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A YTHDF-PABP interaction is required for m6A-mediated organogenesis in plants

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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. 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.)

Dear Peter,

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports as well as referee cross-comments that are all pasted below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, referee 2 also suggests more experiments to strengthen the study, and these might take some time. Given the competing, already published paper, we can only offer to publish your ms if it can be accepted at the very latest in early October, so that it can be published this year. This means that we will need to receive the revised manuscript latest by mid-September.

As you will see, the referees do not agree on whether and how the interaction between ECT2 and PAB should be further confirmed and strengthened. I think some more evidence should be provided to confirm this interaction. If you like, we can also discuss this further, also in a video chat, if you like.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

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3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

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5) a complete author checklist, which you can download from our author guidelines . Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a

reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,

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- the nature of the bars and error bars (s.d., s.e.m.),

- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised form of your manuscript when it is ready.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

Tankmar et al. MS# EMBOR-2023-57741V1
"A YTHDF-PABP axis is required for m6A-mediated organogenesis in plants"

N6-Methyl-Adenosine (m6A) has recently emerged in eukaryotes as a prevalent mRNA modification that controls biological processes through its influence on distinct mRNA properties, such as mRNA stability, mRNA translability, and mRNA processing. m6A functions are usually mediated by members of the YT521-B homology (YTH)-domain protein family, that are well established m6A readers. 13 YTH-type genes (ECT1-12 and CPSF30L) are present in the genome of Arabidopsis, and so far, only 4 of these genes (ECT2,3,4 and CPSF30L) have been characterized. In particular, works from the Brodersen's team previously implicated the m6A modification and the ECT2,3,4 readers in the control of cell proliferation during plant development. This manuscript is a follow-up of these studies, in which Tankmar et al. are applying a large set of approaches and genetic tools to dissect the mode of action of ECT2 in vivo.
In this regard, Tankmar et al. provide evidence that the N-terminal IDRs (intrinsically disordered regions) of ECT2 are essential for the activity of the protein in planta. The authors also demonstrate that two discrete regions within the N-terminal IDR are required for the function of ECT2 and that one of these regions binds the poly(A)-binding protein (PABP). This suggests the existence of a ECT2-PABP functional partnership that could contribute to the reported stabilizing role of m6A on the ECT2,3,4 mRNA targets. The functional significance of this interaction is further supported by the use of an elegant genetic experiment, showing that the overexpression of PABP can compensate for the loss of ECT proteins in vivo, leading the authors to propose a model of action of ECT proteins in Arabidopsis.
The paper is well-written and noteworthy for the technical approach and it addresses problems that are important to the field of plant epitranscriptomics. Although the conclusions drawn in the current study partially overlap with those offered by the Jia's lab in a recent paper (Song et al. 2023), the current work deepens our understanding of the ECT-PABP partnership both at biochemical and functional levels, providing an alternative interpretation for the role of this interaction in vivo. Based on these observations, I think that this is a timely paper whose publication in EMBO Reports would benefit to the field of plant epitranscriptomics.

Minor points
1) PABPs are known to interact with protein partners that contain PAM2-type motifs with a conserved LNP signature. It is striking to notice from the sequence that the small Tyr region identified in ECT2 contains two NP motifs, suggesting the presence of degenerated PAM2-type motifs in this region. Are these motifs also conserved in the IDR region of other ECT2 orthologs?

2) If the proposed model that PAB proteins are sub-limiting in rapidly dividing primordial cells is true, I would expect the ect2-pab2 or ect2-pab3 double mutants to exhibit a te234-type phenotype resulting from synthetic enhancement. Is this correct?

Referee #2:

This paper investigates the molecular mechanism underling the function of N6-methyladenosine readers (ECT2/ECT3/ECT4) in Arabidopsis leaf primordia development. The study can be divided in two parts. In the first part, the authors carried out functional complementation tests with ECT2 deletion constructs to pinpoint the regions outside the m6A binding domain required for the protein function in leaves. In the second part of the study, the authors use IP combined with MS to identify ECT2 associated proteins. The top candidates were the polyA-binding proteins PAB2/4/8 and the study further claims that this interaction is mediated by the N3.2 region. This suggests that ECT2-PAB binding is required for the leaf primordia development. To gain support for the functional relevance of this interaction, the authors showed that overexpression of PAB4 (partially?) suppresses the te234 phenotype.

