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The Retinoblastoma homolog RBR1 mediates localization of the repair protein RAD51 to DNA lesions in Arabidopsis

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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 27 June 2016

Thank you again for submitting your manuscript on Arabidopsis RBR1 and RAD51 in DNA damage responses/repair for our editorial consideration. I am very sorry for the extraordinary delay in its review process - the fact that this was a back-to-back submission involving several major groups in the field however made it difficult to quickly find a sufficient number of unbiased expert referees suitable and available to review these works, and there were unfortunately also additional delays during the reviewing process itself.

We have now received three sets of comments on both of the co-submitted manuscripts. As you will see from the comments on your study copied below, the referees acknowledge the potential interest of defining a gene expression-independent DNA damage response role for RBR1 but are presently not yet convinced that such a direct role is already supported in a sufficiently definitive manner by the present data set. Key concerns pertain to the reliance on a single non-complemented allele (ref 1 pt 1 & ref 3), a lack of quantitation (e.g. ref 1 pt 3), the possibility of spontaneous DNA breaks (ref 1 pt 4), as well as various other technical or presentational issues. Furthermore, it is apparent that further investigation into the nature of the RBR1-RAD51 interplay (see e.g. ref 2) would be important to strengthen the conclusions on a direct, functionally relevant RBR1 role at damage sites.

Given the overall interest of the topic and potential importance of the findings in this study, I would like to give you the opportunity to address these key issues, as well as the various other pertinent experimental and presentational/writing issues in a revised version of the manuscript. I should however point out that it is our policy to only invite a single round of formal major revision, making it important to diligently answer to all points raised at this stage - so should you have any specific

questions/comments regarding the referee reports or your revision work, please do not hesitate to get in touch with me ahead of time, e.g. with a tentative response letter and proposal of how key points might be clarified. We might further arrange for an extended revision period beyond the regular three months, during which time the publication of any competing work (here or elsewhere) would have no negative impact on our final assessment of your own study.

REFEREE REPORTS

Referee #1:

RBs, in mammals, play a routine and essential role in the cell cycle, inhibiting entry into S phase until they are inactivated by cyclin-dependent kinases at the appropriate time. Here the authors present convincing evidence for a second role in some aspect of DSB repair or damage recognition, including the assembly of RAD51 foci. This observation has not been previously published in other eukaryotes, making it especially valuable. The most important results are the effect of the rbr1-2 mutation on the frequency of formation of RAD51 foci and the partial colocalization of RAD51 and RBR1 at gH2AX foci. The decreased frequency of RAD51 foci in the mutant is especially important, as one would predict just the opposite effect if rbr1-2 was only affecting the rate of cell cycle progression. The data on the sensitivity of the mutant to DNA damaging agents is less surprising or novel (conceptually- the experiments themselves are new). I have a few issues with the paper, some related to problems in the writing, but in other cases an experiment needs to be either improved or dropped.

1) In the Arabidopsis literature, two alleles, or restoration of the wild-type phenotype by a transgenic wild-type allele, are required to ascribe a phenotype to the effect of a mutation. This is because mutant lines carry additional mutations in other genes. This standard is also upheld for T-DNA insertion alleles, as insertion mutants carry additional untagged mutations (as shown in the original Feldman paper, the majority of mutant phenotypes in T-DNA insertion lines result from mutations that are not tagged by a T-DNA). The most interesting result presented here is the failure to produce wild-type levels of RAD51 foci in the rbr1-2 homozygote at the "permissive" temperature (which here refers to the mutant state- at the nonpermissive temperature homozygosity is, I assume, lethal). The authors need to show that addition of the wild-type RBR1 gene eliminates this mutant phenotype, or that other- perhaps targeted and subtle?- alleles of RBR1 can produce the same effect. Also, the molecular nature/derivation of rbr1-2 is not described in the reference cited, though its temperature sensitivity is. Given the ms's reliance on the phenotype of this single allele, it needs to be briefly reviewed (and cited) here.
2) The Western blot showing reduced RBR1 expression in the mutant is an important bit of data and should be taken more seriously. Thanks for showing us the entire lane- but please add the size markers, tell us the expected size of the protein (don't just point to what you think is the protein). This western also provides a nice opportunity to show us whether the mCherry tagged protein- which is used to demonstrate localization of Rbr1- is expressed at normal levels in the transgenic line.
3) Although some conclusions are validated by a quantitative analysis of phenotype (i.e., Fig. 4D, Fig. 7 E) there is, often an overreliance on a single microscopic images to support an important conclusion (especially Fig. 3F). We have absolutely no idea what the variation is, from plant to plant, let alone from treatment to treatment, in the number of dead cells. All three seedlings have dead cells, the DE rbr1 double mutant is somewhere between WT and rbr1-2. These nonquantitative experiments don't justify the page of text devoted to their discussion. Also, if the suppressive effect of DE on rbr1's sensitivity to BLM is real, we also don't know if also occurs in the absence of the rbr1 defect- please show us the DE mutant alone. It makes sense to me that anything that slows the cell cycle (other than DNA damage itself) will suppress damage-induced cell death.
4) The constitutive mild upregulation of the five most DSB-sensitive, S phase repair-related transcripts may be due to additional spontaneous breaks in rbr1, rather than a role in repression of expression of these genes. These spontaneous breaks might also be the cause of the spontaneous cell death observed in the mutant. I like to see data on gH2AX foci in untreated rbr1 plants.
5) How real-world is the Al treatment? This is not described at all in Materials and Methods. Given that this this is described several times as relevant to agriculture, please be more specific about the

dose (and pH) vs. soil.

Nit-picky points:

In the abstract:
"...die upon DNA damage dependent on high cyclin..."(just fix this sentence) "Consistent with its canonical role..." in what? Regulation of DNA repair is not RB's canonical role.
Results:
Thanks for clearly stating (most) competing hypotheses, at the top of page 9. However, the third sentence- "RBR1 might sensitize cells to die after inflicted damage and could at the same time involved in DNA repair". I interpret this sentence as saying that RBR1 might be required to activate a programmed cell death in response to damage (given that they are trying to contrast this with the more obvious cell progression related hypothesis). But that wouldn't make sense, as their knockdown line exhibits enhanced cell death in response to damage, not reduced cell death? I guess the authors are erroneously using the term RBR1 refer to the mutant? Please clean up this sentence.
The authors describe a nice experiment in which they artificially slow the cell cycle, using a defective CDKA, and observe that the rbr1 DE double mutant still hyperaccumulates BLM-induced gH2AX foci. This suggests that rbr1 is defective either in repair of DSBs or in the removal of gH2AX from foci after repair. The authors are, I think, too sweepingly general in saying this suggests that rbr1 is "defective in DDR". DDR in the form of gH2AX formation is still going strong.

On p16 second paragraph: BLM-induced DSBs occur independently of cell cycle progression- they're direct breaks, and gH2AX focus formation can occur at any phase of the cell cycle. Therefore it is not "remarkable" that slowing the cell cycle has no effect on the frequency of breaks.
Also, in the sentence at the end of this paragraph, I can't tell whether you're trying to suggest that rbr1 plays a role in both the repair of breaks and (a second role) in the direct suppression of programmed death. While it is possible the Rbr1 plays this second role, you have no evidence to support this notion. If you mean to suggest this, remind the reader that this is pure speculation.
The authors might also mention that fact that the HR repair pathway that's deregulated in rbr1-2 is S/G2 phase specific. Thus it makes sense that these transcripts might routinely be upregulated on entry into S phase. It would be interesting to know whether the extant literature on cell-stage specific gene expression supports this.

In summary, this paper provides data that both further substantiates RBR1 role in genome maintenance and presents data supporting the more conceptually novel idea that that RBR1 plays a direct role in repair by facilitating the formation of RAD51 foci.

Referee #2:

Transcriptional repression of E2F target genes, mostly during the cell cycle, is the best characterization activity of the retinoblastoma protein, both in plants and animals. The authors of this manuscript focus on an apparent role of RBR, the plant Rb homologue, in DNA damage response (DDR), largely based on the observation that a RBR mutation causes cell death upon DNA damage. They elaborate on this result and confirm that RAD51 is actually an E2F target gene. Finally they show a requirement of RBR for correct localization of RAD51 at DNA damage foci. They claim that RBR plays a role in assembling DNA-bound repair complexes.

The connection of RBR with pathways involved in DDR is certainly interesting, due to the known similarities and differences between DDR in plants and animals. The study is of high quality but in my opinion remains short in providing a sufficiently deep set of results fully supporting the major authors' claims. One major question is how is RBR targeted to DNA damaged sites. There are a number of specific points that are listed below.

