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KNO1-mediated autophagic degradation of the Bloom component RMI1 promotes homologous recombination

Po-Yu Chen, Nancy De Winne, Geert De Jaeger, Masaki Ito, Maren Heese, and Arp Schnittger
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Thank you for submitting your manuscript reporting KNO1-mediated regulation of RMI1 during DNA damage repair in Arabidopsis to The EMBO Journal. We have now received three referee reports on your study, which are included below for your information. In light of these comments, we would like to invite you to prepare and submit a revised manuscript.

As you will see, the reviewers are overall positive and appreciate the findings and their interest to the field. The referees nonetheless do raise a number of points, which should be addressed and resolved in the revised version of the manuscript. Referee #2 in particular has three specific concerns (points 1-3), which should be addressed by providing additional data and/or revising the text. Please also ensure that the controls and additional detail on experimental procedures is included as referees #1, #2 (point 4, 6, 7) and #3 (point 1- 5) request. In addition, please also carefully consider all other referee comments and revise the manuscript and figures as needed, in addition to providing a detailed response to each comment. Please also remember that the revised manuscript should fulfill all EMBO Journal formatting requirements when it is next submitted (please see below and: https://www.embopress.org/page/journal/14602075/authorguide#submissionofrevisions)

Referee #1:

Interstrand crosslinks are complex lesions with complex repair pathways, involving template switching and/or strand exchange. Here the authors further characterize the role of KNO1- a plant-specific repair factor identified in a previous paper from this lab-in XL repair/response. They present convincing evidence that KNO1 is regulated at the protein stability level (as well as the previously demonstrated transcriptional level) and directs the regulation, again at the protein level, of the core XL repair factor RMI1. This is a very satisfying story that combines regulation of regulatory proteins by both the proteosome and the phagosome, and is the first description of the involvement of the phagosome in DNA damage response in plants. This particular pathway is not present in animals (although there are many examples of regulation of DDR by the phagosome in animals) and so, at another level, this paper also provides a nice example of the adoption of "off the shelf" preexisting regulatory options in arbitrary, different ways in different clades during the course of evolution.
The writing is also very good, with strong introduction and discussion sections.
I list some very minor suggestions below.
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That's it, really nice work!

Referee #2:

Previous studies have shown that RMI1, a component of the BTR complex, and KNO1 are important regulators of DNA damage responses (DDR). But how RMI1 and KNO1 are regulated during DDR is not well understood. In this manuscript, Chen et al. found that KNO1 is degraded through proteasome and KNO1 facilitates K63-linked ubiquitination of RMI1, which triggers the degradation of RMI1 through autophagy. Therefore, this study provided new mechanistic insights into DDR and revealed a link between autophagy and DDR in plants. Overall, this study is well designed and is of great interest to the plant DDR field.

1. The author claimed that KNO1 directly interacts with RMI1, UBP12 and UBP13 using Y2H assays. However, in Y2H assays, the protein interaction could be indirect because the yeast proteins may mediate their interaction. To confirm the direct interaction, I suggested performing pull-down assay using the purified proteins.
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3. The authors found that both loss of KNO1 and overexpressing of KNO1 lead to CiPt hypersensitivity. How to explain these seemly contradicting results? If KNO1 functions by promoting the degradation of RMI1, can blocking RMI1 degradation or overexpressing RMI1 suppress the hypersensitivity of KNO1ox?
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In this manuscript by Chen et al., the authors investigated the molecular mechanisms regulating homologous recombination upon DNA damage in Arabidopsis plants. The authors analyzed physiological and biochemical properties of the new HR regulator KNO1 and revealed KNO1 induces K63-ubiquitin dependent autophagic degradation of RMI1, a component of Bloom, resulting in promotion of HR in response to DNA damage. Interestingly, KNO1 is also degraded by K48-ubiquitin dependent proteasomal pathway, which is suppressed by deubiquitination enzymes UBP12/13 under DNA damage.
The work is overall well conducted and presented. The complementary approaches provide clear evidence for their conclusions. There are a few concerns regarding the manuscript that are detailed below.

1. The authors showed the dynamics of RMI1 protein level by microscopic analysis of RMI1-GFP under DNA stress in WT or kno1 mutant background independently. But, no data directly comparing the RMI1 protein amounts or stability between WT and kno1 mutant. Please confirm this point by standard immunoblot analysis.