There are two major problems with this study. First, the interaction between ECT2 and PABs is not sufficiently explored and the authors should gain more supporting evidence. Namely, the authors should validate the MS data with IP/western or yeast two hybrid assay to more thoroughly asses the effect of the N3.2 region on the interaction. Taking in consideration that the fine mapping of the ECT2 N-terminal region in terms of PAB interaction and functionality represents the main novel aspect in comparison with the paper recently published by Song et al., the data should be solid and comprehensive. The authors should also include in the analysis the N8 region that seems acts synergistically with N3, indicating its involvement in PAB interaction as well. The paper seems incomplete without the data. Second, the authors rather strongly refute the hypothesis proposed by Song et al that ECTs together with PABs stabilize target transcripts. Instead, they offer an alternative scenario proposing that concentration of PABs is limited in rapidly dividing cells and ECTs serve to recruit PABs to certain transcripts. I must say the study by Song et al is very comprehensive and provides a number of supporting evidence for their model, including the RNA stability data. Model suggested in this paper appears to much less substantiated by presented evidence and more speculative. In fact, the observation that PAB overexpression suppresses ECT-deficient phenotype supports the "RNA stabilization" hypothesis presented by Song, because Gallie et al (2017) show that PUBs are required for RNA stability.

Additional comments
• Figures 1C,F - indicate color code used in charts for different categories of phenotypes
• The authors claim that the N3.2 region is not required for the interaction with RNA. However, the supporting experimental

evidence is very weak (Figure 3A). The claim is based on a single IP reaction, there is no quantification of the signal, and there is no control for specificity of binding - e.g. IP reaction from a sample expressing mCherry. Furthermore, mCherry signals in the input and IP fractions see to differ, so it is not clear how the samples compare in terms of loading
• The rescue of the leaf growth phenotype in PAB4 overexpression lines appears to be only partial (Figure 5B). The authors should use the same quantitative approach as presented in Figure 1C,F to demonstrate the extent of the rescue.
• Were the MS analysis performed in ect2/3/4 mutant background complemented with a mChrerry tagged construct? I assume so, but it should be clearly stated in the result section.
• Do double mutants of PABPs or heterozygous triple mutant generated elsewhere show the leaf primordia development phenotype?
• Fig 1H, W464A should be described in the figure legend.
• Correct enzyme/chemical names such as RNase, DNase, MgCl2 instead of Rnase, Dnase, MgCl2. Authors should thoroughly check for such basic mistakes in the material method section.
• In the Abstract, the authors say that IDR of YTH domain protein may mediate phase separation. Is it already known whether this is indeed the case? For example, in cell localization data by Song et al do not show that ECTs form biomolecular condensates.
• The authors extensive cite work from their own lab, but seem to omit some important articles on ECTs from other labs (e.g. DOI: 10.1105/tpc.17.00934).

Referee #3:

The manuscript submitted for review is interesting. Research on the identification of ECTs as m6A readers in plants has been conducted by several research groups including the authors' group for some time. However, the interactors of these reader proteins and the molecular mechanisms behind such RNA modification recognition functions remain unclear. In this study, Tankmar et al demonstrated two short IDR-elements are important for Arabidopsis organogenesis. In addition, authors identified PAB2/4/8 especially interacted with a short IDR element of ECT2. The experiments are well-thought-out and logical and include the necessary controls. Although some results have been reported by a recently published paper (PMID: 37122016), I think this work should still be published after minor corrections.

Minor comments:
1. Some sentences need to be improved: e.g. the N-terminal IDR of ECT2, as well as its N-terminal and C-terminal halves individually, are essential for function in vivo. N-terminal IDR obviously includes two halves.