Some specific points.
1. Data in Fig 1 are very crude observations of DDR that serve the basis of this study. They could well be placed as Supplementary information.
2. Fig. 3 also has problems. Few conclusions can be extracted from 3A, it is merely descriptive. 3D

and 3E can be combined. Label of the X-axis is missing in 3E.
3. Fig. 4. What is the phenotype of the DE mutation alone?
4. Fig. 5A seems to be also incomplete (DE, DE rbr1?)
5. E2F is a protein that contains several functional domains. It would be highly informative to use several alleles to fully demonstrate a role of the full protein. Also details about whether the allele used is a knock-out or a knock-down? Is a truncated protein produced that could act as a dominant negative?
6. Page 9, line 2 from bottom. Concluding that it RBR1 plays a "direct" role needs further clarification/demonstration.
7. Page 10, Fig. 5. What is the expression profile of E2F targets in the DE rbr1 mutant? Labels in panels C and E are missing, and not explained.
8. Fig. 6. Detailed kinetics studies would likely provide insights to speculate on the functional relevance of the colocalization of RBR and RAD51.
9. It is already known that E2F colocalizes to DNA damaged sites (also in animal cells). Based on this, finding RBR in those sites could be also expected.

Referee #3:

General comment on the two manuscripts:

The authors B. M. Horvath et al. (Scheres and Boegre labs) submitted a manuscript with the title "Arabidopsis RETINOBLASTOMA RELATED is involved in repair and DNA damage response". The authors S. Biedermann et al. (Schnittger lab) submitted a manuscript with the title "The Retinoblastoma homolog RBR1 mediates localization of the DNA repair protein RAD51 to DNA lesions". The manuscripts should be considered for back-to-back publication. While the Scheres/Boegre paper has a lot of data, the Schnittger paper is much less substantial and appears often sloppy (no NGS mRNA analysis, no co-IP interaction data; missing size bars, some statistical analysis missing). Both manuscripts emphasize that RBR not only has a function in cell cycle regulation and transcription but also a direct function in DNA repair. Both studies underpin this latter point by co-localization data between RBR, DNA damage markers (yH2AX) and DNA repair proteins (BRCA1 or RAD51). The Scheres/Boegre study also performed an additional experiment (co-IP) to demonstrate the co-existence of RBR and BRCA1 in the same complex. Unfortunately, both studies suffer from technical short-comings related to afore mentioned key experiments (detailed evaluation below) and it remains unclear if RBR is really targeted to DNA lesions, co-localizing with DNA repair factors and if it has a direct function and not an indirect one (via control of transcription of genes encoding DNA repair proteins and cell cycle factors). The accompanying experiments (cell death studies in root tips, sensitivity assays, epistatic analyses, mRNA expression and promoter control analyses) are not discriminating between an indirect or direct contribution of RBR to DNA damage response. It is important to highlight, that a principle involvement of RBR in DDR and DNA repair is unambiguously shown in both studies. The direct involvement of RBR in DNA repair in plants has already been hypothesized earlier (in a study related to meiotic DNA repair - Chen et al 2011, EMBO J.; in a study by the Scheres lab, Cruz-Ramirez et al., 2013 PLoS Biol.) but not conclusively answered back then. Furthermore, there are conflicting data comparing the given studies and the previous Chen et al. study: now Biedermann et al. report co-localization of RBR with RAD51 in mitotic nuclei, while the previous study of Chen et al. clearly showed no co-localization of these two factors during meiosis; Horvath et al, report co-localization of RBR with BRCA1, a factor needed for DNA repair and speculate about a failure of DNA repair in the mitotic nuclei with reduced RBR levels, yet the previous study by Chen et al. did not observe any DNA repair defects (only defects in connecting to the homologous partner). None of these conflicts are further discussed in the two given manuscripts. In this sense, the two given manuscript fail to provide a strong and non-ambiguous answer for the interesting question if RBR in plants is directly involved in DNA damage repair.

In principle the addressed questions and the submitted findings are interesting and the authors should be given a chance to address all raised points of criticism. Special attention should be given to data quality and the key question of RBR co-localization and co-existence in the same complex together with established DNA repair factors.

General comments on the S. Biedermann et al. manuscript:
The authors S. Biedermann et al. (Schnittger lab) submitted a manuscript with the title "The Retinoblastoma homolog RBR1 mediates localization of the DNA repair protein RAD51 to DNA lesions". The authors state that their data indicates that RBR not only is involved in cell cycle control but also in safeguarding DNA integrity. This latter insight is certainly new and has not been studied in depth before in plants. It should be noted though, that in 2011, a joint paper of the Franklin and Berger labs analyzed the importance of RBR in meiosis (Chen et al., 2011; EMBO J.). Not much reference is given to this study, yet certain key findings in the given manuscript appear not in line with the previous study (see details above and below). It is interesting to mention that Chen et al. did not find a co-localization between RBR and RAD51, but the authors of the given manuscript report that in mitotic cells there is co-localization. This conflicting data is not further discussed.

Furthermore, the authors emphasize that their data indicates a direct involvement of RBR in plant DNA repair, acting together with DNA repair factors, localizing to chromatin/DNA to promote DNA repair. In mammalian cells, the direct involvement of (the mammalian homologue of RBR) pRb in DNA repair has been suggested by co-IP experiments, especially highlighted in Cook et al. (2015 Cell Rep.) with evidence of pRb interacting with proteins involved in cNHEJ (Ku70/80/DNA-Pk; in Xiao and Goodrich (2005 Oncogene) with evidence of interaction between pRB and BRCA1 and Top2...etc... Conversely, Lang et al. (2012 New Phyt.) published that in Arabidopsis E2F, a binding partner of RBR involved in transcriptional control, co-localizes with yH2AX. This latter results would rather suggest that RBR is not directly involved in DNA repair but possibly targeted together with E2F to DNA lesion sites (to integrate the DNA damage signals and release repression of genes encoding DNA repair factors globally). Indeed the authors provide very solid data on RBR dependent DNA repair gene de-repression upon genotoxic stress - rather supporting an indirect role of RBR in DNA damage response. No doubt, it is certainly intriguing to speculate about a direct role of RBR in DDR, but the data in the literature comes from different model systems, is partly conflicting and/or not convincing. In this sense, any statement on RBR's role in plant DDR has to be very solid and beyond any doubt. Unfortunately, the authors fail to make this point (see below).

Specific comments on the S. Biedermann et al. manuscript:

Title:
The title has to be changed, since it is overstating the findings. RAD51 localization is questionable (see detailed comments below) and there is no direct proof provided that the large and few RBR foci are actually at DNA lesion sites (just questionable co-loclisaiton with yH2AX, see comments below).

Abstract:
Pl rephrase in clearer English 2nd and 3rd sentence.

According to the criticism below and to potentially new data to be added pl re-phrase or delete the following sentence: "Further analysis revealed that RBR1, independently of E2FA, is required for the correct localization of RAD51 to DNA lesions. We show that RBR1 is targeted to DNA breakage sites where it partially co-localizes with RAD51...."

Introduction:
..."point mutations" is not the correct term in this context...pl correct.

Page 4: reference is given to the Cruz-Ramirez 2013 study but not to the meiotic study of Chen et al 2011.....pl include information and reference.

Last sentence of introduction: please make sure that the sentence is read in a manner that an indirect RBR effect on RAD51 foci numbers is meant (if no further data is added).

Results:
Please make sure to describe the nature of the used mutants very well and justify why they have been chosen. Also compare to other RBR mutant alleles (e.g. the rbr-2 mutant allele used in the Chen et al study, or the RBR RNAi line from the Gruissem lab....).

Pl revise first sentences of 1st and 2nd paragraphs and use better English.

First paragraph, Figure S1: please provide quantification in addition to picture to better evaluate the RBR protein levels.

Reference for ATR and WEE should be Culligan 2004, pl correct.

Page 7, first and last sentence of first paragraph; last sentence of page: ...pl revise and use better English.

Page 8:

Please include more explanation for the observation of meristem size in rbr1 mutants. How do the authors interpret this observation? Smaller meristems since cells are undergoing more, faster and pre-mature divisions?

Please include a sentence on the effect of the used drugs in the context of G1, S and G2 cells (HU, BLM and CisPt). CisPt is a ICL drug and will be only effective from S onwards, BLM will lead to ss and ds DNA scission in any context.....etc...

Possibly move Fig. 3A to supplements and just mention in text to make Figure 3 smaller and easier to digest.

Figure 3E: label missing, pl complete....

Figure 3F only relevant later ....please move to Figure 4.

Figures 4, 6, 7 and S3: pl provide size bars (!!!).

Figure 4C and Figure 3F include the "DE rbr1" line ...but it has not been introduced at that stage.....pl re-arrange manuscript accordingly.

Page 8: yH2AX experiment (Fig. 4C) not sufficiently labelled or explained....BLM treatment for how long...etc...pl change label in Figure and amend text.

Page 9: Pl revise first sentence: for sure more than three interpretations can be found.... Following the data of the given manuscript (and the accompanying one) it is more than likely that RBR is involved in many processes (see also Figure 8!), among them cell cycle control, transcription of DDR genes and control of cell death (by an unknown mode) .....this makes the study of RBR certainly very difficult.

Page 9, 2nd paragraph: pl revise 1st sentence to allow more possibilities ....

Pl revise sentence:..." exchanged to Asp and Glu...."....

Last sentence of paragraph: "Moreover, the increased appearance of γH2AX
foci in rbr1 DE independent of cell death indicates that RBR1 plays a direct role in DDR.". Please revise sentence: RBR certainly appears involved in DDR, but still unclear if directly or indirectly!

Figure 5 D and page 11:
It would be good to test further E2F factors for redundancy.....

