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The conserved factor De-Etiolated 1 cooperates with CUL4-DDB1^{DDB2} to maintain genome integrity upon UV stress

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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 07 September 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. These reviewers all consider your findings on a CUL4-DDB-associated DET1 role in NER in Arabidopsis potentially interesting, but raise a number of important issues that would need to satisfactorily addressed before publication may be warranted. Among the most salient of those concerns are the need for a better follow-up investigation on the surprisingly similar phenotypes of DET1 gain- and loss-of-function (see referees 1 & 2); more definitive experimental support for the notion of DET1 degradation taking place and being required during NER (referees 1 & 3); and analysis of other DNA damage response defects in plants with altered DET1 levels (referee 3). In the absence of significant further insights on this points, we must consider the study currently too preliminary to be a good candidate for an EMBO J paper, but should you be able to satisfactorily address these main criticisms (as well as the more specific concerns e.g. regarding controls, quantifications etc.) then we should be happy to consider a revised manuscript further for publication. I have to however remind you that it is EMBO Journal policy to allow a single round of major revision only, and that it will thus be important to diligently answer to all the various experimental and editorial points raised at this stage. When preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). Finally, please make sure to carefully assemble and proofread the final version also from an editorial point of view, paying attention to the completeness of the bibliography (various references cited in the text are currently missing there), to language and writing issues, and also briefly indicate the individual author's contributions, either in the acknowledgements section or in an adjacent separate section. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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):

In this manuscript, Castells et al., report the involvement of DET1 in the Nucleotide Excision Repair (NER), where DET1 cooperates with the CUL4-DDB1-DDB2 complex in mediating this process. The authors first show that both the partial loss-of-function mutant, det1-1, and DET1-overexpression lines displayed hypersensitivity to UV-C in root growth inhibition assays. Additionally, the authors find that in det1-1, UV-induced DNA photoproducts accumulated at a higher level, and extracts of the mutant has a reduced ability to perform synthesis-dependent repair of DNA in vitro. Further, the authors show that upon UV-C exposure, DET1 protein levels decreased in a CUL4-dependent manner, and that degradation of DDB2 was impaired in det1-1. Finally, the authors report the occurrence of a transient UV-induced high-molecular weight DET1 complex, which depends on DDB2.

Overall, the finding that DET1 is involved in the DNA repair process is novel and interesting. However, the manuscript is at a rather preliminary stage with few solid conclusions and has not offered more insights into the molecular interaction and mechanism of the proposed cooperation of DET1 and the CUL4-DDB1-DDB2 complex.

Major Points:
1. The seemingly contradicting observation that under- and over-expression of DET1 lead to similar hypersensitive effect to UV-C is very intriguing. Although the authors have suggested possible reasons in the discussion, this issue should be further explored experimentally to strengthen the notion that DET1 indeed participates in the repair process and cooperates with the other factors. This is particularly important because due to the nature of the examined UV-C response, the mutant phenotypes are not as obvious as one could hope (also see point #4).
Inclusion of the DET1-OE lines in both the in vitro synthesis DNA repair assay (Fig. 2B) and the analysis of DDB2 degradation profile (Fig. 5A) should be initial experiments that could yield some insights.
Does DDB2 interact less with CUL4 in DET1-OE? Does the CUL4 activity change in det1-1 vs. the DET1-OE lines? Addressing these may allow the authors to gain some mechanistic understanding of DET1 in this process (see point #3).

2. Based on the present data, the claim that DET1 protein is degraded by the CUL4 E3 ligase upon UV-C irradiation is an overstatement. The authors should first show that DET1 is actually degraded by examining its protein level with the addition of a proteasome inhibitor. Further, evidence for CUL4 directly involves in DET1 degradation should be tested for the claim to be valid. Ultimately, an in vitro ubiquitination assay, probably with the use of a recombinant or in vivo-pulled down CUL4 E3 ligase complex, is necessary to confirm this.

3. The nature of the "cooperation" between DET1 and the CUL4-DDB1-DDB2 complex is still very obscure and should be better defined. First, is DET1 present on the same complex as the DDB2 ligase after UV exposure? Does DET1 interact with DDB2? Although the gel-filtration data provide some insights, direct interaction assays are needed to address this. Second, the steady state and UV-C irradiated protein level of DDB2 in WT and det1-1 should be directly compared (on the same western), so as the level of DET1 in WT and ddb2 mutant (as DET1 level appears to be higher in ddb2 mutant upon UV irradiation in the "Input" panel of Fig. 6D). Third, it has been well established that UV induces the association of the DDB2 complex to the chromatin. This should also be tested

on DET1. Finally, since a model of DET1 facilitating CUL4-DDB1 -mediated DDB2 degradation is presented, additional data from in vitro ubiquitination assays is highly desirable in supporting this point.

4. In most of the graphs presented in Figs. 1-5, the scaling start of the Y-axis was not set at 0, but rather at an arbitrary value that amplifies the differences visually. This would mislead readers and should be avoided. A broken Y-axis should be used instead.

Minor points:
1. In Fig. 2B, adding loading controls for the protein amount added would be more desirable.

2. In Fig. S1, is the up-right angle of the cotyledons of det1-1/DET1 OE-3 an artifact in preparation? If it is not, this may indicate that the complementation is only partial.