2. In the study, the authors revealed the important function role of N3.2 and N8 fractions from ECT2 in Arabidopsis. It will interesting to examine the amino acids of these regions from different ECT proteins and/or ECTs from different organisms.

3. Employing the IP-MS, the authors identified that three PAMPs proteins interact with the N3.2 region of ECT2. Please provide more direct experimental evidence to show such interaction.

Cross-comments from referee 1:

Concerning Rev2 P1: The interaction between ECT and PABP has been validated by several quantitative proteomics and I believe that these data are highly convincing. Doing co-IP, as suggested by Rev2, could be informative and should not take a long time (less than a month). In contrast, I am not a big fan of yeast two hybrid assay, since these experiments are prone to artifact and will require more time to be set up. Concerning the N8 region, doing a sequence analysis as suggested by rev3 could be a good idea. I will not go for more experiments on this particular region.

Concerning Rev2 P2: The authors should easily answer to this point by toning-down their discussion.

regarding the Rev2 P1, are you aware of the attached correspondence recently published in Nature Plants concerning the incongruity of using Co-IP to validate quantitative proteomics?
https://doi.org/10.1038/s41477-022-01314-8

Cross-comments from referee 3:

I totally agree with Referee 2's concerns. This manuscript seems not incomplete. Especially, the first concern from Referee 2, which I also raised, was that authors should provide more evidence supporting the interaction between ECT2 and PABs, e.g. Y2B, Co-IP, and Pulldown...

Response to Reviewers

We thank all three reviewers for evaluating our manuscript in such a timely fashion. Overall, we take from the reviews that the importance and novelty of our work are appreciated. Criticisms tend to touch on different aspects with only limited convergence on common points. In our view, the most important such points of convergence are

(1) Requests for more experimentation on the ECT2-PABP interaction (reviewers 2/3)
(2) Request for better analysis of conserved amino acids within the region of the ECT2 IDR required for PABP association (reviewers 1/3).

We have carefully considered these points. We think that the results of our efforts to address them have substantially improved the paper: it is better and more interesting in its present form, and we wish to thank all reviewers for that.

Please note that there are also cases in which we disagree with reviewers. In these cases, we explain our reasons to do so in detail in this letter. We hope that the reviewers will find our explanations fair, if at times stated rather firmly, and that they will take no offense at our clear way of stating our disagreements. Disagreements, argumentation and discussion are, after all, a necessary part of scientific advance, and can be rather joyful as long as we maintain basic respect for one another. And we do: there is no doubt in our minds that the evaluation of our work by all reviewers has helped improve the paper which is, everything considered, the most important point.

Our revised version contains the following improvements, some of which were not directly asked for.

(1) We include two variants of *in vitro* binding assays. In one, we use heterologously expressed GST and GST-PAB8 as an affinity matrix and show that it efficiently retains ECT2-mCherry from total plant lysates. ECT2-mCherry containing the small N3.2.2 deletion is retained much less efficiently from total lysates. In the other, we use an *in vitro* binding assay with purified components: heterologously expressed GST/GST-PAB8 and a fragment of the ECT2 IDR. The results show clear, specific binding of the ECT2 fragment to GST-PAB8 at a concentration of 10µM, but less binding at 2.5 µM, indicating that the interaction is of low affinity.
(2) We include results of a bacterial two-hybrid assay with PAB8 and the N3.2 fragment of the ECT2 IDR. The results show direct interaction between the two in bacterial cells, and, therefore, indicate that the N3.2 fragment is both necessary and sufficient for PABP interaction. Together, (1) and (2) satisfy the various reviewer requests for more analyses on the ECT2-PABP interaction, including an assessment of whether it is direct.
(3) We show results of extensive sequence analysis of the N3.2 region. Interestingly, it does contain a novel, conserved motif with high information content across sequences from many plant species. Gratifyingly, this motif is situated in the small region that we identified as functionally important, and the Tyr residues that we mutated stand out as the ones with highest information content. Quite remarkably, a similar motif is also found in metazoan YTHDF proteins, a result that we discuss in the light of reported associations between human YTHDF3 and PABC1.