Page 11, Section headline "RBR1 accumulates at DNA lesions after Bleomycin treatment". Please tame down statement: it is unclear if the few RBR foci are localizing to DNA damage sites. At the most, a partial co-localization with yH2AX could be envisioned.

Page 11, last paragraph; Figure 6:
Cytology of somatic nuclei exposed to BLM:
RBR localizes only as a few, large and diffuse foci per nucleus but no quantitative analysis is provided how many of which class of foci are observed and how many cells show staining. Are the RBR pos. cells in a specific cell cycle state...eg.: EdU pos cells? The foci areas take up about 1 micrometer in width, which is about 25% of the entire width of the somatic nuclei. Similarly, the yH2AX foci reside as few, large foci in the nuclei (also no quantification ...please provide data). Interestingly, the observed RBR and yH2AX foci appear in some cases side-by-side in some cases overlapping. Please provide a tight definition of "co-localization" and also a solid statistical analysis. Is there any correlation with the intensely stained DAPI regions (it looks, there is). If so, are these centromeric regions? The reviewer points out that Coschi et al. 2014 (Cancer discovery; not a plant study) found a protein complex associating with pericentromeric repeats comprised of E2F1, condensin and pRb. If this is also true in plants, the nature of the presented staining (a few massive foci of RBR) would be in accordance with previous findings in mammalian cells. In general, the possibly low amount of cells that show a staining altogether after BLM treatment and the diffuse / low amount of yH2AX foci in those few cells may reflect different technical short-comings: BLM stability and penetration; over-fixation of cells/proteins; limited permeability for antibodies to entering the cells/nuclei during the staining procedure...etc...

Please re-do and extend the analysis and re-write the paragraph accordingly.
The authors also provide a graph of measured fluorescence intensity in the respective channels, to underline their statement of co-localization.

The experimental section does not explain how the pictures are acquired: are this single stacks or are these (max. intensity?) projections? Why not performing a 3D re-construction with the (most likely) available z-layers. How is co-localization defined? Please provide a definition? Are these foci in the same z-level? Has the picture acquisition been done in a manner that wave length shifts has been considered? Furthermore, to argue for co-localization (according to a definition yet to be provided) a statistical test and a comparison to a random situation is needed. Preferentially this test should be done in 3D (and not on a projection!) using the actually measured nuclei volume, exclude the volume of the nucleolus and use the average size of the foci volumes.....

Page 12: "The finding that mCherry.....". This sentence needs revision according to the newly acquired data....in the current form it is neither backed by data, nor do the chosen experiments address the question if RBR localizes to DNA lesions.

Why is the first row of panels in Fig. S3A identical with Fig. 6B....pl fix.

Page 12/13 and Figure 7: IF with RAD51 etc...see comments above! Statistical tests needed!

Page 12: "This finding suggests that RBR has a local role...." This statement is not justified. Pl. delete.

Figure 8....is fine, but "D" is speculative at the current moment....

A further short-coming of the manuscript is that direct interaction is insinuated from the (weak) co-localization data, but not corroborated by any additional experiment. The manuscript of the Boegre/Scheres lab provides a co-IP for RBR and BRCA1, but the experiment also does not give a solid result (yet).

1st Revision - authors' response 08 December 2016

Overview over the major changes incorporated in this revision:

- Use of a second *rbr1* allele, i.e. an *RBR1* knock-down line via RNAi (amiGO); use of this line confirmed the reduction of RAD51 foci when RBR1 activity is reduced.

- Use of the CDK inhibitory drug Roscovitine; application of this drug confirmed our previous results obtained with the double mutant of *rbr1* with a hypomorphic *cdka;1* mutant (DE), i.e. reduction of cell proliferation activity rescues the cell death phenotype of *rbr1* but not the increased level of DNA damage as revealed by gH2AX foci. Furthermore, we have now carefully quantified the cell death phenotype in *rbr1* mutants.

- Repetition and detailed analysis of the co-localization of RBR1 with gH2AX and RAD51. This work confirms that our previous conclusion that RBR1 partially co-localizes with gH2AX and RAD51. Quantitative data on the co-localization studies are provided. Moreover, we have calculated the Pearson's Coefficient (with Costes randomization) and the Manders Coefficients to provide statistical evidence for the co-localization and the definition of co-localization.

- Elaboration of the question how RBR1 is targeted to DNA lesions: We show now that the activity of the previously identified B1-type kinases (CDKB1), which play a major role in DDR in plants (Weimer et al., 2016), is required for the recruitment of RBR1 to gH2AX foci. In further support, we find that mutants in the cyclin partner of CDKB1, the B1-type cyclins, have also reduced gH2AX foci. Finally, we have generated and analyzed the triple mutant *cdkb1;1 cdkb1;2 rbr1* revealing that both RBR1 and CDKB1 function in one genetic pathway.

Detailed response to the reviewers:

Referee #1:

RBs, in mammals, play a routine and essential role in the cell cycle, inhibiting entry into S phase until they are inactivated by cyclin-dependent kinases at the appropriate time. Here the authors present convincing evidence for a second role in some aspect of DSB repair or damage recognition, including the assembly of RAD51 foci. This observation has not been previously published in other eukaryotes, making it especially valuable. The most important results are the effect of the rbr1-2 mutation on the frequency of formation of RAD51 foci and the partial colocalization of RAD51 and RBR1 at gH2AX foci. The decreased frequency of RAD51 foci in the mutant is especially important, as one would predict just the opposite effect if rbr1-2 was only affecting the rate of cell cycle progression. The data on the sensitivity of the mutant to DNA damaging agents is less surprising or novel (conceptually- the experiments themselves are new).

We like to thank this reviewer for his/her positive and constructive evaluation of our work.

I have a few issues with the paper, some related to problems in the writing, but in other cases an experiment needs to be either improved or dropped.

1) In the Arabidopsis literature, two alleles, or restoration of the wild-type phenotype by a transgenic wild-type allele, are required to ascribe a phenotype to the effect of a mutation. This is because mutant lines carry additional mutations in other genes. This standard is also upheld for T-DNA insertion alleles, as insertion mutants carry additional untagged mutations (as shown in the original Feldman paper, the majority of mutant phenotypes in T-DNA insertion lines result from mutations that are not tagged by a T-DNA). The most interesting result presented here is the failure to produce wild-type levels of RAD51 foci in the rbr1-2 homozygote at the "permissive" temperature (which here refers to the mutant state- at the nonpermissive temperature homozygosity is, I assume, lethal). The authors need to show that addition of the wild-type RBR1 gene eliminates this mutant phenotype, or that other- perhaps targeted and subtle?- alleles of RBR1 can produce the same effect. Also,
the molecular nature/derivation of rbr1-2 is not described in the reference cited, though its temperature sensitivity is. Given the ms's reliance on the phenotype of this single allele, it needs to be briefly reviewed (and cited) here.

We have now repeated the key experiments of our work with an RNAi RBR1 knock-down line, called amiGO, published by Cruz-Ramirez et al. (2013). We show now that the number of RAD51 foci is also significantly reduced in this allele (presented in Figure S4) providing independent experimental

support for our initial observation using the rbr1-2 allele. We have also added the citation to Chen et al., who have shown that the molecular nature rbr1-2 is a splicing defect but did not become aware of its temperature sensitivity. In addition, we like to point out to the work by Horvath et al., submitted back-to-back with our work that also shows that RBR1 has cell-cycle independent role in DNA damage in Arabidopsis.

2) The Western blot showing reduced RBR1 expression in the mutant is an important bit of data and should be taken more seriously. Thanks for showing us the entire lane- but please add the size markers, tell us the expected size of the protein (don't just point to what you think is the protein). This western also provides a nice opportunity to show us whether the mCherry tagged protein- which is used to demonstrate localization of Rbr1- is expressed at normal levels in the transgenic line.

We have revised this figure and show now the size markers. In addition, we provide quantification of the protein levels.

3) Although some conclusions are validated by a quantitative analysis of phenotype (i.e., Fig. 4D, Fig. 7 E) there is, often an overreliance on a single microscopic images to support an important conclusion (especially Fig. 3F). We have absolutely no idea what the variation is, from plant to plant, let alone from treatment to treatment, in the number of dead cells. All three seedlings have dead cells, the DE rbr1 double mutant is somewhere between WT and rbr1-2. These nonquantitative experiments don't justify the page of text devoted to their discussion. Also, if the suppressive effect of DE on rbr1's sensitivity to BLM is real, we also don't know if also occurs in the absence of the rbr1 defect- please show us the DE mutant alone. It makes sense to me that anything that slows the cell cycle (other than DNA damage itself) will suppress damage-induced cell death.

We have carefully taken this comment into account and have carried out quantitative analyses, which are now presented in our revised figure 4. To this end we have applied the drug Roscovitine that is often used to inhibit cdc2-type CDK activity. We quantify cell death in wt and rbr1 mutants in untreated conditions with plants treated with BLM alone, with Roscovitine alone, and with both drugs at the same time. The data obtained fully supports our previous finding that reduction of CDK activity does suppress the cell death in rbr1 mutants but does not reduced the level of DNA damage as judged by the number of gH2AX foci.

