors' response 19 May 2009

The authors would like to thank the referees for their helpful comments and suggestions that have enormously increased the quality and impact of our manuscript. We have addressed the different issues raised by the referees with additional experiments and data displayed in the new Figs. 1C, 4D, 4E & 7B, the new supplementary Figs. 1, 2, 4, 5 & 6, and the new supplementary Table I. In detail, the changes in the revised manuscript are as follows:

Referee #1

1. The presence of Rad23B and centrin 2 in the XPC complex is discussed throughout the revised manuscript (see for example p. 3, L. 22-23; p. 15, L. 11-20). In relation to the referee’s specific question regarding Rad23B, we point out that XPC fragment 607-940 fails to interact with Rad23B (as demonstrated by Uchida et al, 2002). Thus, the reduced damage recognition capacity of this fragment is supportive of a direct stimulating role of Rad23B in DNA damage sensing (p. 15, L. 14-16).
2. The contribution of UV-DDB has been examined in XP-E cells that are completely devoid of UV-DDB activity due to a mutation in the DDB2 gene, generating an inactive product that is not expressed to detectable levels (new Figs. 1C, 4D & 4E). In view of these additional experiments, it can be concluded beyond any doubt that "the damage-specific accumulation of XPC truncates as well as the effect of the beta-turn structure take place in the absence of DDB2 protein" (p. 8, L. 29-30).
3. The first part of the "Results" section has been shortened. The superfluous part of Fig. constitutive distribution of GFP and XPC-GFP) has been deleted. The paper by Hoogstraten et al. is referenced on three occasions in the "Results" section. Also, the paper by Politi et al. is included. The high-resolution multiphoton laser has not been used before for systematic studies of the XPC function. Thus, it is important to document rigorously the results obtained with full-length XPC, including the co-localization with CPDs and (6-4) photoproducts and the kinetics of protein redistribution. The black-and-white images facilitate recognition of the accumulation patterns in the print version.
4. On the same line, Fig. 2A (and supplementary Movie 2) are important to demonstrate that we obtained different results than in the study of Yasuda et al. (2007) (see below).
5. The discrepancy with the findings of Yasuda et al (2007) is discussed in detail in the revised manuscript (p. 5, L. 29-33 & p. 6, L. 1-7). The main explanation is that we have used an XP-C cell line with inherently low DDB2 expression (new supplementary Fig. 4). Thus, in contrast to the report by Yasuda et al, the fibroblasts of our study provide a cellular context where the damage recognition defect of XPC mutants becomes evident without the need for down-regulation of endogenous DDB2.
6. We agree with the reviewers' concern that CHO cells may contain a low residual DDB activity and, in fact, the same criticism would apply to the proposed gene silencing approach. Therefore, in the revised manuscript, the accumulation of XPC fragment 1-766 and the critical role of the beta-turn structure have been confirmed in XP-E cells carrying the R273H mutation (new Figs. 4D & 4E). These cells express non-detectable levels of an inactive DDB2 mutant and, as a consequence, are absolutely devoid of any remaining DDB activity. There is now no doubt that XPC fragment 1-766 is able to detect DNA lesions in the absence of DDB and the new Figs. 4D & 4E yield more conclusive results than what down-regulation by silencing may have provided.
7. There is no discrepancy between XPC fragments 1-766 and 1-831. In fact, we show that both truncates display a common UV-sensitive response as they are both retarded upon UV irradiation (Figs. 5D & 5E; indicated by the arrow in Fig. 5D). In the revised manuscript, this conclusion is confirmed by quantitative measurements of diffusion coefficients and kon/koff ratios (new supplementary Table I). The finding that only fragment 1-831 results in an immobile fraction is discussed in relation to its unique single-stranded DNA-binding activity on p. 14 (L. 22-32) of the revised manuscript. The reviewer is right in asking us to explain the observation that fragment 1-831 does not support more GGR activity than fragment 1-766. As indicated in the revised manuscript (p. 13, L. 9-11), this is likely due to the absence of at least some components of the TFIIH-recruiting domain in their C-terminal region.
8. The typo on page 9 has been corrected.
9. The DNA-binding profile of full-length XPC protein, using exactly the same substrates as in Fig. 6, is displayed in the new supplementary Fig. 6. The paper by Kusumoto et al (2001) has been added to the references.