(4) We include characterization of a chromosomal in-frame mutant of *ECT2* corresponding to the region encoding the 37-amino acid N3.2 region necessary for physical association with PAB2/4/8 *in vivo*. The in-frame deletion allele, named *ect2-4*, was induced by CRISPR-Cas9 engineering in the *ect3 ect4* background, and we present analyses of three families homozygous for *ect2-4* after loss of the Cas9 transgene and backcrossing to the *ect3 ect4* background. The results show that the *ect2-4* mutation is a partial loss-of-function mutation, consistent with the lower frequency of complementation observed in the transgenic approach that identified the N3/N3.2 region. They also show that the protein encoded by the *ect2-4* allele accumulates to levels comparable to wild type ECT2. We view these additional results as a stamp of quality that fully corroborates the conclusion that the N3.2 region of the IDR of ECT2 is important for leaf formation *in vivo*.
(5) We include additional control experiments to show that while overexpression of PAB4-Venus in leaf primordia accelerates leaf formation in *ect2 ect3 ect4* mutants as reported in the original version, it does not do so in a genetic background with ECT2/ECT3/ECT4 expression and normal leaf formation. Thus, overexpression of PAB4 does not merely accelerate leaf formation in any genetic background, supporting the significance of the dosage-dependent suppression of *ect2 ect3 ect4* mutant phenotypes that we report.
(6) We have constructed *ect2 pab2* mutants and include a brief mention of their phenotypes which are not detectably different from wild type.
(7) We have rewritten parts of the discussion to further clarify how our study differs from the one by Song et al. (2023). This also includes an account on what the two studies allow one to conclude on the *in vivo* importance of ECT-PABP association and what it might do molecularly.

Below, we provide a point-by-point answer (in blue) to the points raised by each reviewer (reproduced here in black).

Referee #1:

Tankmar et al. MS# EMBOR-2023-57741V1 "A YTHDF-PABP axis is required for m6A-mediated organogenesis in plants"N6-Methyl-Adenosine

(m6A) has recently emerged in eukaryotes as a prevalent mRNA modification that controls biological processes through its influence on distinct mRNA properties, such as mRNA stability, mRNA translability, and mRNA processing. m6A functions are usually mediated by members of the YT521-B homology (YTH)-domain protein family, that are well established m6A readers. 13 YTH-type genes (ECT1-12 and CPSF30L) are present in the genome of Arabidopsis, and so far, only 4 of these genes (ECT2,3,4 and CPSF30L) have been characterized. In particular, works from the Brodersen's team previously implicated the m6A modification and the ECT2,3,4 readers in the control of cell proliferation during plant development. This manuscript is a follow-up of these studies, in which Tankmar et al. are applying a large set of approaches and genetic tools to dissect the mode of action of ECT2 in vivo. In this regard, Tankmar et al. provide evidence that the N-terminal IDRs (intrinsically disordered regions) of ECT2 are essential for the activity of the protein in planta. The authors also demonstrate that

two discrete regions within the N-terminal IDR are required for the function of ECT2 and that one of these regions binds the poly(A)-binding protein (PABP). This suggests the existence of a ECT2-PABP functional partnership that could contribute to the reported stabilizing role of m6A on the ECT2,3,4 mRNA targets. The functional significance of this interaction is further supported by the use of an elegant genetic experiment, showing that the overexpression of PABP can compensate for the loss of ECT proteins in vivo, leading the authors to propose a model of action of ECT proteins in Arabidopsis. The paper is well-written and noteworthy for the technical approach and it addresses problems that are important to the field of plant epitranscriptomics. Although the conclusions drawn in the current study partially overlap with those offered by the Jia's lab in a recent paper (Song et al. 2023), the current work deepens our understanding of the ECT-PABP partnership both at biochemical and functional levels, providing an alternative interpretation for the role of this interaction in vivo. Based on these observations, I think that this is a timely paper whose publication in EMBO Reports would benefit to the field of plant epitranscriptomics.