4) The constitutive mild upregulation of the five most DSB-sensitive, S phase repair-related transcripts may be due to additional spontaneous breaks in rbr1, rather than a role in repression of expression of these genes. These spontaneous breaks might also be the cause of the spontaneous cell death observed in the mutant. I like to see data on gH2AX foci in untreated rbr1 plants.

We provide now quantitative data on the number of gH2AX foci in rbr1 mutants grown on agar without genotoxic drugs (new Fig. S4). These experiments show that indeed untreated rbr1 mutants have already increased number of gH2AX foci in comparison to the wildtype. However, after treatment with BLM, the number of these foci is dramatically increased. As raised by this reviewer below, the DDR genes up-regulated in rbr1 do indeed show an expression peak once wild-type cells enter S-phase (synchronization by sucrose starvation). Taken together, these finding support a role for RBR1 in preparing a cell not only for replication but also for the potential damage, which may occur during the cell cycle.

5) How real-world is the Al treatment? This is not described at all in Materials and Methods. Given that this this is described several times as relevant to agriculture, please be more specific about the dose (and pH) vs. soil.

This is a good point and we have added a few sentences about the abundance of Al when we introduce our Al experiments, i.e. it is the 3rd most common element in the crust of the earth and

present at toxic or at least plant growth reducing levels in approximately 50% of all arable land. Typical concentrations of mobile Al found in soil range between micromolar to millimolar when the pH is lower than 5. Please note that our experiments were conducted with an Al concentration of 0.75 to 2.0 mM, thus in a range, which occurs in nature. The pH of our medium is 4.2, again in the range of what can be found in nature.

Nit-picky points:

In the abstract:
"...die upon DNA damage dependent on high cyclin..."(just fix this sentence)

This sentence was re-written to also take into account that treatment with the CDKA inhibitor roscovitine resembles the restoration of cell viability seen in rbr1 cdka double mutants.

"Consistent with its canonical role..." in what? Regulation of DNA repair is not RB's canonical role.

We have added here "canonical role as transcriptional repressor".

Results:
Thanks for clearly stating (most) competing hypotheses, at the top of page 9. However, the third sentence- "RBR1 might sensitize cells to die after inflicted damage and could at the same time involved in DNA repair". I interpret this sentence as saying that RBR1 might be required to activate a programmed cell death in response to damage (given that they are trying to contrast this with the more obvious cell progression related hypothesis). But that wouldn't make sense, as their knockdown line exhibits enhanced cell death in response to damage, not reduced cell death? I guess the authors are erroneously using the term RBR1 refer to the mutant? Please clean up this sentence.

We apologize for making ourselves not clear enough. Our third hypothesis is a combination of hypothesis one and hypothesis two, i.e. rbr1 mutants undergo cell death due to defects in cell cycle progression and at the same time RBR1 might be important for DNA repair. However, the repair aspect could be covered by the cell death phenotype. We have rephrased this now and hope that the three possibilities become clear now.

The authors describe a nice experiment in which they artificially slow the cell cycle, using a defective CDKA, and observe that the rbr1 DE double mutant still hyperaccumulates BLM-induced gH2AX foci. This suggests that rbr1 is defective either in repair of DSBs or in the removal of gH2AX from foci after repair. The authors are, I think, too sweepingly general in saying this suggests that rbr1 is "defective in DDR". DDR in the form of gH2AX formation is still going strong.

We thank the reviewer for this careful comment. This is of course right and we conclude now: "...Taken together, the rbr1 cell-death phenotype is largely dependent on CDK activity/cell-cycle progression. Moreover, the elevated levels of gH2AX foci in rbr1 DE and in rbr1 mutants treated with Roscovitine in comparison with the wildtype indicate that RBR1 has a cell cycle independent function in DNA repair. ...".

On p16 second paragraph: BLM-induced DSBs occur independently of cell cycle progression- they're direct breaks, and gH2AX focus formation can occur at any phase of the cell cycle. Therefore it is not "remarkable" that slowing the cell cycle has no effect on the frequency of breaks.

We have removed this sentence.

Also, in the sentence at the end of this paragraph, I can't tell whether you're trying to suggest that rbr1 plays a role in both the repair of breaks and (a second role) in the direct suppression of programmed death. While it is possible the Rbr1 plays this second role, you have no evidence to support this notion. If you mean to suggest this, remind the reader that this is pure speculation.

We have underlined that this is only one possible explanation.

The authors might also mention that fact that the HR repair pathway that's deregulated in rbr1-2 is S/G2 phase specific. Thus it makes sense that these transcripts might routinely be upregulated on entry into S phase. It would be interesting to know whether the extant literature on cell-stage specific gene expression supports this.

We thank this reviewer for this helpful comment. Indeed, when we checked the available transcriptomics data sets (e.g. Menges et al. 2003), we found that all five genes, which are upregualted in rbr1 mutants (BRCA1, PARP2, RAD51 and TSO2) have their expression maximum in S-phase. This is indeed consistent with our hypothesis that RBR1 links the expression of genes involved in DNA replication with genes participating in DNA repair. Hence, when cells enter S-phase they also prepare for possible DNA damage. We have included this point in our discussion.

In summary, this paper provides data that both further substantiates RBR1 role in genome maintenance and presents data supporting the more conceptually novel idea that that RBR1 plays a direct role in repair by facilitating the formation of RAD51 foci.

Referee #2:

Transcriptional repression of E2F target genes, mostly during the cell cycle, is the best characterization activity of the retinoblastoma protein, both in plants and animals. The authors of this manuscript focus on an apparent role of RBR, the plant Rb homologue, in DNA damage response (DDR), largely based on the observation that a RBR mutation causes cell death upon DNA damage. They elaborate on this result and confirm that RAD51 is actually an E2F target gene. Finally they show a requirement of RBR for correct localization of RAD51 at DNA damage foci. They claim that RBR plays a role in assembling DNA-bound repair complexes.

The connection of RBR with pathways involved in DDR is certainly interesting, due to the known similarities and differences between DDR in plants and animals. The study is of high quality but in my opinion remains short in providing a sufficiently deep set of results fully supporting the major authors' claims. One major question is how is RBR targeted to DNA damaged sites. There are a number of specific points that are listed below.

We also like to thank this reviewer for his/her positive evaluation of our work. While we completely agree that it is very interesting and important to understand how RBR1 is targeted to DNA lesions, it is also clear that this question is not so easy to experimentally address. None-the-less, we provide now in this revised manuscript version an important step forward to answer this question by showing that CDKB1 kinases, which we have recently identified as key regulators of HR in plants (Weimer et al., 2016), are also important for the correct RBR1 localization. We show that the number of RBR1 foci is strongly reduced in cdkb1;1 cdkb1;2 double mutants. In addition, we show that RBR1 foci are also lowered in mutants of the cyclin partner of CDKB1s (CYCLIN B1) during DNA damage. Furthermore, we have generated the cdkb1;1 cdkb1;2 rbr1 triple mutant and can show that there is no additional reduction in root growth on media with BLM with respect to the cdkb1 and rbr1 mutants providing genetic evidence that CDKB1 and RBR1 act in the same regulatory pathway.

Some specific points.

1. Data in Fig 1 are very crude observations of DDR that serve the basis of this study. They could well be placed as Supplementary information.

We agree that these analyses are rough and only provide an organismic overview over the DNA damage response. For the moment, we have kept them in the main figure section since we felt that they make the experimental set up more palpable for those readers not so familiar with plants. However, we are also happy to place these figures in the supplement if this reviewer and the editor find them better suited for that section.

2. Fig. 3 also has problems. Few conclusions can be extracted from 3A, it is merely descriptive. 3D and 3E can be combined. Label of the X-axis is missing in 3E.

With respect to the comment of the other reviewers, we have restructured Fig 3. First, we have removed several time points and the HU results and placed them into a supplementary figure. Then, we have the former panel F into Fig. 4. Finally, we have double checked all labels and corrected the missing labels.

3. Fig. 4. What is the phenotype of the DE mutation alone?

DE does not show cell death and is not hypersensitive to DNA damage. More details on DE can be found in a recent publication from our lab (Weimer et al., 2016). Please note that the work with the DE mutant is complicated since it is fully sterile (due to meiotic defects as described in Dissmeyer et al., 2009). In addition, the transmission of the mutants allele is reduced, thus the percentage of DE plants is always low. For that reason we have also now included a chemical suppression of CDK activity by applying the CDK inhibitor Roscovitin (please see new figure 4). The results of this chemical downregulation of CDK activity supports the genetical downregulation provided in the first version of the manuscript.

4. Fig. 5A seems to be also incomplete (DE, DE rbr1?)

Since CDKA;1 is the major regulator of RBR1 (please see paper by Nowack et al., 2012), a transcriptional analysis of DE-rbr1 is very complex. Please note that rbr1 is not a null allele (as we have also mentioned in our manuscript). Thus, we feared that a reduction of the counter player of RBR1 in a line where RBR1 has reduced activity gives ambiguous results in terms of quantitative transcriptional analyses. The analysis of the double mutant in terms of DNA damage and cell death defects is still valid since we clearly see that we can uncouple cell death from damage.

5. E2F is a protein that contains several functional domains. It would be highly informative to use several alleles to fully demonstrate a role of the full protein. Also details about whether the allele used is a knock-out or a knock-down? Is a truncated protein produced that could act as a dominant negative?