2. Translocation of RMI1 from nuclear to cytoplasm was evaluated by microscopic analysis of RMI1-RFP (Fig. 6, Fig EV7). The reviewer is also interested in this regulation and basically agree the authors conclusion. But how did the authors distinguish the RMI1 localization and quantify the nuclear-cytoplasm ratio by only observing RMI1-RFP signal? Please describe detailed methods in the manuscript. Alternatively, it seems better to analyze the nuclear-cytoplasm ratio by biochemical approach (fractionation and immuno blotting).

3. Through the manuscript, the ubiquitination signals of RMI1-GFP (IP product) was faint, especially Fig. 7 Apu3/K63 blot. Please consider the improvement of these data. It is recommended to add deubiquitination inhibitors into the extraction buffers for IP.

4. Page 10 line21-26, The authors mentioned the results using 35S:GFP-KNO1, but no data in Fig. 3D, instead, rmi1 data shown in the Figure.

5. Although the authors performed TAP-MS analysis and identified putative KNO1 interactors, the reviewer couldn't find the materials and method information about this analysis (plasmid preparation, growth condition, TAP and MS analysis). Also please add the material information about KNO1 OX plant.

6. Fig. EV1A. The photo image and graph for CiPt treatment look very similar to that used in previous publication by Bouyer et al (Bouyer et al, 2018, PLoS Genetics). If same, please remove the CiPt data from the figure and only show MMC data in this manuscript.

Mainor concerns

1. Page 9 line 5-10 (Fig. EV2C). It's difficult to understand DUF1767 domain site and structure of the deletion construct used in Y2H. Please add schematic structure image in the figure or detailed explanation in legends.

2. Page 9 line 11-16. Please insert the corresponding Fig number about Y2H results.

3. Page 6 "immunno-detection of vH2aX (Fig. EV2B)". Not shown in EV2B but in EV1B.

4. Figure 5. Mislabeled, maybe 5B should be 5C, vice versa.

5. Page 16 line 14 "E64d (Fig. 6A)" should be Fig. 7A. Line 21 "Apu3 antibodies (Fig. 4B and 7C)" should be Fig. 4C and 7C.

6. Typo: Page 3 line 18, delete "no"; Page 10 line 17, 19, 35:GFP-KNO1 (35S:GFP-KNO1).

Detailed response to the reviewer comments on
Chen et al. "**KNO1-mediated autophagic degradation of the Bloom component RMI1 promotes homologous recombination"** (EMBOJ-2022-111980)

Reviewer #1:

Interstrand crosslinks are complex lesions with complex repair pathways, involving template switching and/or strand exchange. Here the authors further characterize the role of KNO1- a plant-specific repair factor identified in a previous paper from this lab- in XL repair/response. They present convincing evidence that KNO1 is regulated at the protein stability level (as well as the previously demonstrated transcriptional level) and directs the regulation, again at the protein level, of the core XL repair factor RMI1. This is a very satisfying story that combines regulation of regulatory proteins by both the proteosome and the phagosome, and is the first description of the involvement of the phagosome in DNA damage response in plants. This particular pathway is not present in animals (although there are many examples of regulation of DDR by the phagosome in animals) and so, at another level, this paper also provides a nice example of the adoption of "off the shelf" preexisting regulatory options in arbitrary, different ways in different clades during the course of evolution.
The writing is also very good, with strong introduction and discussion sections.

We heartily thank this reviewer for his/her very positive and encouraging feedback.

I list some very minor suggestions below.

INCLUDE LINE NUMBERS NEXT TIME PLEASE- it's for your benefit

Agreed and done.

P3 What does "potent" mean more XLs per gram?

According to Lopez-Martinez et al. (Lopez-Martinez, Liang, and Cohn 2016) the % of ICLs among all lesions induced by mitomycin C is higher (15%) than the % of ICLs among all lesions induced by cisplatin (5-8%). We agree that "more potent" is not a clear term to convey this information, so we rephrased the sentence and thank the reviewer for bringing this to our attention.

"different lines of research" in what sort of organisms?

We apologize for being unclear here. The overview given here relates to what is known from yeast and animals. We now state in the text which organisms we are referring to. We also added a general sentence to explain the situation in plants for which knowledge is still very patchy and therefore difficult to sum up briefly, so a reference to the most recent review is given.