Referee #2

1. We fully agree that it is eminently important to monitor protein expression in the nuclei subjected to live-cell imaging. The new supplementary Fig. 2 demonstrates by immunocytochemistry that we selected only those nuclei containing low levels of XPC protein comparable to that of normal cells. The new supplementary Fig. 5 shows that we have also taken care of this important issue by comparing the expression of different truncates by the measurement of overall fluorescence.
2. It is clearly stated in the revised manuscript that the XPC fragments 1-766 and 1-831 display only limited complementing activity in the host-cell reactivation assay comparable to the pathogenic mutant W690S (p. 14, L. 7-9). There is no contradiction
with the study of Uchida et al. because in vitro excision assay are notoriously less sensitive and, hence, unable to detect such low activities. This view is supported by the fact that, in the assay of Uchida et al., the functional XPC fragment 118-940 is more than 60% reduced in GGR activity compared to the full-length protein.

3. In the revised manuscript, the accumulation of XPC fragments 1-741, 1-766 and 1-831 has been tested in XP-E cells carrying the R273H mutation in DDB2 (new Figs. 4D & 4E). These cells express non-detectable levels of a completely inactive DDB2 mutant and, accordingly, are devoid of any DDB activity. On the basis of these additional experiments, it can be concluded that the key results of our manuscript, i.e., that the damage-specific accumulation of XPC truncates as well as the effect of the -turn structure take place in the absence of DDB2 (p. 8, L. 29-30). As stated before, these experiments using XP-E cells completely devoid of DDB activity yield more conclusive results than what could have been achieved by gene silencing.

4. With regard to the host-cell reactivation assay, it is important to point out the W690S mutant is not completely inactive in DNA damage recognition in living cells. A pattern of accumulation is clearly visible in the black & white image of Fig. 2A. The fact that there is some accumulation of W690S is also demonstrated in the quantification of Figure 2C. Accordingly, there is a residual capacity in removing DNA lesions, which is detected in the sensitive host-cell reactivation assay of Fig. 7. In the revised discussion, we make clear that the residual activity detected with W690S (as well as with fragment 1-766) are not sufficient to complement the repair defect of XP-C cells (p. 14, L. 7-9).

5. The idea that E550 in Rad4 may adopt the function of E755 in XPC has not been further pursued because inspection of the Rad4 structure shows that E550 is not in close proximity to the DNA. Thus, the interpretation of results would be difficult. The fact that E755 is not conserved in yeast is pointed out in the legend to Fig. 7 with the following statement: "This residue is not conserved in the Rad4 sequence, suggesting that the yeast ortholog may have different dynamic properties". Nonetheless, the expanded Fig. 7 provides conclusive evidence (based on three lines of experimentation: DNA-binding, GGR assay and FRAP) demonstrating that E755 in human XPC protein enhances the dynamics of XPC protein searching for DNA lesions.

6. The typo in Figure 3A has been corrected.

Referee #3

1. We like the proposed link between the beta-hairpins of UvrB and XPC that, unfortunately, has not been discussed by Min & Pavletich. This analogy should be the subject of a future review paper also taking into consideration other beta-hairpins involved in nucleotide excision repair (for example those of RPA). Considering the imposed space limitation, we believe that it is not adequate to include this beta-hairpin analogy in the present manuscript, which is actually focused on molecular events taking place before the beta-hairpin insertion. The crystal structure of Rad4, in relation to the homologous human domains, the beta-turn structure carrying the critical E755K mutation and the constructs of this study, is shown in the new supplementary Fig. 1.

2. The legend to Fig. 2 has been improved by indicating that the substrate consists of single-stranded DNA. Further experiments, shown in the new supplementary Fig. 6, demonstrate that full-length XPC protein binds to single-stranded DNA with higher affinity than to double-stranded DNA or heteroduplexes. Exactly the same trend is observed with the previously (Maillard et al., 2007) reported mutants of Figure 2. These known mutants have been used to demonstrate the correlation between DNA-binding in vitro and DNA damage recognition in living cells (Fig. 2C) but their detailed biochemical characterization is beyond the scope of the present study.

3. As discussed in the revised manuscript (p. 15, L. 14-16), the interaction with Rad23B is not necessary for DNA damage detection. Western blots, as suggested by the reviewer, would not add new information because they measure the overall expression across heterogeneous populations of transfected cells, including cells with no expression at all and cells that produce very high levels of the fusion protein. Instead, as shown in the new supplementary Fig. 2, it is important to make sure that those individual nuclei subjected to imaging contain "physiologic" levels of XPC constructs, comparable to XPC...
expression in normal fibroblasts. In addition, the new supplementary Fig. 5 shows how we compared the expression of XPC truncates. It is clear from the data of supplementary Figs. 2 & 5 that the observed differences in protein mobility and DNA damage recognition cannot be attributed to differential expression.

4. The section describing the results of Fig. 4 has been rephrased with quantitative descriptors as requested by the reviewer (p. 8, L. 11-17).