This is a good point. We provide now additional information on the allele we used, i.e. e2fa-2 in which the transactivation domain is missing. Since the question of E2F involvement has been a focal point in the paper by the Scheres and Bogre labs (Horvath et al.) that has been submitted back to back to our work, we have not elaborated this further.

6. Page 9, line 2 from bottom. Concluding that it RBR1 plays a "direct" role needs further clarification/demonstration.

We have rephrased this sentence, also with respect to the comments of the other reviewers and write now: "…indicate that RBR1 has a cell cycle independent function in DNA repair. …".

7. Page 10, Fig. 5. What is the expression profile of E2F targets in the DE rbr1 mutant? Labels in panels C and E are missing, and not explained.

We have added the labels, our apologies for not providing them in the first case. As explained above, our previous work (Nowack et al. 2012) has shown that loss of CDK activity and reduction of RBR function can partially compensate for each other. Hence we feared that the results of such expression analyses are ambiguous and we have not perused these experiments. In any case, our main statement here is that DNA damage genes are under the control of the RBR1-E2F module.

8. Fig. 6. Detailed kinetics studies would likely provide insights to speculate on the functional relevance of the colocalization of RBR and RAD51.

We agree that kinetics studies would likely be helpful. Probably the best way to do this is by live imaging of single cells. However, such a system is currently not set up in our laboratory and given the other experiments that we needed to conduct for this revision, we did not manage to establish these kinetics analyses in the given time frame. We apologize for this but hope that the reviewer appreciates our other attempts to respond to the comments raised by this and the other reviewers.

9. It is already known that E2F colocalizes to DNA damaged sites (also in animal cells). Based on this, finding RBR in those sites could be also expected.

We are aware of the paper by Lang et al. that show partial co-localization of E2F and gH2AX. As requested by reviewer 3, we have now put a lot of effort in documenting the co-localization of RBR1 and gH2AX as well as RAD51 has he/she was not so convinced by these data.

Please also note that we found that the number of RAD51 foci is not altered in e2fa mutants (although these mutants are hypersensitive of genotoxic stress). We are also aware of the fact that Rb and E2F have been implicated in DDR in animals and have also discussed this. To our knowledge, however, it is even in the animal and yeast field new that RAD51 foci are decreased in mutants with lower Rb activity. In addition, we have added now experiments that show that CDKB1 are required for efficient targeting of RBR1 to DNA damage sites and we hope that our work is helpful to push our understanding of DDR forward.

Referee #3:

We also like to thank this reviewer for taking his/her time to critically read and comment both manuscripts. The points raised are very important and addressing them has helped us to improve our manuscript.

General comment on the two manuscripts:

The authors B. M. Horvath et al. (Scheres and Boegre labs) submitted a manuscript with the title "Arabidopsis RETINOBLASTOMA RELATED is involved in repair and DNA damage response". The authors S. Biedermann et al. (Schnittger lab) submitted a manuscript with the title "The Retinoblastoma homolog RBR1 mediates localization of the DNA repair protein RAD51 to DNA lesions". The manuscripts should be considered for back-to-back publication. While the Scheres/Boegre paper has a lot of data, the Schnittger paper is much less substantial and appears often sloppy (no NGS mRNA analysis, no co-IP interaction data; missing size bars, some statistical analysis missing). Both manuscripts emphasize that RBR not only has a function in cell cycle regulation and transcription but also a direct function in DNA repair. Both studies underpin this latter point by co-localization data between RBR, DNA damage markers (yH2AX) and DNA repair proteins (BRCA1 or RAD51). The Scheres/Boegre study also performed an additional experiment (co-IP) to demonstrate the co-existence of RBR and BRCA1 in the same complex. Unfortunately, both studies suffer from technical short-comings related to afore mentioned key experiments (detailed evaluation below) and it remains unclear if RBR is really targeted to DNA lesions, co-localizing with DNA repair factors and if it has a direct function and not an indirect one (via control of transcription of genes encoding DNA

repair proteins and cell cycle factors). The accompanying experiments (cell death studies in root tips, sensitivity assays, epistatic analyses, mRNA expression and promoter control analyses) are not discriminating between an indirect or direct contribution of RBR to DNA damage response. It is important to highlight, that a principle involvement of RBR in DDR and DNA repair is unambiguously shown in both studies. The direct involvement of RBR in DNA repair in plants has already been hypothesized earlier (in a study related to meiotic DNA repair - Chen et al 2011, EMBO J.; in a study by the Scheres lab, Cruz-Ramirez et al., 2013 PLoS Biol.) but not conclusively answered back then.

Furthermore, there are conflicting data comparing the given studies and the previous Chen et al. study: now Biedermann et al. report co-localization of RBR with RAD51 in mitotic nuclei, while the previous study of Chen et al. clearly showed no co-localization of these two factors during meiosis; Horvath et al, report co-localization of RBR with BRCA1, a factor needed for DNA repair and speculate about a failure of DNA repair in the mitotic nuclei with reduced RBR levels, yet the previous study by Chen et al. did not observe any DNA repair defects (only defects in connecting to the homologous partner). None of these conflicts are further discussed in the two given manuscripts. In this sense, the two given manuscript fail to provide a strong and non-ambiguous answer for the interesting question if RBR in plants is directly involved in DNA damage repair.

To our knowledge Chen et al. did not suggest that RBR1 plays a role in DNA damage repair but rather in recombination, on page 8 they write: "...there is no evidence of DNA fragmentation in rbr-2. This suggests that despite reduced CO formation, the DSBs are efficiently repaired, either through non-CO recombination or via repair using a sister chromatid as the repair template...".

Please also note that Chen et al. did not show co-localization of RBR1 with RAD51 but with DMC1 (Figure 6 in Chen et al.). Kurzbauer et al. published a very careful analysis in Plant Cell (2012) in which they showed by immuno-cytology that RAD51 and DMC1 are actually spatially separated in meiosis. Hence, it is not clear at the moment whether RBR1 and RAD51 co-localize in meiosis or not.

Interestingly, Chen et al. reported that the number of RAD51 foci is not reduced in male meiocytes of rbr1-2 mutants and the reviewer raises a very important point here. Apparently we made our discussion on the difference concerning the number of RAD51 by Chen et al. and our work not clear enough. We actually found this difference very intriguing and have even concluded with this point our paper, please see page 17 (last paragraph) till page 18 (end of first paragraph) in our first submission. Interestingly, a different role and regulation of RAD51 in meiosis versus mitosis was revealed by a recently published separation-of-function allele of RAD51. This allele did not display meiotic defects (rescue of the sterility of rad51 mutants) but was dominantly sensitizing mitotic cells to DNA damage (Da Ines et al. 2013). A different function of RAD51 in mitosis and meiosis is not a plant specific feature and Cloud et al. (2012) could distinguish different RAD51 features in yeast. Thus, to link this difference of RAD51 function to RBR1 might contribute to an understanding of the regulatory mechanisms behind.

In principle the addressed questions and the submitted findings are interesting and the authors should be given a chance to address all raised points of criticism. Special attention should be given to data quality and the key question of RBR co-localization and co-existence in the same complex together with established DNA repair factors.

General comments on the S. Biedermann et al. manuscript:
The authors S. Biedermann et al. (Schnittger lab) submitted a manuscript with the title "The Retinoblastoma homolog RBR1 mediates localization of the DNA repair protein RAD51 to DNA lesions". The authors state that their data indicates that RBR not only is involved in cell cycle control but also in safeguarding DNA integrity. This latter insight is certainly new and has not been studied in depth before in plants. It should be noted though, that in 2011, a joint paper of the Franklin and Berger labs analyzed the importance of RBR in meiosis (Chen et al., 2011; EMBO J.). Not much reference is given to this study, yet certain key findings in the given manuscript appear not in line with the previous study (see details above and below). It is interesting to mention that Chen et al. did not find a co-localization between RBR and RAD51, but the authors of the given manuscript report that in mitotic cells there is co-localization. This conflicting data is not further discussed.

Please see our comments above: Chen et al. did not analyze RBR1 and RAD51 but DMC1. Furthermore, we have discussed at the very end of our paper the results of Chen et al. concerning the unaltered localization of RAD51 in male meiocytes in rbr1-2 plants in the light of a different role and/or regulation of RAD51 in mitosis versus meiosis at the very end of our manuscript (p17 and 18).

Furthermore, the authors emphasize that their data indicates a direct involvement of RBR in plant DNA repair, acting together with DNA repair factors, localizing to chromatin/DNA to promote DNA repair. In mammalian cells, the direct involvement of (the mammalian homologue of RBR) pRb in DNA repair has been suggested by co-IP experiments, especially highlighted in Cook et al. (2015 Cell Rep.) with evidence of pRb interacting with proteins involved in cNHEJ (Ku70/80/DNA-Pk; in Xiao and Goodrich (2005 Oncogene) with evidence of interaction between pRB and BRCA1 and Top2...etc... Conversely, Lang et al. (2012 New Phyt.) published that in Arabidopsis E2F, a binding partner of RBR involved in transcriptional control, co-localizes with yH2AX. This latter results would rather suggest that RBR is not directly involved in DNA repair but possibly targeted together with E2F to DNA lesion sites (to integrate the DNA damage signals and release repression of genes encoding DNA repair factors

globally). Indeed the authors provide very solid data on RBR dependent DNA repair gene de-repression upon genotoxic stress - rather supporting an indirect role of RBR in DNA damage response. No doubt, it is certainly intriguing to speculate about a direct role of RBR in DDR, but the data in the literature comes from different model systems, is partly conflicting and/or not convincing. In this sense, any statement on RBR's role in plant DDR has to be very solid and beyond any doubt. Unfortunately, the authors fail to make this point (see below).