Please note that the situation in plants with respect to the players relevant for our results is more detailed later in the introduction.

"...(HR) machinery" again, is this something confirmed in plants? Maybe add a planty ref?

Agreed. Please see response above.

P6 no untreated control for EV1A. I'd like to know if the mutants grow more slowly anyway

We have added pictures of untreated mutants and controls along with a graph showing the quantification of their root growth into Fig EV1A. In short, the kno1 mutants have a similar root growth as the WT in control condition.

Want to talk about the RAD51 localization photos in EV2B?

We have added the following description of RAD51 localization (now EV1B)
"We also analyzed the localization of the DNA repair recombinase RAD51 as a marker for the assembly of the HR repair machinery upon treatment with cisplatin and MMC (Fig EV1B). In the WT, RAD51 and γH2AX foci co-localize, indicating that RAD51-mediated homology search takes place in both chemical treatments. However, no clear RAD51-foci were seen in *kno1* mutants, suggesting a requirement of KNO1 for chemical induced HR repair.*"*

P7 is the primary data presented somewhere? like, how many nuclei were scored, reproducibility, was the study blinded (guess not as it isn't mentioned)- did the authors score just a single (2D) image or take a 3-D image of the nucleus

We apologize for omitting this information. In each experiment 100 γH2AX foci were scored (from 10-12 nuclei, sampled from 6-8 roots), the experiment was done in triplicate, standard deviations are shown. The study was not blinded and 2D images were analyzed.
We have now added a clear description of what was measured to the text and changed the label of the y axis of Fig 1C to "Frequency of γH2AX foci co-localizing with KNO1 foci (%)"

in this legend, and in all similar figures, please clarify what "relative" means. Presumably relative to the untreated sample of the same genotype? But possible relative to untreated wt?

We have compared in this and other figures the treated with the untreated samples of the same genotype. We have now made this clear in the legend and also modified all similar instances.

Similarly, are the statistics on effects vs "growth in wt" a comparison of the computed value for "relative growth" in one genotype (treated vs untreated) or simply of root length vs untreated wt?

This is a comparison of the computed relative values between different genotypes. We have made this also clear now in all legends.

p8 correct supp vs EV figure labels

We carefully double checked all labels.

p9 TAP tagging methods could use more detail

We added a detailed description of the applied TAP method to the manuscript.

rmi1 mutant is.. null?

We have used the rmi1-2 allele described by Hartung et al. (see reference below). According to Hartung et al., rmi1-2 is a null allele.

Hartung, Frank, Stefanie Suer, Alexander Knoll, Rebecca Wurz-Wildersinn, and Holger Puchta. 2008. "Topoisomerase 3alpha and RMI1 Suppress Somatic Crossovers and Are Essential for Resolution of Meiotic Recombination Intermediates in Arabidopsis Thaliana." PLoS Genetics 4 (12): e1000285.

"non-additive phenotype is consistent..." can we also conclude from this that KNO1 is upstream of rmi1, as it is irrelevant in the absence of rmi1?

We think the genetics is somewhat complicated here. An epistasis argument works best if the phenotypes are opposite to each other. Our case is more difficult since both mutants are sensitive to DNA damage although, what we think, due to different reasons. Mutants in RMI1 have a high level of genome instability presumably due to somatic cross-overs. Adding then a cross-linker enhances this instability by having even more unwanted somatic DNA connections. Mutants in KNO1 are sensitive to cross-linkers because they cannot (or to a large extent) repair this type of DNA damage (by homologous recombination). Thus, we would like to keep this statement here and hope that the epistatic relationship will become clear by our additional experiments as presented in the next sections of our paper.

P10 this expression isn't limited to the root tip meristem, I bet it isn't limited to primary roots either. I'm guessing this is expressed in all cell types? Please be more accurate here

Indeed, *35S-KNO1* is broadly expressed. We only thoroughly analyzed expression in the root, so we do not want to claim it can be expressed all over the sporophyte (which we would assume, however, since 35S activity is indeed not confined to roots)

So we write now:
“Complementing our loss of function approach, we constructed a transgenic line that stably expressed *KNO1* under the control of the broadly active cauliflower mosaic virus (CaMV) *35S* promoter, which resulted in very strong expression of *KNO1* in the entire root including the root apical meristem even in the absence of DNA damage treatments (Fig 3A).”