3. The sub-figure order in the Fig. 5 legend is not correct.

4. All Arabidopsis protein names throughout the manuscript should be upper case, i.e. De-Etiolated 1 -> DE-ETIOLATED 1 (refer to the TAIR guideline).

5. Please check the writing for better fluency and grammatical accuracy.

Referee #2 (Remarks to the Author):

The manuscript "The conserved factor De-Etiolated 1 cooperates with CUL4-DDB1DDB2 to maintain genome integrity upon UV stress" by Castells et al is part of a series of interesting works dealing with the mechanisms of DET1 in plant UV response. Previously Castells et al (2010) reported that DET1 regulated plant UV-C tolerance by controlling the expression of the PHR1 and UVR3 photolyase genes. In this study, the authors further demonstrate that DET1 works together with CUL4-DDB1DDB2 complex for efficient removal of UV-induced DNA lesions through the GGR pathway. It has been shown that Arabidopsis DDB1A and DET1 co-purify with the E2 Ub conjugase COP10 (Yanagawa et al. 2004) and these proteins have been found to form a complex with AtCUL4 and RBX1 (ROC1) (Bernhardt et al. 2006; Chen et al. 2006). In this manuscript, the authors showed that DET1 is required for CUL4-DDB1-mediated DDB2 degradation, and its own is also a target of a CUL4 E3 ligase. Interestingly plants either knockdown or overexpression of DET1 exhibited an enhanced UV-C sensitivity, suggesting appropriate dosage of DET1 protein is necessary for DET1 precisely function in DNA damage repair. This manuscript provides a new insight on how DET1 regulates plant UV response.

General comments
1. The authors discussed a lot about the possibilities of the similar effect of DET1 overexpression and knockdown on plant UV-sensitivity. The authors have to show native DET1 protein level comparing to your three overexpression lines. It often disrupts function of a protein complex if one component changed in this complex. It may help if the authors perform size-exclusion chromatography analysis of DDB2/CUL4 complex and determine the protein levels of CUL4-DDB1-DDB2 from both DET1 overexpression and det1-1 mutant plants.

2. det1-1 is in Col-0 ecotype and ddb2-2 is in Nossen ecotype. Different genetic backgrounds often display different UV sensitivities. It is hard to judge det1-1 ddb2-2 double mutant phenotype before several backcrosses.

3. DDB1A mutation enhances det1 mutant phenotypes (Wesam et al., 2007). Is it also the truth for UV response? Why cul4 mutation does not?

Minor points,
1. I do not see the "Asterisks" in Fig 4.

2. Figure 5, "A" and "B" are mislabeled. I also do not see "Asterisks indicate t-test significant differences" in this figure. The protein loading controls are unacceptable.

3. Fig S1, "A" need a significant difference test. I am not sure det1-1 mutant phenotype is complemented by overexpression of DET1. At least we still see the open cotyledons in "B".

4. Some typos should be corrected.

Referee #3 (Remarks to the Author):

Castells et al. suggest a novel role for the conserved DET1 protein in DNA damage repair in Arabidopsis. The manuscript is well written and provides interesting new data on DET1 function, a protein known as a repressor of photomorphogenesis in plants. However, there are few experiments that are required to significantly strengthen the work and some minor comments that have to be considered before publication.

1) The authors postulate a role of DET1 in NER. Similar to other Arabidopsis mutants impaired in NER, it is expected that det1-1 and DET1-Ox are altered in response to other DNA-damaging agents as well. Such data has to be provided, including appropriate controls for comparison. This would also further support a role of DET1 in DNA damage responses independent of the regulation of photolyase expression and "sunscreen" biosynthesis published before (Castells et al., Plant J, 2010).

2) If relative root lengths are given, provide the length of the non-treated controls in the figure or figure legends. Otherwise it is not clear, for example, if non-treated det1-1 roots were already different under the experimental conditions without treatment. Generally, also provide n (number measured) and repetitions in figure legends and not only M&M section.

3) Fig.4A is not convincing to me. This experiment should be repeated with a larger number of roots. This should provide more convincing data answering whether the double mutant is like the single mutants vs. showing an additive effect. The conclusion to be drawn would be certainly different.

4) The authors repeatedly argue that DDB2 and DET1 degradation is required for NER. This is not supported by data, even though NER activity correlates with turnover. E.g. p10; the DDB2 WDxR mutation does not only block its degradation but likely also interaction with DDB1. Thus, it is not clear what of the two is causing the failure to rescue the mutant phenotype. The degradation data should be supported by data using proteasome inhibitors, in both Col and det1-1. Similarly, it would be informative to perform the NER experiment in extracts containing proteasomal inhibitors (again det1-1 and WT) (Fig. 2B).

5) Data published by Dohmann, Development 2008 and Zhang and Schroeder, Planta, 2009 should be discussed.

6) Fig. 1A should be provided using the anti-DET1 antibody. Otherwise the relative amounts to wt Col-0 cannot be estimated.

1st Revision - authors' response 06 December 2010

Please find below a detailed description of the main changes made, followed by a point-by-point answer to the reviewers' comments.

In the Introduction part, we referred to a recent publication by Dohmann et al (2008) describing the accumulation of single/double-strand DNA breaks in several photomorphogenic mutants and notably in det1-1 mutant (page 5).