We fully agree with the reviewer that RBR has multiple functions and does not only act as transcriptional repressor. Our work together with the paper by Horvath et al. indicates that RBR's role during DNA damage is likely very complex as well. While we provide evidence that RBR1 does transcriptionally control (repress) DNA damage repair genes such RAD51 as acknowledged by this reviewer, it is not clear why then rbr1 mutants should be hypersensitive to DNA damage. The reduction of RAD51 foci (despite the fact that RAD51 is upregulated in rbr1) argues for at least one other function of RBR1 in DNA damage. This together with the partial co-localization data, which we have substantiated in this revised version, hints at a local role. None-the-less, we agree with the reviewer that additional aspects of RBR1, which we are not aware of at the moment, may play a role. Hence, as suggested we have down-tuned our conclusions and make the readers aware of potentially other mechanisms of RBR1, which could play a role in DDR.

Specific comments on the S. Biedermann et al. manuscript:

Title:
The title has to be changed, since it is overstating the findings. RAD51 localization is questionable (see detailed comments below) and there is no direct proof provided that the large and few RBR foci are actually at DNA lesion sites (just questionable co-loclisaiton with yH2AX, see comments below).

The title emphasizes the main finding of our work, i.e. that RAD51 foci are reduced in rbr1 mutants. As far as we can tell from the reviewer comments, our localization studies of RAD51 in rbr1 have not been questioned. Moreover, since we could find independent support for this reduction in RBR1 knock-down lines (amiGO, please see comments to reviewer 1), we hope that we have convincing data that justify this title.

Abstract:
Pl rephrase in clearer English 2nd and 3rd sentence.

The sentences have been re-written to improve readability.

According to the criticism below and to potentially new data to be added pl re-phrase or delete the following sentence: "Further analysis revealed that RBR1, independently of E2FA, is required for the correct localization of RAD51 to DNA lesions. We show that RBR1 is targeted to DNA breakage sites where it partially co-localizes with RAD51...."

Following the advice of this reviewer, we have down-tuned our conclusion and write now: "…We show that RBR1, dependent on cyclin-dependent kinase B1 (CDKB1) activity, is targeted to DNA breakage sites where it partially co-localizes with RAD51, indicating at a role of RBR1 in assembling DNA-bound repair complexes in addition to its function as a transcriptional regulator…."

Introduction:
..."point mutations" is not the correct term in this context...pl correct.

We have corrected this.

Page 4: reference is given to the Cruz-Ramirez 2013 study but not to the meiotic study of Chen et al 2011.....pl include information and reference.

We have specified our sentence and write now "…However, the role of Rb-type proteins in DDR outside of the stem-cell niche is currently not clear….". Since Chen at al. have concluded that RBR1 has a role in recombination (see comment above), we think that a reference to their work here is misleading. However, since Chen et al. have unraveled the likely (or at least a part of the) molecular nature of the rbr1-2 allele we cite them few lines below when we discuss the use of this allele. We hope that the reviewer agrees with this procedure.

Last sentence of introduction: please make sure that the sentence is read in a manner that an indirect RBR effect on RAD51 foci numbers is meant (if no further data is added).

We down-tuned our statement and write now "…Importantly, RBR1 is required for DNA repair since in rbr1 but not e2fa mutants, the number of RAD51 foci is strongly reduced…."

Results:
Please make sure to describe the nature of the used mutants very well and justify why they have been chosen. Also compare to other RBR mutant alleles (e.g. the rbr-2 mutant allele used in the Chen et al study, or the RBR RNAi line from the Gruissem lab....).

The allele we use here is the same as the one used by Chen et al., named there rbr-2. However, Ebel et al. (2004) have first named this allele rbr1-2 and hence we like to follow the nomenclature of the initial characterization. We provide now references to Ebel et al., Chen et al., and our own work by Nowack et al. in which we discovered that rbr1-2 has actually a temperature-sensitive behavior.

Pl revise first sentences of 1st and 2nd paragraphs and use better English.

We have revised these sentences.

First paragraph, Figure S1: please provide quantification in addition to picture to better evaluate the RBR protein levels.

We now provide a quantification in Figure EV1.

Reference for ATR and WEE should be Culligan 2004, pl correct.

We thank the reviewer for spotting this mix-up and have corrected our mistake.

Page 7, first and last sentence of first paragraph; last sentence of page: ...pl revise and use better English.

We have re-phrased these sentences.

Page 8:

Please include more explanation for the observation of meristem size in rbr1 mutants. How do the authors interpret this observation? Smaller meristems since cells are undergoing more, faster and pre-mature divisions?

We interpret the reduction in meristem size as a consequence of the massive cell death seen in rbr1 mutants when exposed to DNA-damaging drugs, please see Figure 3. Due to loss of stem cells, cell production cannot keep pace with the root-ward differentiation process. Reduction of meristem size after DNA damage has often been observed, see for instance the recent paper by Chen and Umeda (2015). We have added this interpretation to the text. Later in our work, we show that the rbr1-dependent cell death can be largely rescued by slowing down the cell cycle.

Please include a sentence on the effect of the used drugs in the context of G1, S and G2 cells (HU, BLM and CisPt). CisPt is a ICL drug and will be only effective from S onwards, BLM will lead to ss and ds DNA scission in any context.....etc...

Since we have introduced the drugs and their way of action in the previous paragraphs, we are not sure whether we should repeat this information here at the end of this paragraph. We are happy to do so if the reviewer and editor think that this increases the readability of the text.

Possibly move Fig. 3A to supplements and just mention in text to make Figure 3 smaller and easier to digest.

The reviewer is right that figure 3 was very crowded and difficult to read. We have restructured this figure, also with respect to the comments of the other reviewer. Additional time points and the HU data set have been shifted into supplementary files. The last panel has been moved into Figure 4.

Figure 3E: label missing, pl complete....

This was corrected.

Figure 3F only relevant later ....please move to Figure 4.

Has been moved, thank you for this suggestion.

Figures 4, 6, 7 and S3: pl provide size bars (!!!).

Size bars were added.

Figure 4C and Figure 3F include the "DE rbr1" line ...but it has not been introduced at that stage.....pl re-arrange manuscript accordingly.

Panel F of figure 3 was shifted into figure 4. The panel of figure have now been arranged in the order of their mentioning in the text.

Page 8: yH2AX experiment (Fig. 4C) not sufficiently labelled or explained....BLM treatment for how long...etc...pl change label in Figure and amend text.

We have now added always the duration of the treatment into the figure to increase readability.

Page 9: Pl revise first sentence: for sure more than three interpretations can be found.... Following the data of the given manuscript (and the accompanying one) it is more than likely that RBR is involved in many processes (see also Figure 8!), among them cell cycle control, transcription of DDR genes and control of cell death (by an unknown mode) .....this makes the study of RBR certainly very difficult.

The reviewer is right and we have adjusted the text accordingly.

Page 9, 2nd paragraph: pl revise 1st sentence to allow more possibilities ....

We write now: "…To narrow down the function of RBR1 in DNA damage,…".

Pl revise sentence:..." exchanged to Asp and Glu...."....

Has been changed.

Last sentence of paragraph: "Moreover, the increased appearance of γH2AX foci in rbr1 DE independent of cell death indicates that RBR1 plays a direct role in DDR.". Please revise sentence: RBR certainly appears involved in DDR, but still unclear if directly or indirectly!

We have revised this sentence and write now: "…Taken together, this demonstrates that the rbr1 cell-death phenotype is largely dependent on CDK activity/cell-cycle progression. Moreover, the elevated levels of gH2AX foci in rbr1 DE in comparison with the wildtype indicates that RBR1 has a cell cycle independent function in DNA repair. …"

Figure 5 D and page 11: It would be good to test further E2F factors for redundancy.....

We like to reference here to the work by Horvath et al. (back to back paper) who have focused on the role of E2F in DNA damage repair. Our main conclusion is that RBR1 has a key role in DDR.

Page 11, Section headline "RBR1 accumulates at DNA lesions after Bleomycin treatment". Please tame down statement: it is unclear if the few RBR foci are localizing to DNA damage sites. At the most, a partial co-localization with yH2AX could be envisioned.

We have changed this to the more descriptive statement: "RBR1 accumulates in nuclear foci after Bleomycin treatment".

Page 11, last paragraph; Figure 6:
Cytology of somatic nuclei exposed to BLM:
RBR localizes only as a few, large and diffuse foci per nucleus but no quantitative analysis is provided how many of which class of foci are observed and how many cells show staining. Are the RBR pos. cells in a specific cell cycle state...eg.: EdU pos cells?