"more dead cells than in untreated conditions were observed in all genotypes" I'm not seeing this death in your wt example. Given how few dead cells are observed without or with this treatment in kno1 and wt, you need to analyze this statistically- not with just one picture. For example maybe: "20 root tips were scored and X/10 exhibited dead cells in the root tip meristem...".

A graph presenting the frequency of roots with cell death was included in the new manuscript version, please check Appendix Fig S1B. 30 roots per genotype were analyzed in total.

Would a somatic crossover- an exchange of chromosome arms between homologs- result in instability? Maybe you mean ectopic HR?

Yes, indeed this is what we meant. We explain now on page 4:
“However, the relevance of the plant analog of the BTR complex, called RTR (Recq4A, TOP3α and RMI1) for restricting somatic crossovers seems conserved (Knoll, Schröpfer, and Puchta 2014). Mutant analysis shows that somatic recombination repair is in part suppressed by RTR function (Hartung et al. 2008), likely to avoid inappropriate recombination between non-homologous sites which would lead to chromosomal instability.”

P11 GFP (GFP sans fusion) signal isn't shown in these figures- please add, maybe supplemental.

We are not sure if we correctly understand the reviewer’s concern here, but we think that he/she is looking for additional confirmation that our results relate to protein degradation and not KNO1 promoter activity only. We have now performed qPCR analysis of KNO1 in WT and ubp12 ubp13 mutants to support our conclusion that KNO1 is continuously degraded at non stress conditions. Please find these results in Appendix Fig S3C.

We write now
*“*Consistently, the amount of GFP-KNO1 did not increase in ubp12 ubp13 double mutant plants after CiPt treatment (Fig 5C) although KNO1 transcript accumulated as in WT (Appendix Fig S3C), showing that UBP12 and UBP13 are crucial for DNA damage-induced KNO1 protein accumulation regulated at a post-transcriptional level*.”*

P17 "are even hardly known"- not sure what you mean here, do we know about some or not?

We rephrased. Like BLM, RMI1 has been shown to be phosphorylated in mitosis, however, this PTM has been attributed to SAC function. To our knowledge there is no publication showing that PTMs of RMI1 lead to modification of BTR/RTR amount or activity upon DNA damage.

That's it, really nice work!

We very much appreciate this feedback and thank the reviewer his/her time to help improving our manuscript.

Reviewer #2:

Previous studies have shown that RMI1, a component of the BTR complex, and KNO1 are important regulators of DNA damage responses (DDR). But how RMI1 and KNO1 are regulated during DDR is not well understood. In this manuscript, Chen et al. found that KNO1 is degraded through proteasome and KNO1 facilitates K63-linked ubiquitination of RMI1, which triggers the degradation of RMI1 through autophagy. Therefore, this study provided new mechanistic insights into DDR and revealed a link between autophagy and DDR in plants. Overall, this study is well designed and is of great interest to the plant DDR field.

We also like to thank this reviewer for his/her positive judgment of our work and the time and energy invested in the review process.

1. The author claimed that KNO1 directly interacts with RMI1, UBP12 and UBP13 using Y2H assays. However, in Y2H assays, the protein interaction could be indirect because the yeast proteins may mediate their interaction. To confirm the direct interaction, I suggested performing pull-down assay using the purified proteins.

We tried to express KNO1 and RMI1 in E. Coli. However, we couldn't obtain enough recombinant protein for in vitro experiments (perhaps due to protein instability and/or potential toxicity for bacteria). To still follow this reviewer's comment and strengthen our conclusions, we performed BiFC analyses in Arabidopsis protoplasts to check for very close proximity (which is usually interpreted as interaction of both proteins) in vivo. Indeed, these experiments also strongly suggest KNO1 and RMI1 interact directly. Please find the new results in Appendix Fig S2.
However, we still see Y2H as a binary interaction assay. If yeast proteins are involved, this would be a false positive result, which of course might happen. We think the scientific community is aware of possible false positives (as well as false negatives) in Y2H assays, so we don't elaborate on this. But we now also restrain from explicitly stressing that the interaction is direct.

2. One of the novelties of this study is that autophagy is involved in HR. The authors have shown that the atg mutants were hypersensitive to DNA-damaging agents. If the authors want to emphasize this, they need to show that the HR efficiencies in these atg mutants are

reduced. In addition, if RMI1 is degraded through autophagy, it is suggested to test whether RMI1 is colocalized with autophagosome (e.g.GFP-ATG8a).