In the Results section, we introduced several new data. These include the estimation of

mycDET1 protein levels in OE-1, OE-2 and OE-3 transgenic lines using a DET1 antibody that detects both endogenous and transgene-driven mycDET1 (Figure S1). The UV-C induced degradation of the mycDET1 protein in DET1 OE-1 line is now introduced in the Figure 4D. We further added the observation that mild overexpression of mycDET1 in the OE-1 line does not affect the UV-induced degradation of endogenous DET1 (Figure 4D and S7A) while it is visibly affected in OE-3, the strongest overexpression line (Figure S7). Finally, the DET1 OE-3 overexpressing line was further analyzed through size-exclusion chromatography showing that mycDET1 form high-molecular weight complexes in this line, possibly indicating protein aggregation. As suggested by two reviewers, the effect of DET1 overexpression was tested in vitro for synthesis-dependent DNA repair (new Figure S5) showing that, like in det1-1, this function is affected in the DET1 OE-3 line.

The capacity of the mycDET1 fusion protein to complement the phenotype of plants grown under normal light conditions, similar to the ones used for most sensitivity and protein-based assays, has been introduced in Figure S2A. We also modified the statement that this fusion protein can fully complement the dark-grown phenotype of the det1-1 mutant and mentioned that formation of the apical hook typical of etiolated det1-1 seedlings is only partially complemented (page 6).

As requested by a reviewer, we better investigate the protein contents of DET1, DDB2 and CUL4 in UV-C irradiated plants and in the det1-1 mutant (Figure 6F). This was completed by a novel series of size-exclusion chromatography analyses of DET1, DDB2 and CUL4 complexes, altogether showing the overall stability of DDB2 and CUL4 protein complexes in det1-1 and/or DET1 OE lines (Figures S7, S10 and Figure 1 for Reviewers).

We also performed additional sensitivity assays of the det1-1 mutant and of the DET1 OE-3 transgenic line with other damaging agents (cisplatin and H2O2). This is presented in Figure S6.

The genetic analysis for det-1 and ddb2-2 interactions in UV-C sensitivity was repeated using a higher dose of UV-C exposure. This new set of experiments incorporates null segregant plants from the det1-1ddb2-2 cross as a control for potential effects of genetic backgrounds between Col-0 and Nossen (Figure S9).

The authors also modified significantly the conclusions raised upon the demonstration that UV-C induced DET1 protein degradation is inhibited by the cul4-1 mutation. It is now concluded that DET1 degradation is CUL4-dependent, but it is not stated that CUL4 ubiquitin-ligase targets DET1 for degradation.

The Discussion part has been slightly modified, mainly correcting typos and aiming to improve reading fluency. Missing references have been introduced.

Point-by point answer to the reviewers

REVIEWER 1

In this manuscript, Castells et al., report the involvement of DET1 in the Nucleotide Excision Repair (NER), where DET1 cooperates with the CUL4-DDB1-DDB2 complex in mediating this process. The authors first show that both the partial loss-of-function mutant, det1-1, and DET1-overexpression lines displayed hypersensitivity to UV-C in root growth inhibition assays. Additionally, the authors find that in det1-1, UV-induced DNA photoproducts accumulated at a higher level, and extracts of the mutant has a reduced ability to perform synthesis-dependent repair of DNA in vitro. Further, the authors show that upon UV-C exposure, DET1 protein levels decreased in a CUL4-dependent manner, and that degradation of DDB2 was impaired in det1-1. Finally, the authors report the occurrence of a transient UV-induced high-molecular weight DET1 complex, which depends on DDB2.

Overall, the finding that DET1 is involved in the DNA repair process is novel and interesting. However, the manuscript is at a rather preliminary stage with few solid conclusions and has not offered more insights into the molecular interaction and mechanism of the proposed cooperation of DET1 and the CUL4-DDB1-DDB2 complex.

Major Points:
1. The seemingly contradicting observation that under- and over-expression of DET1 lead to similar hypersensitive effect to UV-C is very intriguing. Although the authors have suggested possible reasons in the discussion, this issue should be further explored experimentally to strengthen the notion that DET1 indeed participates in the repair process and cooperates with the other factors.

This is particularly important because due to the nature of the examined UV-C response, the mutant phenotypes are not as obvious as one could hope (also see point #4).

- We partially disagree with this first comment and therefore we aim to answer it in a complete way. Throughout the manuscript, measurements of relative root growth of mutants are claimed to be affected only when they reveal significant differences with respective wild-type controls. We also included positive controls with known defects in UV responses in each assay, e.g. cul4-1, ddb2-2 and rad10 mutants, which allow comparing the amplitude of the responses. Most notably, det1-1 is usually affected to a similar extent as cul4-1 or ddb2-2.

Futhermore, we have previously observed that the det1-1 mutant accumulates UV-absorbing pigments such as flavonoids in the roots and accumulates less CPD and 6,4-PPs in genomic DNA (Castells et al, 2010). The observation that det1-1 seedlings still exhibit enhanced UV-C sensitivity, while being less damaged, is therefore highly meaningful. The in vitro repair assay is particularly important because it mostly interrogates the NER pathway, independently of any in vivo pleiotropic effects, and shows in an unambiguous way that det1-1 plants are defective in synthesis-dependent DNA repair.