5.1. The term "N-terminal extension" had been introduced by Min & Pavletich (2007). Following the reviewer’s recommendation, this subdomain of XPC protein is now referred to as the "beta-turn structure" throughout the revised manuscript.

5.2. Diffusion coefficients derived from the FRAP experiments of pp. 9-10 are provided in the new supplementary Table I, as requested. The diffusion coefficient measured for wild-type XPC protein (0.3 micrometer2/s) corresponds to that predicted in the model of Politi et al (2005). The data of Fig. 5 and supplementary Table I are evaluated in detail in the revised "Discussion" section, but the proposed theoretical calculation ("how long a complete search of the genome would take") does not appear feasible because too many parameters are still unknown. For example, the time it takes to search the entire genome is greatly influenced by the variable accessibility of condensed chromatin regions.

6. The paragraph describing the DNA-binding assays of Fig. 6 has been rephrased. In the revised manuscript, this section contains quantitative binding constants in support of the conclusion that the beta-turn structure exerts a DNA-repellent effect (p. 11, L. 2-6). Here, the reviewer’s interpretation of the biochemical results is not correct because the beta-turn structure (Fig. 6F) has a distinctly different effect than BHD3 (Fig. 6G). The beta-turn exerts a general DNA repulsion, irrespective of conformation, whereas BHD3 (containing the beta-hairpin) exerts a repulsion only towards homo- and heteroduplexes. The strand opening previously observed by Evans et al (1997) and Mocquet et al (2007) is included in the revised discussion (p. 13, L. 30-31). The reviewer may have confused beta-turn with beta-hairpin. In fact, the position E755 is located in the beta-turn motif and, hence, is not involved in the beta-hairpin-induced strand opening.

7. A biochemical analysis of E755K has been included as requested. The new Fig. 7B clearly shows that the E755K mutation increases the binding to double-stranded DNA (not just to single-stranded DNA as suspected by the reviewer). This additional data fits with the FRAP experiments of Fig. 7C and supports the model of Fig. 8.

8. It has never been our intention not to credit Wim for his pioneering work in the field. In the revised manuscript, we make clear that the fluorescence-mediated analysis of nucleotide excision repair proteins has been established by Houtsmuller, Vermeulen and collaborators (p. 4, L. 17-20). Overall, the expanded reference list contains a total of 8 papers co-authored by Wim Vermeulen.

In summary, we are confident that the reviewers’ concerns have been adequately addressed with appropriate experiments and that we have incorporated all their comments in the extensively revised manuscript.

Additional correspondence

16 June 2009

I have now heard back from both referees regarding your revised manuscript EMBOJ-2009-70529 - apologies again for the delay. The comments of referees 1 and 2 are enclosed below, and as you will see, reviewer 1 finds that the paper is now acceptable for publication without further changes. Reviewer 2 does highlight a couple of remaining concerns and suggests additional experiments. Having looked at these points myself and discussed them with referee 1, my feeling is that - although the suggested experiments might be useful - they were not asked for during the first round of review and are not essential to the main message of the paper. Therefore, I do not feel it would be fair to ask you to perform extra work at this stage, and so am happy to tell you that we will be able to accept the manuscript for publication without further revision.

You will receive the formal acceptance e-mail shortly, but I just wanted to transmit this message, along with the referees' reports, without any further delay.
Referee 1 (remarks to author):

The authors have satisfactorily rebutted most (not all) issues raised by the reviewers and therefore this reviewer thinks that after condensing the manuscript to a more concise manuscript is suitable for publication in EMBO J.

Referee 2 (remarks to author):

The authors address some of this reviewer's comments properly. One of the concerns that still remain is evaluation of the results from host-cell reactivation assays. Considering the DNA binding activity shown in Fig. 2B, host-cell reactivation in the cells expressing XPC W690S or W690A mutant (Fig. 7D) seems too high anyway. As they state, this type of assay could be more 'sensitive' than in vitro assays, but it's nonetheless an indirect way to assess cellular global genome NER activity. It would be far more convincing if the authors could directly measure repair kinetics of some lesions, like (6-4) photoproducts, with the cells expressing the various mutant XPC proteins.

Another point is the accumulation of mutant XPCs in XP-E cells (Fig. 4). These results seem to indicate that the mutant XPC proteins could be recruited to UV-laser-damaged areas in a DDB-independent manner. However, the XP-E cells must express endogenous wild-type XPC, so it's even surprising that the co-existence of wild-type XPC did not at all affect accumulation of the mutant XPCs (Fig. 4C). This reviewer would suggest that siRNA knockdown of DDB2 must provide more proper controls, even though the knockdown efficiency might not reach 100%.