We thank the author for appreciating our finding that autophagy in plants is involved in DNA damage response pathways. We also think that this finding opens up many new and important research questions. At this level, however, we only want to present the general sensitivity of plants that are mutant for central autophagy components, i.e., atg 2, atg5 and atg7. We found, as presented in the manuscript, that these mutants are sensitive to HU, which causes replicative stress, Zeocin, which induces DNA double strand break and cisplatin, which cross-links DNA. Thus, we believe that autophagy will be involved in many DNA damage response pathways, not only HR. We think that a detailed analysis of autophagy in DNA damage response needs further and in-depth analysis. Here, we really only want the general statement of the importance of autophagy in DNA damage response and have adjusted the text to this central and important message.

3. The authors found that both loss of KNO1 and overexpressing of KNO1 lead to CiPt hypersensitivity. How to explain these seemly contradicting results? If KNO1 functions by promoting the degradation of RMI1, can blocking RMI1 degradation or overexpressing RMI1 suppress the hypersensitivity of KNO1ox?

This is a good comment, and we believe that the underlying situation is complex. However, these finding fit very well to the situation found for RMI1. Please see also our response to reviewer 1 on a related point.

If we overexpress KNO1, we create a situation similar to an rmi1 mutant. However, if we would block RMI1 degradation or overexpress RMI1, we should phenocopy a kno1 mutant. And both situations result in hypersensitivity to CiPt, but due to different reasons, as explained below.

Without RMI, plants have a high level of HR leading to genome instability because of recombination between non-homologous (only locally homologous) sites, corresponding to the situation in patients with Bloom disease. If a DNA cross-linker is added, rmi1 mutants show even higher levels of genome instability presumably through the additional damage caused by these drugs. Now, in kno1 mutants, the RTR complex cannot be inactivated (due to the lack of RMI degradation as shown here) and thus, HR, which is needed for the repair of ICLs cannot be launched. As a consequence, kno1 mutants are hypersensitive to cisplatin and other DNA cross-linkers. Conversely, KNO1 overexpression causes the constant inactivation of the RTR complex leading to a high general level of genomic instability because of recombination between "wrong" sites (similar to rmi1 mutants). Thus, these two types of hypersensitivities have different origins. This becomes visible by our finding that rmi1 and KNO1 overexpressing plants but not kno1 mutant show already high levels of cell death (likely reflecting the high level of genomic instability) in the absence of cisplatin.

4. In several cases, the control samples were missing. For example, in Fig. 2C and 3B, the authors need to show the plants growing on MS medium.

Pictures of control samples were now added to each figure.

5. In Fig. 3D, I could not find the data for 35S:GFP-KNO1.

We apologize for this omission. Pictures of the 35S:GFP-KNO1 were added to figure 3D.

6. In Fig 5D, the level of GFP-KNO1 in the untreated plants was much lower than in the other samples. To better compare the ubiquitination levels, the levels of GFP-KNO1 should be adjusted to the similar level.

We thank this reviewer for this comment. In the presented experiment, we want to show the *in-vivo* level of protein amount before and after treatment, so we didn't adjust the protein levels to the untreated sample. The point we want to make is that, if we inhibit protein degradation, we get more KNO1 protein which is highly ubiquitinated. If we add CiPt only, we get a similar amount of KNO1 but which now is not/less ubiquitinated (because of UBP12/13 action).

7. In Fig 7C, RMI1-GFP in WT background should be used as a control instead of kno1.

We used *kno1* as a negative control to confirm that there is no endogenous ubiquitination signal. As we show in Fig 7A, the ubiquitination signal is increased after Cisplatin and E-64D treatment in WT background. However, in Fig 7C, i.e., in *kno1* background, we don't find a change in RMI1 ubiquitination level when treating with Cisplatin and E64-D (compared to non-treatment).

8. Page 15, Line 1, "Fig EV2D" should be "Figure EV2E".

Thanks for this comment, we have corrected the citation.

9. In Fig. EV6 (now Appendix Fig S4), the Zeocin concentration should be 2 µM, not 2 mM.

We have revised this.

10. The formats of the reference list were not consistent.

We have double checked the references and hope that everything has the same format now.