From a technical point of view, because the root growth assay is quite simple and is very sensitive to the physiological state of the plants, all root growth assays have been performed independently by the two first authors in different laboratories. Although the amplitude of the defects of det1-1 and control lines were variable in different experiments, presumably because of the physiological states of the plants and variations in the effectiveness of UV-C irradiations from different equipment, the results were perfectly reproducible over all replicate experiments. Finally, it is important to mention that in most experiments the plants were irradiated with different UV doses (600 to 900 J/m2) and only those conditions giving significant but nonetheless only partial reductions in relative root growth in wild-types were used for accurate quantification. This explains why the reduction in relative root growth can have different amplitudes in different experiments, as in Figure S9, but always shows the same trend.

Inclusion of the DET1-OE lines in both the in vitro synthesis DNA repair assay (Fig. 2B) and the analysis of DDB2 degradation profile (Fig. 5A) should be initial experiments that could yield some insights.

- Following the reviewer's suggestion, we tested the DET1 OE-3 line for in vitro synthesis DNA repair. This is now included as Figure S5 and discussed in the main text.

Does DDB2 interact less with CUL4 in DET1-OE? Does the CUL4 activity change in det1-1 vs. the DET1-OE lines? Addressing these may allow the authors to gain some mechanistic understanding of DET1 in this process (see point #3).

- Such observations would indeed be highly informative. However these mechanistic aspects still require important investigations that we were not able to complete during the time provided for manuscript revision. We nonetheless performed new size-exclusion chromatography analyses, and could show that DDB2 complex size was not affected in the det1-1 mutant nor in DET1 OE-3 overexpressing lines. As shown in the Figure 1 for the reviewers, we also observed that CUL4 complex size is maintained in det1-1, as was already shown in the cop10 CDD mutant by Chen et al., Plant Cell (2010) 22: 108ñ123;). Investigating further the effect of DET1 on CUL4 activity might represent a challenging task because CUL4-DDB1 associates with WDxR proteins (DCAFs) to form a multitude of specific E3 ligases. The specific effect of DET1 might not be detectable.

2. Based on the present data, the claim that DET1 protein is degraded by the CUL4 E3 ligase upon UV-C irradiation is an overstatement. The authors should first show that DET1 is actually degraded by examining its protein level with the addition of a proteasome inhibitor. Further, evidence for CUL4 directly involves in DET1 degradation should be tested for the claim to be valid.

- Considering that DET1 protein decay is fast, independent of DET1 gene expression, and abolished in the cul4 E3 ligase mutant, we considered that it would be an overstatement to conclude that DET1 protein is degraded. We reasoned, as in Molinier et al (PLoS Genet 2008), that showing the implication of a CULLIN was more targeted and would allow us to draw more specific conclusions

than if we used proteasome inhibitors, which have wide and indirect cellular effects. We nonetheless made several experiments with proteasome inhibitors, with partially conclusive results. To our knowledge, there are no available protocols that combine plant uptake of proteasome inhibitors in vivo and UV irradiation. This turned out to be technically difficult in our hands, because the incubation of the seedlings in liquid medium (with DMSO or inhibitors) reproducibly affected the subsequent UV irradiation and DET1 degradation (Figure 2 for reviewers). Nonetheless, in the revised version, we show that constitutively expressed mycDET1 fusion protein also decreases upon UV exposure (Figure 4C), providing additional evidence for DET1 protein being post-translationally degraded upon UV irradiation.

Based on the well established property of DET1 to interact with a CUL4-DDB1 complex in plants and mammals, that we also confirm by showing DET1 interaction with DDB1a and DDB1b, and based on our observation that DET1 protein decay is CUL4-dependent, we consider that concluding on the direct role of CUL4 CRL in DET1 degradation is reasonable. We nonetheless realize that our conclusions could be considered as being overstated. We therefore modified the text to simply state that DET1 degradation is CUL4-dependent. We also removed any statement of DET1 being directly targeted by CUL4 CRL. Based on our data and previous knowledge, we nonetheless propose a working model in which CUL4 CRL triggers DET1 protein degradation, similar to the well-established DDB2 degradation pathway.

Ultimately, an in vitro ubiquitination assay, probably with the use of a recombinant or in vivo-pulled down CUL4 E3 ligase complex, is necessary to confirm this.
- In vitro ubiquitylation using purified complexes is a challenging task. To our knowledge, such an assay is only handled by a handful of plant biology laboratories, and has not been reported in the particular case of CUL4. Unfortunately we were not able to set it up during the time of the revision. We also question its necessity in view of the fact that we show CUL4-dependent protein degradation using in vivo approaches.

3. The nature of the "cooperation" between DET1 and the CUL4-DDB1-DDB2 complex is still very obscure and should be better defined. First, is DET1 present on the same complex as the DDB2 ligase after UV exposure? Does DET1 interact with DDB2? Although the gel-filtration data provide some insights, direct interaction assays are needed to address this.

- We made intense efforts to experimentally test possible DET1-DDB2 interaction following UV-C exposure. This turned out to be technically difficult using classical approaches such as co-immunoprecipitation and BiFC, as one can imagine for attempting to detect interactions that may occur transiently between two proteins in the process of being degraded.