The team of Ben Scheres and Lazlo Bögre have spent a lot of effort to untangle a possible cell cycle phase dependency of RBR1 and gH2AX foci. To not repeat or overlap more with their work, we like to reference to their back-to-back paper for this analysis.

The foci areas take up about 1 micrometer in width, which is about 25% of the entire width of the somatic nuclei. Similarly, the yH2AX foci reside as few, large foci in the nuclei (also no quantification ...please provide data).

We agree that some foci are rather large. We have repeated this analysis several times and we usually did not see that the foci are 25% of the width of the nucleus (please see our new figure 6 and 7 as well as supplementary figure S5 and S6). We show now several examples of nuclei with RBR1 foci in figure S5 to give the reader the chance to develop a better feeling about the actual phenotypes. In addition, we provide now quantitative data (Venn diagram in figure 7) that show the overlap between gH2AX, RAD51 and RBR1 foci in more than 10 nuclei analyzed.

Interestingly, the observed RBR and yH2AX foci appear in some cases side-by-side in some cases overlapping. Please provide a tight definition of "co-localization" and also a solid statistical analysis. Is there any correlation with the intensely stained DAPI regions (it looks, there is). If so, are these centromeric regions? The reviewer points out that Coschi et al. 2014 (Cancer discovery; not a plant study) found a protein complex associating with pericentromeric repeats comprised of E2F1, condensinand pRb. If this is also true in plants, the nature of the presented staining (a few massive foci of RBR) would be in accordance with previous findings in mammalian cells.

We have taken this point of this reviewer very seriously and think that our paper has profited with this a lot. First of all, we have improved the quality of the image acquisition, please see our new figure 6 and 7 next to the supplementary figure S5 and S6. Then we have calculated the Pearson's Coefficient (was for the example provided 0.821) and the Manders Coefficients, was M1=1.0 (fraction of gH2AX overlapping RBR1), M2=0.995 (fraction of RBR1 overlapping gH2AX). Next we did Costes randomization (200 rounds) based colocalization with r=0.82. These data are presented in Fig 6D and Fig 7D, E.
Typically, Pearson's Coefficient of 0.8 and higher is considered to be strongly co-localized. For Manders, values above 0.9 are considered to be strong indication for co-localization, for Costes values above 0.8 are considered to indicate a strong relationship.

An overlap between chromocenters and RBR1 foci was not apparent to us. We clearly can have foci that do not overlap with chromocenters. Please see our picture in the new figures 6 and 7 as well as S5 and S6, in which we have scanned through two nuclei demonstrating that the co-localizing foci come from one optical plane and are not an artifact of a pseudo 3D projection. At the same time this question was very difficult to push to a more quantitative level, e.g. are these dots more frequent in higher condensed parts of the chromatin. Because of the experimental difficulties in answering this question unambiguously, we prefer to make no statement at this moment and focus on the question whether RBR1 binds to foci and is co-localized to gH2AX and/or RAD51.

In general, the possibly low amount of cells that show a staining altogether after BLM treatment and the diffuse / low amount of yH2AX foci in those few cells may reflect different technical short-comings: BLM stability and penetration; over-fixation of cells/proteins; limited permeability for antibodies to entering the cells/nuclei during the staining procedure...etc... Please re-do and extend the analysis and re-write the paragraph accordingly.

As laid out above, we have carefully re-analyzed the localization aspects and provide now several additional data sets, which confirm that RBR1 localizes to foci on DNA, that RBR1 and RAD51 partially overlap, that RBR1 and gH2AX partially overlap, and that even all three foci can overlap. These data are presented in Fig 6, 7, S5, and S6.

The authors also provide a graph of measured fluorescence intensity in the respective channels, to underline their statement of co-localization.
The experimental section does not explain how the pictures are acquired: are this single stacks or are these (max. intensity?) projections? Why not performing a 3D re-construction with the (most likely) available z-layers. How is co-localization defined? Please provide a definition? Are these foci in the same z-level? Has the picture acquisition been done in a manner that wave length shifts has been considered? Furthermore, to argue for co-localization (according to a definition yet to be provided) a statistical test and a comparison to a random situation is needed. Preferentially this test should be done in 3D (and not on a projection!) using the actually measured nuclei volume, exclude the volume of the nucleolus and use the average size of the foci volumes.....

The intensities scans were done in one single optical section. We also provide with Appendix figure S3 a scan in z-dimension through a nucleus clearly showing that the overlapping signals come from the same optical section and are not produced by pseudo 3D constructions or projections.

Page 12: "The finding that mCherry.....". This sentence needs revision according to the newly acquired data....in the current form it is neither backed by data, nor do the chosen experiments address the question if RBR localizes to DNA lesions.

As the reviewer may be aware of, it is very difficult to directly visualize broken DNA stands. Hence, we have used gH2AX as a close proxy for DNA lesions that has been used by many others. We will now make the reader aware of this read-out system. As now underpinned by our statistical analyses RBR1 does partially overlap with gH2AX and RAD51 foci. A similar finding, at least with respect to gH2AX has been obtained by Horvath et al. in independent experiments. Thus, we hope that the reviewer agrees that this conclusion is backed up by our combined revised data.

Why is the first row of panels in Fig. S3A identical with Fig. 6B....pl fix.

Figure 6 and S3 were completely revised. However, the pictures that were used in Fig. 6B and 7A were added to provide better comparison. A note of this was made in the figure legend.

Page 12/13 and Figure 7: IF with RAD51 etc...see comments above! Statistical tests needed!

We have calculated the Pearson and the Manders coefficient, please see above.

Page 12: "This finding suggests that RBR has a local role...." This statement is not justified. Pl. delete.

We have tuned this statement down.

Figure 8....is fine, but "D" is speculative at the current moment....

We make the reader aware in our figure legends that this is only a hypothesis.

A further short-coming of the manuscript is that direct interaction is insinuated from the (weak) co-localization data, but not corroborated by any additional experiment. The manuscript of the Boegre/Scheres lab provides a co-IP for RBR and BRCA1, but the experiment also does not give a solid result (yet).

We have tested the interaction between RBR1 and RAD51 in yeast two hybrid assays but did not find interaction in this assay. In addition, we have tried by to analyze RBR-containing protein complexes after IP with mass spec. However, these are difficult experiments and we could unfortunately not detect any proteins for the moment. It seems likely that posttranslational modifications are important here, especially since we show not that the localization of RBR1 into foci does depend on

the activity of CDKB1-CYCB1 complexes. Thus, further work is required to address these points in molecular detail in future.

2nd Editorial Decision 30 January 2017

Thank you again for your patience during the re-evaluation of your revised manuscript on Arabidopsis RBR and DNA repair. We have now received the below comments from two referees that had agreed to re-review it, and in their light I am pleased to say that we shall be happy to accept your manuscript, pending a number of remaining minor modifications as detailed below:

- All referees retain a few minor points that should be addressed by clear responses text modifications, and possible (referee 2?) also figure modifications.

REFEREE REPORTS

Referee #1:

Biedermann et al re-review

Overall summary-
1) The authors carefully document many phenotypes presented by plants with a partial RBR1 defect, clearly indicating that RBR1 plays an important role in maintaining genomic stability. We already knew that RBR1 in plays a role in regulating progression into S phase, so this is not a surprise.
2) They also find that RBR1 forms foci in response to DNA damage, and that these foci sometimes overlap with gH2AX and/or RAD51 foci. This had been observed in animals but not previously in plants.
3) Most interestingly (to me) they find that the frequency of RADS51 foci is reduced in the rbr1 mutant, a phenotype that can't be explained by unrestricted progression into S phase (quite the opposite would be predicted). Together with the colocalization to (some) RAD51 and gH2AX foci, this suggests that RBR1 plays a role in genome maintenance beyond cell cycle regulation- perhaps in the assembly or activity of RAD51 foci. That's novel.

General writing suggestion:
Interpretive remarks in the Results section still repeatedly state that a certain phenotype "suggests a repair defect" (= new news) when that phenotype is also entirely consistent with a checkpoint defect (= old news). On page 9, the authors (finally) clearly present these two not necessarily mutually exclusive hypotheses. I'd move these two hypotheses up to the front of the Results section, and at the end of the presentation of each type of data, tell us if this allows us to distinguish between hypotheses. Usually it doesn't, so these comments should be corrected.

Specific issues:

P10 and 11- The discussion of the effects of roscovitine and BLM on gH2AX production is incorrect- or I'm crazy. The conclusion is correct, but the statement of the frequency of lesions in wt is wrong. Please correct this, comparing this paragraph (top of p 11) to data presented in fig. 4I.

Upregulated transcriptional response in rbr1 might be due entirely to the (demonstrated) higher levels of both spontaneous damage- not because RBR1 is a classical transcriptional repressor. Extensive additional upregulation by damaging agents still occurs in rbr1 (though I recognize that the rbr1 mutant employed is not a KO). I think the authors are on shaky ground when they propose a new role for RBR1 as a director repressor of DDR-induced transcripts, based only on the fact that they see it binds upstream of RAD51. It's possible, but this just seems a little thin.