Reviewer #3:

In this manuscript by Chen et al., the authors investigated the molecular mechanisms regulating homologous recombination upon DNA damage in Arabidopsis plants. The authors analyzed physiological and biochemical properties of the new HR regulator KNO1 and revealed KNO1 induces K63-ubiquitin dependent autophagic degradation of RMI1, a component of Bloom, resulting in promotion of HR in response to DNA damage. Interestingly, KNO1 is also degraded by K48-ubiquitin dependent proteasomal pathway, which is suppressed by deubiquitination enzymes UBP12/13 under DNA damage. The work is overall well conducted and presented. The complementary approaches provide clear evidence for their conclusions. There are a few concerns regarding the manuscript that are detailed below.

We are very happy that the third reviewer is also very supportive of our work and we acknowledge his/her energy and time to help improving our manuscript.

1. The authors showed the dynamics of RMI1 protein level by microscopic analysis of RMI1-GFP under DNA stress in WT or kno1 mutant background independently. But, no data directly comparing the RMI1 protein amounts or stability between WT and kno1 mutant. Please confirm this point by standard immunoblot analysis.

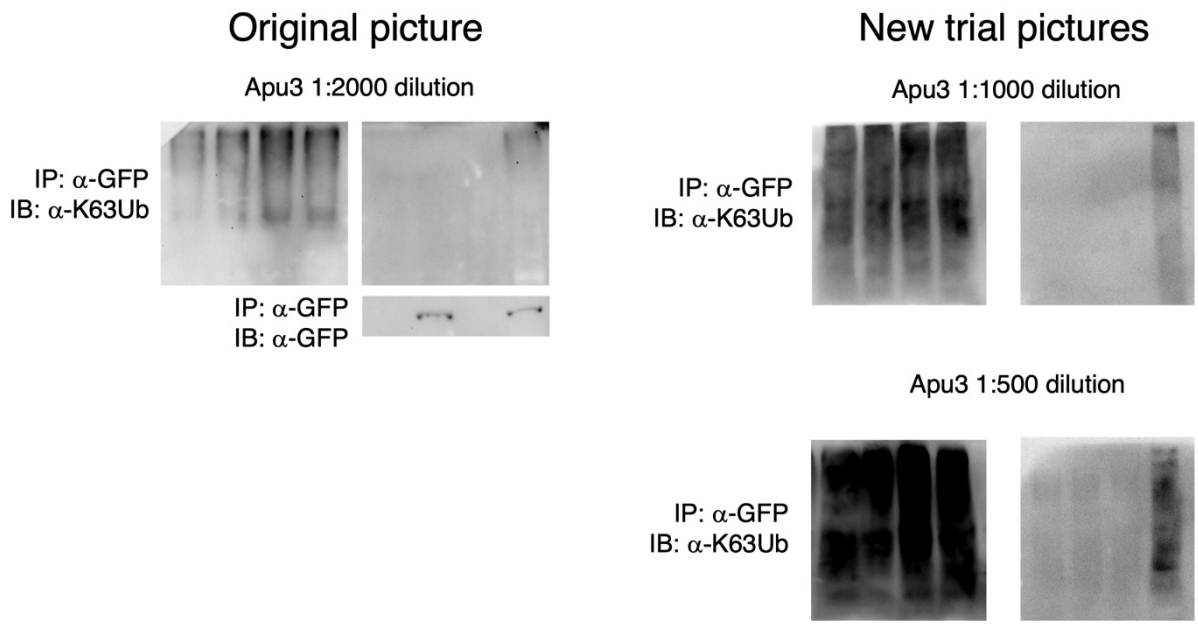
We have added an immunoblot analysis in the revised manuscript version. This result obtained matches our microscopic results. Please check Fig. EV3D.

2. Translocation of RMI1 from nuclear to cytoplasm was evaluated by microscopic analysis of RMI1-RFP (Fig. 6, Fig EV7). The reviewer is also interested in this regulation and basically agree the authors conclusion. But how did the authors distinguish the RMI1 localization and quantify the nuclear-cytoplasm ratio by only observing RMI1-RFP signal? Please describe detailed methods in the manuscript. Alternatively, it seems better to analyze the nuclear-cytoplasm ratio by biochemical approach (fractionation and immuno blotting).