Considering the transient nature of the putative interaction, we first used a BiFC approach using our functional split YFP-tagged DET1 and DDB2 constructs. However, exposure of plant tissues to different doses of UV-C rapidly induced strong photobleaching of the YFP and CFP signals. This was clearly observed on YFP positive controls obtained with known DDB1A-DDB2 and DDB1A-DET1 interactions. As shown in Figure 3 for reviewers, we nonetheless could observe appearance of faint nuclear signals upon UV-C exposure when co-expressing YC-DDB2 and YN-DET1, but considering potential background signals in BiFC experiments we felt the signal was not strong enough for publication.

Interaction was also tested by co-immunoprecipitation of GFP-DDB2 with DET1 protein, again resulting in reproducible but very faint signals. A possible way to succeed in these experiments might be to stabilize DET1 and DDB2 proteins following UV irradiation (eg, using proteasome inhibitors or expressing mutated proteins), but these approaches might also affect the interaction. An alternative would be to stabilize the putative interactions using chemical crosslinkers, but this would require further intense efforts to adapt protocols to plant UV-assays.

At the moment then, our gel-filtration data represent our main evidence for an association between DET1 and DDB2, although we realize that it does not demonstrate a direct interaction.

Second, the steady state and UV-C irradiated protein level of DDB2 in WT and det1-1 should be directly compared (on the same western), so as the level of DET1 in WT and ddb2 mutant (as DET1 level appears to be higher in ddb2 mutant upon UV irradiation in the "Input" panel of Fig. 6D).

- This was the case. The wt and det1-1 samples from Figure 5 were on the same blot, and have been split to fit with the other mutants in the figure. We now also introduced another blot in

supplementary material, which shows the DDB2 and CUL4 protein levels in det1-1 and in WT upon UV-C irradiation.

Third, it has been well established that UV induces the association of the DDB2 complex to the chromatin. This should also be tested on DET1. Finally, since a model of DET1 facilitating CUL4-DDB1 -mediated DDB2 degradation is presented, additional data from in vitro ubiquitination assays is highly desirable in supporting this point.

- Again, we agree that mechanistic aspects still require additional investigations, but we consider that a complete investigation of the novel role of DET1 in NER is beyond the scope of this manuscript.

4. In most of the graphs presented in Figs. 1-5, the scaling start of the Y-axis was not set at 0, but rather at an arbitrary value that amplifies the differences visually. This would mislead readers and should be avoided. A broken Y-axis should be used instead.

- This has now been modified accordingly.

Minor points:
1. In Fig. 2B, adding loading controls for the protein amount added would be more desirable.

- To our knowledge, protein loading controls are never given for this assay, as for example in the reference method publication for this assay in plants (Li et al, Plant Cell 2002) and in our recent publication (Molinier et al, PLoS Genet 2008). The output from the synthesis-dependent DNA repair assay mainly relies on the amount of UV-damaged plasmid added as a function of time. Moreover, upon repair, DNA is separated on agarose gels, and therefore loading control for the protein amount added can only be indirect, by running separately part of the input protein on PAGE. In any case, we are confident that the marked differences of DIG incorporation observed between wild-type and det1 mutant in Figure 2B could hardly result from biases in the quantity of input protein extracts: for each time point 20 µg of extracts have been used based on protein concentrations determined by Bradford assay. Moreover, before submission of this manuscript, this assay had been repeated twice using wild-type and det1-1 Arabidopsis extracts, as well as using tomato wild-type and hp2 DET1 mutant (for other purposes). It always reproducibly gave the same result.

Nonetheless, because the revised manuscript introduces a new in vitro repair assay to test synthesis-dependent DNA repair in the DET1 OE-3 line (Supplementary Figure 5), a SDS-PAGE was performed on similar amounts of input wild-type and OE-3 protein extracts. As requested, coomassie staining of this gel is included as a loading control.

2. In Fig. S1, is the up-right angle of the cotyledons of det1-1/DET1 OE-3 an artifact in preparation? If it is not, this may indicate that the complementation is only partial.
- This was not an artifact of preparation. As previously observed in Zhang & Schroeder (Planta, 2010, 231: 337-348), a defect in apical hook formation was observed for many of the complemented seedlings with mycDET1, while few others display a normal one. We now mention in the text that complementation of det1-1 skotomorphogenic phenotype is partial.
The apical hook defect is unlikely to affect the conclusions drawn on the functionality of the mycDET1 fusion protein in DNA repair. We nonetheless introduced a new figure showing the phenotype of 2-week-old complemented det1-1/DET1 OE-3 line seedlings grown under normal light conditions, showing that they exhibit a wild type phenotype (Figure S2A). With regard to the question of Reviewer 1 and 3, we felt it was necessary to show that det1-1/DET1 OE-3 line has a wt phenotype under the growth conditions of most of the UV-C responses tested (in vitro DNA repair, protein content, etc).

3. The sub-figure order in the Fig. 5 legend is not correct.
- We apologize for this error. This is now corrected.

4. All Arabidopsis protein names throughout the manuscript should be upper case, i.e. De-Etiolated 1 -> DE-ETIOLATED 1 (refer to the TAIR guideline).

- We first aimed to respect the original spelling from Chory et al (Cell 1989). However, this has now been corrected.