We thank the reviewer for the accurate summary of our findings and for making the point that our study goes deeper in the analysis of the functional relevance of the ECT2-PABP interaction than the study by (Song *et al*, 2023); an important, and, in our view, indisputable point.

Minor points

1) PABPs are known to interact with protein partners that contain PAM2-type motifs with a conserved LNP signature. It is striking to notice from the sequence that the small Tyr region identified in ECT2 contains two NP motifs, suggesting the presence of degenerated PAM2-type motifs in this region. Are these motifs also conserved in the IDR region of other ECT2 orthologs?

We have checked the sequence we identified as necessary for PABP interaction for similarity to the two known PAM1 and PAM2 motifs originally identified by the Sonenberg lab (Roy *et al*, 2002), and did not notice any similarity originally. The refined consensus motif of PAM2 is L/P/F—X—P/V—X—A—X—X—F/W—X—P, but it is true that X in many cases is N and that ECT2 has two NP dipeptides in the region, we identify as important. We have now derived a consensus sequence by alignment of IDRs of plant YTHDF proteins. This approach actually does yield a motif with high information content, but it does not look like PAM2; rather, what it underscores as important is around 6 closely spaced tyrosine residues as well as a few other residues that do not conform to PAM2. Remarkably, vertebrate YTHDF proteins, for which there is also evidence of PABP interaction, contain a very similar motif. We have now included this information in Figure 4J and Figures EV3B and EV4. We think this is useful information and thank the reviewer for pushing us to do more analyses to derive a motif.

2) If the proposed model that PAB proteins are sub-limiting in rapidly dividing primordial cells is true, I would expect the ect2-pab2 or ect2-pab3 double mutants to exhibit a te234-type phenotype resulting from synthetic enhancement. Is this correct?

It is definitely a possibility, but it is difficult to know exactly by how much the concentrations *in vivo* must be changed to see an effect as it depends on equilibrium constants, steady state concentrations and perhaps even rate constants that we do

not know. Since the first submission, we isolated *ect2 pab2* double mutants and analysed their phenotypes. The result is that the double mutant, just like both single mutants, has no appreciable developmental phenotype. We have included this negative result in a new supplementary figure. Given the known compensatory effects of other ECTs (in particular ECT3) and PABs (PAB4/8), and the unknown thermodynamic and kinetic parameters discussed above, this negative result does not disprove the idea of the importance of ECT-PAB interactions to overcome limiting PAB concentrations for a subset of poorly competing mRNAs.

Referee #2:
This paper investigates the molecular mechanism underling the function of N6-methyladenosine readers (ECT2/ECT3/ECT4) in Arabidopsis leaf primordia development. The study can be divided in two parts. In the first part, the authors carried out functional complementation tests with ECT2 deletion constructs to pinpoint the regions outside the m6A binding domain required for the protein function in leaves. In the second part of the study, the authors use IP combined with MS to identify ECT2 associated proteins. The top candidates were the polyA-binding proteins PAB2/4/8 and the study further claims that this interaction is mediated by the N3.2 region. This suggests that ECT2-PAB binding is required for the leaf primordia development. To gain support for the functional relevance of this interaction, the authors showed that overexpression of PAB4 (partially?) suppresses the te234 phenotype.

We do not understand why the reviewer feels the need to add a question mark here. We certainly do not think it can be because the manuscript is not clear enough.

The abstract reads:
"Remarkably, overexpression of PAB4 in leaf primordia partially rescues the delayed leaf formation in ect2 ect3 ect4 mutants,…"

The title of figure 5 that shows the relevant data is:
"*Figure 5. Overexpression of PAB4 partially suppresses defective leaf formation caused by loss of ECT2/3/4 function*

The legend to Figure 5 reads:
"B Partial suppression of the delay in leaf formation (top) and defective leaf morphology (bottom) of te234 plants in two independent transgenic lines (L1, L2) overexpressing PABVenus in actively dividing cells."