It's clear that DDR-induced PCD at the stem cell niche requires cell cycle progression in wild-type

as well as rbr1. Do not state that it is required for PCD in rbr1 without reminding us that its required for PCD in wt too (ie, top of page 19).

Very minor issues:
Delete comma in abstract after "activity", replace "temporally" with "temporarily" in introduction

Plant materials: save the reader some effort and tell us that all mutants are in a Col background- If this is correct. Saying Col is used "as wt" is not necessarily the same thing.

Bottom p 8- insensitivity to HU could also be interpreted as HU itself artificially replacing the G1/S checkpoint that's defective in rbr1. Just something to think about, I'm not requesting anything here. This is how cell cycle checkpoint genes were originally characterized in yeast- they could be rescued by chemicals that directly arrest the cell cycle.

Referee #2:

This is revised version of a manuscript entitled "The Retinoblastoma homolog RBR1 mediates localization of the repair protein RAD51 to DNA lesions". Authors have made a significant effort to address most, if not all, the points outlined in my report. I accept that the main conclusions that (1) RBR1 has a direct role in DNA damage response (DDR), (2) RBR1 is required for RAD51 localization, and (3) RBR1 is targeted to DNA breakage sites after phosphorylation by CDKB1, are supported by the results obtained. The use of a triple cdkb1;1 cdkb1;2 rbr1 mutant, among others, provides genetic evidence that CDKB1 and RBR1 act in the same regulatory pathway.

Most of the points included in my report have been addressed satisfactorily. I suggest that a discussion paragraph is included to expand/speculate on:
- What is the expression profile of E2F targets in the DE rbr1 mutant?
- Discuss on possible mechanism for RBR1 recruitment to damaged sites.

I still have a concern regarding data in Fig 8. Differences between panels C-E and F-H are not clear at all. Based on this information one would say that differences do not exist. The pattern in panels F-H should be comparable to that Fig 6. This should be corrected.

2nd Revision - authors' response 17 February 2017

Reviewer 1 comments:
Overall summary-

1)The authors carefully document many phenotypes presented by plants with a partial RBR1 defect, clearly indicating that RBR1 plays an important role in maintaining genomic stability. We already knew that RBR1 in plays a role in regulating progression into S phase, so this is not a surprise.

2) They also find that RBR1 forms foci in response to DNA damage, and that these foci sometimes overlap with gH2AX and/or RAD51 foci. This had been observed in animals but not previously in plants.

3) Most interestingly (to me) they find that the frequency of RADS51 Foci is reduced in the rbr1 mutant, a phenotype that can't be explained by unrestricted progression into S phase (quite the opposite would be predicted). Together with the colocalization to (some) RAD51 and gH2AX

foci, this suggests that RBR1 plays a role in genome maintenance beyond cell cycle regulation- perhaps in the assembly or activity of RAD51 foci. That's novel.

We once more thank the reviewer for his/her time and are happy to see that he/she finds that our paper holds new and interesting data.

General writing suggestion:
Interpretive remarks in the Results section still repeatedly state that a certain phenotype "suggests a repair defect" (= new news) when that phenotype is also entirely consistent with a checkpoint defect (= old news). On page 9, the authors (finally) clearly present these two not necessarily mutually exclusive hypotheses. I'd move these two hypotheses up to the front of the Results section, and at the end of the presentation of each type of data, tell us if this allows us to distinguish between hypotheses. Usually it doesn't, so these comments should be corrected.

We agree with the reviewer and have changed the text now. However, we found that the text is easier to read when we start with the description of the mutant phenotype rather than presenting abstract hypotheses (for the people not so familiar with the cell cycle) in the beginning. Thus, we have removed all suggestive statements till p9 of the results part and simply report the hypersensitivity of rbr mutants. Then we present, as suggested the different hypotheses in this part as suggested by the reviewer, followed by the discriminative experiment, i.e. reduction of CDK activity, which should at least partially restore the defects if they were only due unrestricted progression through the cell cycle.

Specific issues:

P10 and 11- The discussion of the effects of roscovitine and BLM on gH2AX production is incorrect- or I'm crazy. The conclusion is correct, but the statement of the frequency of lesions in wt is wrong. Please correct this, comparing this paragraph (top of p 11) to data presented in fig. 4I.

Wild-type plants treated with BLM and Roscovitine have slightly more gH2AX foci then wild-type plants treated with BLM alone, please compare Figure 4l forth column from the left with the second column from the left. This is what we have stated in the text. To enhance the readability we have now explicitly spelled out whether or not BLM and Roscovitine were applied at the same time. In addition, we compare now other classes of foci numbers. Our previous description was apparently a bit confusing and we the revised descriptions we think our points are made very clear now.

Upregulated transcriptional response in rbr1 might be due entirely to the (demonstrated) higher levels of both spontaneous damage- not because

RBR1 is a classical transcriptional repressor. Extensive additional upregulation by damaging agents still occurs in rbr1 (though I recognize that the rbr1 mutant employed is not a KO). I think the authors are on shaky ground when they propose a new role for RBR1 as a director repressor of DDR-induced transcripts, based only on the fact that they see it binds upstream of RAD51. It's possible, but this just seems a little thin.

The reviewer is right and we have included now a warning for the reader by writing: "While we currently cannot exclude that these DNA damage genes are up-regulated in *rbr1* mutants due to the occurring cell death and elevated levels of DNA fragmentation, our ChIP data suggest that RBR1 functions as a conventional (negative) regulator of RAD51 and likely four additional DDR genes…."

It's clear that DDR-induced PCD at the stem cell niche requires cell cycle progression in wild-type as well as rbr1. Do not state that it is required for PCD in rbr1 without reminding us that its required for PCD in wt too (ie, top of page 19).

We thank the reviewer for pointing this out and have included the conclusion in our discussion.

Very minor issues:
Delete comma in abstract after "activity", replace "temporally" with "temporarily" in introduction

Corrected.

Plant materials: save the reader some effort and tell us that all mutants are in a Col background- If this is correct. Saying Col is used "as wt" is not necessarily the same thing.

All mutants used are indeed in the Col-0 background and we have adopted the suggestion of the reviewer.

Bottom p 8- insensitivity to HU could also be interpreted as HU itself artificially replacing the G1/S checkpoint that's defective in rbr1. Just something to think about, I'm not requesting anything here. This is how cell cycle checkpoint genes were originally characterized in yeast- they could be rescued by chemicals that directly arrest the cell cycle.

We agree with the reviewer and appreciate this comment. However, as rbr1 mutants are not sensitive to HU and as we are not going to go further into the question whether HU could possibly re-introduce a G1-S checkpoint in rbr1, we have not commented on this in the paper.

Referee #2:

This is revised version of a manuscript entitled "The Retinoblastoma homolog RBR1 mediates localization of the repair protein RAD51 to DNA lesions". Authors have made a significant effort to address most, if not all, the points outlined in my report. I accept that the main conclusions that (1) RBR1 has a direct role in DNA damage response (DDR), (2) RBR1 is required for RAD51 localization, and (3) RBR1 is targeted to DNA breakage sites after phosphorylation by CDKB1, are supported by the results obtained. The use of a triple cdkb1;1 cdkb1;2 rbr1 mutant, among others, provides genetic evidence that CDKB1 and RBR1 act in the same regulatory pathway.

We also thank this reviewer again for taking his/her time to re-read and comment on our work. We are glad that this reviewer is also largely satisfied with additional experiments we have provided in the revised version.

Most of the points included in my report have been addressed satisfactorily. I suggest that a discussion paragraph is included to expand/speculate on:
- What is the expression profile of E2F targets in the DE rbr1 mutant?
- Discuss on possible mechanism for RBR1 recruitment to damaged sites.

We have now included a discussion on a possible feedback between RBR1 and CDKA;1 which interferes with a conclusive analysis of RBR1 target genes in DE rbr1 as explained in our previous response letter. In addition, we cite work in Chlamydomonas that has CDKA implicated in transcriptional control.

However, since we have no data on the actual translocation process of RBR1 to damaged sites (beyond a genetic requirement of CDKB1s and in vitro kinase data), we are worried such a discussion beyond what we have already would be too speculative and perhaps even misleading as we can not discuss of all possible mechanisms. Hence, we propose to leave such a debate for an opinion paper.

I still have a concern regarding data in Fig 8. Differences between panels C-E and F-H are not clear at all. Based on this information one would say that differences do not exist. The pattern in panels F-H should be comparable to that Fig 6. This should be corrected.

The row C-E indeed shows no accumulation of RBR1 foci (untreated plants). In panel F, one can see foci while in G and H these foci are not present. However, the reviewer is right that the magnification is lower in this figure than in Figure 6 and we present now a second inlay that shows the foci in F and their absence in

G and H with higher magnification. The new inlays are indeed consistent with figure 6.

3rd Editorial Decision 20 February 2017

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Arp Schnittger
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2016-94571R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All samples were treated the same way, each genotype was analyzed at least with three independent biological replicates
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not applicable.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No.
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes.
Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Only previously published ab were used, their use is fully referenced in our manuscript.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not applicable.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Not applicable.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not applicable.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Not applicable.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not applicable.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not applicable.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable.

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	All mutants used in this study were already previously published.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Not applicable.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	Not applicable.
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Not applicable.
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G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.