5. Please check the writing for better fluency and grammatical accuracy.

- This has been done

REVIEWER 2

The manuscript "The conserved factor De-Etiolated 1 cooperates with CUL4-DDB1DDB2 to maintain genome integrity upon UV stress" by Castells et al is part of a series of interesting works dealing with the mechanisms of DET1 in plant UV response. Previously Castells et al (2010) reported that DET1 regulated plant UV-C tolerance by controlling the expression of the PHR1 and UVR3 photolyase genes. In this study, the authors further demonstrate that DET1 works together with CUL4-DDB1DDB2 complex for efficient removal of UV-induced DNA lesions through the GGR pathway. It has been shown that Arabidopsis DDB1A and DET1 co-purify with the E2 Ub conjugase COP10 (Yanagawa et al. 2004) and these proteins have been found to form a complex with AtCUL4 and RBX1 (ROC1) (Bernhardt et al. 2006; Chen et al. 2006). In this manuscript, the authors showed that DET1 is required for CUL4-DDB1-mediated DDB2 degradation, and its own is also a target of a CUL4 E3 ligase. Interestingly plants either knockdown or overexpression of DET1 exhibited an enhanced UV-C sensitivity, suggesting appropriate dosage of DET1 protein is necessary for DET1 precisely function in DNA damage repair. This manuscript provides a new insight on how DET1 regulates plant UV response.

General comments
1. The authors discussed a lot about the possibilities of the similar effect of DET1 overexpression and knockdown on plant UV-sensitivity. The authors have to show native DET1 protein level comparing to your three overexpression lines.

- We appreciated this comment from two reviewers, and this has now been done. Different blots have been performed using the DET1 antibody that allows comparison of the overexpression levels. The results are now shown in Figure S1 and mentioned in the text. We show that DET1 overexpression at the protein level is highly significant, and was estimated to reach more than 10 fold the endogenous levels in the OE-3 line.

It often disrupts function of a protein complex if one component changed in this complex. It may help if the authors perform size-exclusion chromatography analysis of DDB2/CUL4 complex and determine the protein levels of CUL4-DDB1-DDB2 from both DET1 overexpression and det1-1 mutant plants.

- We agree with Reviewer 2 that a better follow-up on this aspect is important. We therefore investigated further the DDB2 and CUL4 complex sizes in the DET1 knock-down and overexpressing lines. As expected for a protein that can assemble a multitude of complexes, we confirmed that the CUL4 profile in size-exclusion chromatography is not affected in det1-1 (Figure 3 for reviewers), as was already shown in the cop10 CDD mutant by Chen et al. (Plant Cell, Vol. 22: 108ñ123, 2010). DDB2 complex size is not modified upon UV exposure nor in the det1-1 mutant, and we further show that it is also not affected in the DET1 OE-3 overexpressing line before and after UV-C exposure (new Figure S10).

Upon this request, we also further tested DET1 and mycDET1 complex profiles in the OE-3 transgenic line. Figure S7 shows that in addition to the DET1-like 350 kDa complex, mycDET1 also forms a high-molecular weight complex, as was previously shown in a GFP-DET1 transgenic line by Dr Chory's laboratory (Schroeder et al, Curr. Biol., 2002). The profile of this HMW is not similar to the UV-induced DET1 HMW complex, is not dependent on UV irradiation, and could therefore represent a form of aggregated DET1.

Finally we also tested the different levels of DET1, CUL4 and DDB2 proteins in the det1-1 mutant, and observed no visible differences (Figure 6F).

2. det1-1 is in Col-0 ecotype and ddb2-2 is in Nossen ecotype. Different genetic backgrounds often display different UV sensitivities. It is hard to judge det1-1 ddb2-2 double mutant phenotype before several backcrosses.

- We first considered this could not be a problem since Col-0 and Nossen wild-type accessions exhibit similar UV-C sensitivity in the root growth assay. Introgression of the ddb2-2 mutation in the Col-0 background would require at least 4 generation (>1 year) and therefore the time-lapse for revision was obviously not long enough to obtain these plants. We nonetheless performed a null segregant analysis, by testing F3 seeds from the det1-1 X ddb2-2 cross. This is presented in the new Figure S9 in which the wild-type null segregant (DET1 DDB2) and ddb2-2DET1 have been used as controls. DET1DDB2 null segregant plants exhibit the same UV sensitivity as the wild-type Col 0 plants, while ddb2-2DET1-1 plants exhibit the same UV hypersensitivity as the original ddb2-2 mutant plants, confirming the accuracy of our original data.
Note that in the new experiments the UV-C dose has been adjusted to increase the amplitude of the response, which is higher than in Figure 5C, allowing to better differentiate the phenotype of wild-type lines.

3. DDB1A mutation enhances det1 mutant phenotypes (Wesam et al., 2007). Is it also the truth for UV response?

- Indeed, it has been reported by Schroeder et al (2002) and then by Al Khateeb & Schroeder (2007) that ddb1a enhances some det1-1 plant development phenotypes. This is true for the skotomorphogenic phenotype, but for some other aspects such as chlorophyll content the authors reported a partial suppression of det1-1 phenotype by ddb1a. It would therefore be interesting to test it also for DNA repair. Unfortunately, the time for revision did not allow us to perform the experiment since obtaining homozygous plants from the det1 X ddb1a cross would require about 6 months.

Why cul4 mutation does not?