The data presented in Fig6D are the frequency of cells with a cytoplasmic RMI1-RFP signal, not a ratio of protein amounts. We describe now more clearly what was done. We used this result to globally describe the increase of cytoplasmic accumulation under CiPt with the concomitant application of e64D. The obtained images fit very well to our hypothesis that RMI1 is degraded by autophagy in DNA stress conditions.
In addition, we now also performed cell fractionation assays and analyzed the protein amount by immune blotting in WT versus kno1 background. In these experiments, we also found a strong cytoplasmic (i.e. non-nuclear) accumulation of RMI1 in kno1 mutants when CiPt was applied consistent with a requirement of KNO1 for autophagic degradation of RMI1. Please find the results of the cell fractionation assay in Appendix Figure S5.

3. Through the manuscript, the ubiquitination signals of RMI1-GFP (IP product) was faint, especially Fig. 7 Apu3/K63 blot. Please consider the improvement of these data. It is recommended to add deubiquitination inhibitors into the extraction buffers for IP.

Since we don't know which DUBs act on RMI1 and since there is no general DUB inhibitor (at least to our knowledge), we would have to test many different deubiquitination inhibitors. Since many of these inhibitors have not been used with plants/plant extracts and since we are missing many positive controls, we were worried that establishing them would take a long time. To none-the-less, respond to the point raised by this reviewer, we have tried to refine the experiment by using higher concentration of the Apu3 antibody. However, this resulted in increased background staining as well as less sharp and more smearing bands than before (see image below). Therefore, we prefer to use the original results for the manuscript.



4. Page 10 line21-26, The authors mentioned the results using 35S:GFP-KNO1, but no data in Fig. 3D, instead, rmi1 data shown in the Figure.

This was left out by mistake. Pictures of the 35S:GFP-KNO1 were added now to figure 3D. Please find this in the revised manuscript.

5. Although the authors performed TAP-MS analysis and identified putative KNO1 interactors, the reviewer couldn't find the materials and method information about this analysis (plasmid preparation, growth condition, TAP and MS analysis).

We added detailed TAP methods to the manuscript.

Also please add the material information about KNO1 OX plant.

We added the missing information to the plasmid construction section of material and methods.

6. Fig. EV1A. The photo image and graph for CiPt treatment look very similar to that used in previous publication by Bouyer et al (Bouyer et al, 2018, PLoS Genetics). If same, please remove the CiPt data from the figure and only show MMC data in this manuscript.

We apologize for this mistake. These figures were stored in the same folder. We use now a picture from a different experiment in the revised manuscript.

Mainor concerns

1. Page 9 line 5-10 (Fig. EV2C). It's difficult to understand DUF1767 domain site and structure of the deletion construct used in Y2H. Please add schematic structure image in the figure or detailed explanation in legends.

We added the DUF1767 domain to (now) Figure EV2A and describe exactly which amino acids are deleted in each deletion construct in the figure legend.

2. Page 9 line 11-16. Please insert the corresponding Fig number about Y2H results.

We now reference the correct figure number in the revised manuscript.

3. Page 6 "immunno-detection of vH2aX (Fig. EV2B)". Not shown in EV2B but in EV1B.

Thanks for this comment. We have corrected this.

4. Figure 5. Mislabeled, maybe 5B should be 5C, vice versa.

We have double checked the labelling of the figures and corrected them where necessary.

5. Page 16 line 14 "E64d (Fig. 6A)" should be Fig. 7A. Line 21 "Apu3 antibodies (Fig. 4B and 7C)" should be Fig. 4C and 7C.

We now cite the correct figure number in revised manuscript.

6. Typo: Page 3 line 18, delete "no"; Page 10 line 17, 19, 35:GFP-KNO1 (35S:GFP-KNO1).

Thanks for this comment, we have corrected these issues in new manuscript.

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that in light of the positive re-review by two of the original referees (copied below), we have now accepted it for publication in The EMBO Journal!

Referee #2:

The manuscript was improved through revision. My concerns were addressed and I have no other comments.

Referee #3:

The authors have adequately addressed all of my concerns.

EMBO Press Author Checklist

Corresponding Author Name: Arp Schnittger and Maren Heese
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2022-111980

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Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

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The data shown in figures should satisfy the following conditions:
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- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
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Each figure caption should contain the following information, for each panel where they are relevant:
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Please complete ALL of the questions below.
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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	All primer sequences used in this study are listed in Appendix Table S1.

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