Based on our data and on several published works, we observe that photomorphogenic mutants in the CUL4/DDB1, DET1 and COP genes exhibit complex and interesting interactions, and genetic analyses give different clues depending on the phenotype analysed.
For etiolated development in darkness, all of them seem to aggravate the phenotype of det1-1. As reported for CUL4 co-suppressor lines (Chen et al., 2006), we described that cul4-1 mutation aggravates the det1-1 skotomorphogenic phenotype, similarly to the ddb1a mutation. The same occurs with the det1-1ddb2-2 mutant (Castells et al, 2010), as already observed using a DDB2 hypomorph mutation introduced in det1-1 (Al Khateeb & Schroeder, 2007).
When grown under normal light conditions, it is fascinating to observe that the cul4-1 mutation partially suppresses some aspects of det1-1 adult plant development, and notably its severe dwarf phenotype. For example, leaves of the cul4-1det1-1 double mutant are about 3-fold bigger than det1-1 leaves. This observation suggests that complex effects are presumably linked to different DET1 targets, and opens the possibility that DET1 could have antagonistic roles with CUL4, as suggested by the role of DET1 in the inhibition of the degradation of LHY in plants (Song & CarrÈ, PMB 2005) and of CDT1 in human (Pick et al, Mol Cell Biol 2007). These aspects are clearly beyond the scope of this manuscript, but will deserve our future attention to decipher the molecular roles of DET1 in plants.
For the specific aspect of DNA repair, we observed that combination of det1-1 and cul4-1 alleles did not aggravate the respective defects in relative root growth upon UV-C exposure, and this epistatic interaction indicates that DET1 and CUL4 act in the same pathway for light-independent DNA repair.

Minor points,
1. I do not see the "Asterisks" in Fig 4.
- This has now been corrected.

2. Figure 5, "A" and "B" are mislabeled. I also do not see "Asterisks indicate t-test significant differences" in this figure. The protein loading controls are unacceptable.

- We apologize for these errors. The loading controls are given with better resolution pictures, and now should be acceptable.

3. Fig S1, "A" need a significant difference test. I am not sure det1-1 mutant phenotype is complemented by overexpression of DET1. At least we still see the open cotyledons in "B".

As previously answered to Reviewer 1, a defect in apical hook formation was observed for many of the seedlings complemented with mycDET1, while few others display a normal one. We note this was already observed by Zhang & Schroeder (Planta, 2010, 231: 337-348) using the same type of construct. We now mention in the text that complementation of the det1-1 skotomorphogenic phenotype is partial.
This partial complementation is unlikely to affect the conclusions drawn on the functionality of the mycDET1 fusion protein in DNA repair. We nonetheless introduced a new figure showing the phenotype of 2-week-old complemented det1-1/DET1 OE-3 line seedlings grown under normal light conditions, showing that they exhibit a wild type phenotype (Figure S2A). With regards to the question of Reviewers 1 and 3, we felt it was necessary to show that the det1-1/DET1 OE-3 line has a wt phenotype under the growth conditions of most of the UV-C responses analysed (in vitro DNA repair, protein content, etc).

4. Some typos should be corrected.
- This has been done.

REVIEWER 3

Castells et al. suggest a novel role for the conserved DET1 protein in DNA damage repair in Arabidopsis. The manuscript is well written and provides interesting new data on DET1 function, a protein known as a repressor of photomorphogenesis in plants. However, there are few experiments that are required to significantly strengthen the work and some minor comments that have to be considered before publication.

1) The authors postulate a role of DET1 in NER. Similar to other Arabidopsis mutants impaired in NER, it is expected that det1-1 and DET1-Ox are altered in response to other DNA-damaging agents as well. Such data has to be provided, including appropriate controls for comparison. This would also further support a role of DET1 in DNA damage responses independent of the regulation of photolyase expression and "sunscreen" biosynthesis published before (Castells et al., Plant J, 2010).

- As suggested by Reviewer 3, we performed a sensitivity assay using cisplatin, a genotoxic agent causing DNA inter/intra crosslinks (Figure S6). In contrast to ddb2-2, we observed that det1-1 plants do not exhibit hypersensitivity to this genotoxic agent. Surprisingly, the DET1 OE-3 overexpressor was found to be sensitive, which we can only explain through possible indirect effects, possibly perturbing DDB2 function. As previously shown for ddb2-2, we also observed that det1-1 plants do not exhibit hypersensitivity to hydrogen peroxide, in agreement with H2O2 induced DNA lesions being not primarily repaired by the NER pathway but by the BER pathway involving specific DNA glycosylases.
We believe that our data clearly distinguish between the DNA damage responses linked to photolyases and ìsunscreenî compounds and NER, as the phenotypes are opposite. While sunscreens and photolyases diminish UV-C sensitivity, the defect in DNA repair reported here in the absence of light and in vitro enhances this sensitivity.

2) If relative root lengths are given, provide the length of the non-treated controls in the figure or figure legends. Otherwise it is not clear, for example, if non-treated det1-1 roots were already different under the experimental conditions without treatment. Generally, also provide n (number measured) and repetitions in figure legends and not only M&M section.

- This assay actually measures relative root growth, and does not compare root lengths. It therefore allows mutants to be compared because it excludes initial differences in root length, and data are presented as percentages like in a reference publication (Jiang et al, PNAS 1997, 94: 7441ñ7445). Nonetheless, we confirmed that root length of det1-1 and wild type exhibit no statistical difference in the absence of UV-irradiation in the conditions of the assay (Castells et al, Plant J 2010). Therefore differences in root length can only result from UV-C responses. We did not include this in the revised version for space constraints.
We now indicate in each figure legend that n>20 individuals for each biological replicate.

3) Fig.4A is not convincing to me. This experiment should be repeated with a larger number of roots. This should provide more convincing data answering whether the double mutant is like the single mutants vs. showing an additive effect. The conclusion to be drawn would be certainly different.
- All relative root growth assays have been performed at least in triplicates. See also the previous answer.

4) The authors repeatedly argue that DDB2 and DET1 degradation is required for NER. This is not supported by data, even though NER activity correlates with turnover. E.g. p10; the DDB2 WDxR mutation does not only block its degradation but likely also interaction with DDB1. Thus, it is not clear what of the two is causing the failure to rescue the mutant phenotype. The degradation data should be supported by data using proteasome inhibitors, in both Col and det1-1.

- The first aspect concerns DDB2 degradation. CUL4-DDB1 targeted DDB2 proteasome degradation is well established in mammals and was published in plants in Molinier et al (2008). As Reviewer 3 mentions, in vivo the WDxR mutation is expected to affect both DDB2 binding to DDB1 and degradation. Because DDB2 degradation requires direct interaction with DDB1 as part of the CUL4-DDB1 E3 ligase, the two aspects are thus indistinguishable. The role of this degradation during NER has been shown in previous studies in human cells (reviewed in Huang & D'Andrea, 2006), and our data are fully compatible with this model being true also in plants.
However, we agree with Reviewer 1 that this mutation might also affect other aspects of DDB2 function during NER, and therefore our data are compatible with, but do not accurately demonstrate, the requirement of DDB2 degradation for NER. Consequently we removed this statement from the text and modified our conclusions accordingly.
Concerning DET1 decay, we observed that it is fast, independent of DET1 gene expression, and abolished in the cul4 E3 ligase mutant. We therefore considered it reasonable to conclude that the DET1 protein is degraded. We reasoned, as in Molinier et al (PLoS Genet 2008), that showing the implication of a CULLIN was more targeted and allowed us to draw more specific conclusions than using proteasome inhibitors, which have wide and indirect cellular effects. We nonetheless performed several attempts, with only partially conclusive results. To our knowledge, there are no available protocols combining plant uptake of proteasome inhibitors in vivo and UV irradiation. This turned out to be technically difficult in our hands, because the incubation of the seedlings in liquid medium (with DMSO or inhibitors) reproducibly affected the subsequent UV irradiation and DET1 degradation (Figure 2 for reviewers). Nonetheless, in the revised version, we show that constitutively expressed mycDET1 fusion protein also decreases upon UV exposure (Figure 4C), providing additional evidence for DET1 protein being post-translationally degraded upon UV irradiation. Finally, we do not claim that DET1 degradation is necessary for the NER process.

Similarly, it would be informative to perform the NER experiment in extracts containing proteasomal inhibitors (again det1-1 and WT) (Fig. 2B).
To our knowledge proteasome inhibitors do not function in the in vitro repair assay, due to the loss of proteasome subunits thorough preparation of DNA repair cell extracts. Nonetheless, even though these inhibitors would be active, they are expected to affect many other proteasome-mediated processes during NER, not only DDB2 or DET1 degradation, and we believe that abolishing in vitro DNA synthesis by this means would give no clue on these specific aspects. However, we now show that cul4-1, ddb2-2 and det1-1 are all affected using this assay, altogether clearly indicating a role for CUL4-based E3 ligase in this process (Molinier et al, 2008, this manuscript).

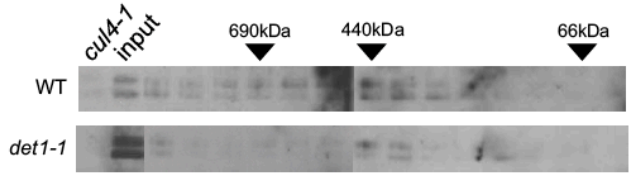
5) Data published by Dohmann, Development 2008 and Zhang and Schroeder, Planta, 2009 should be discussed.

- We first thought that citing data by Dohmann et al (2008) on DNA breaks accumulation in det1-1 mutant might confuse the message of the manuscript. We now realize this omission was incorrect, and therefore we describe these data in the Introduction.
The second reference has now been introduced, at the right place instead of Al Khateeb et al (2010) for the enhancing of det1-1 phenotype by DDB1A overexpression.

6) Fig. 1A should be provided using the anti-DET1 antibody. Otherwise the relative amounts to wt Col-0 cannot be estimated.

- We appreciated this comment from two reviewers, and this has now been done. Two blots using the DET1 antibody are now shown in Supplementary Figure S1, allowing overexpression levels to be compared directly.

Figure 1 for reviewers



CUL4 complex size is not destabilized in the *det1-1* mutant.

Gel filtration of CUL4 protein in wild-type Col-0 and in the *det1-1* mutant. Size-exclusion chromatography was performed as in Figure S10. Note that CUL4 complex size was estimated to peak at the 440 kDa marker in Chen et al Plant Cell (2010) 22: 108–123.

Acceptance letter 10 January 2011

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below), 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

Referee #2

(Remarks to the Author)
The authors have addressed most of my comments from last version. I think that the manuscript is suitable for publication in EMBO J now.

Referee #3

(Remarks to the Author)
The authors did a good job in revising the manuscript. The manuscript is now acceptable for publication.