The description of the results reads:
"…and partial suppression of the defects in the size and shape of the leaves could be appreciated at later stages (Fig 5B)."

The discussion of the results reads:
"…and explain the partial phenotypic rescue by PAB4 overexpression"

There are two major problems with this study. First, the interaction between ECT2 and PABs is not sufficiently explored and the authors should gain more supporting evidence. Namely, the authors should validate the MS data with IP/western or yeast two hybrid assay to more thoroughly asses the effect of the N3.2 region on the interaction. Taking in consideration that the fine mapping of the ECT2 N-terminal

region in terms of PAB interaction and functionality represents the main novel aspect in comparison with the paper recently published by Song et al., the data should be solid and comprehensive.

There is a number of problems with this reviewer criticism.

(1) IP-western is no “validation” for quantitative IP-MS data. It is, in essence, the same basic experiment (immuno-affinity purification of ECT2 from total lysates), but instead of applying advanced technology to directly identify and quantify many proteins present in the immuno-affinity purified fraction, it uses an antibody to get a semi-quantitative readout of one specific protein in such fractions. We thank Reviewer 1 for engaging actively here to clarify that there is no good scientific basis to ask for “validation” by IP-western given the exhaustive series of independent IP-MS experiments carried out here.

(2) The identification of the region of ECT2 necessary for association with PAB2/4/8 is certainly novel compared to the paper by (Song *et al.*, 2023), but it is by no means the only important advance compared to that paper. What is crucial to understand is that our paper provides evidence for the functional importance of the ECT2-PAB interaction. It manages to do so because it defines a small region necessary for function, and establishes that loss of ECT2 function correlates with loss of PAB interaction. We also show that increased dosage of PAB4 is sufficient to partially suppress the *te234* phenotype. The paper by (Song *et al.*, 2023) provides no such evidence. Hence, based on the findings shown by (Song *et al.*, 2023), it is not clear that the ECT2-PAB interaction is even functionally relevant, and the novelty of our paper is not limited to pinpointing the region of the IDR necessary for PAB interaction. It is the use of that knowledge that makes the big difference.

Despite our fundamental disagreement with the reviewer on the basis for the criticism, we see the relevance of further exploration of the ECT2-PABP interaction. We chose PAB8 for such experiments, and now include a battery of biochemical and genetic results on the interaction (Figure 4G-I, Figure EV3A). Together, they establish that the interaction is direct and involves the N3.2 region, as summarized in the beginning of this letter. We hope that this additional evidence satisfies the requirement for more data on the ECT2-PABP interaction.

The authors should also include in the analysis the N8 region that seems acts synergistically with N3, indicating its involvement in PAB interaction as well. The paper seems incomplete without the data.

The summary provided by the reviewer is not accurate here. As a consequence, the criticism is not justified.

(1) We do not only include N3 and N8 in our analysis. Indeed, they are called N3 and N8, because they represent the regions deleted in mutants 3 and 8, not counting the full deletions of N- and C-terminal IDRs (i.e. 10 initial deletions reported in total in this manuscript). These 10 deletions represent a mini-screen for regions of importance for the effect on leaf formation of ECT2. We therefore report the results obtained with all of the deletions initially designed, as we should. It turns out that five of the ten deletions have effects (N, N1, N2, N3, N8), of which two are particularly informative, because they represent relatively small regions of the IDR (N3 and N8). It is normal and correct practice to report

all of the initial results of a screen, and then focus on one of the hits for a deeper understanding subsequently. We cannot see that there is anything "incomplete" in that practice.

(2) The effects of deleting N3 and N8 **are additive, not synergistic,** as shown in what is now **Figure EV1.** The most straightforward interpretation of the additive nature is that the elements act independently of each other, further supporting that it is correct to focus on only one of them at this point, and report the properties of the other subsequently. It may well be that further molecular analyses might reveal some level of convergence in the action of the two elements, but this is a follow-up question that can be answered in a subsequent report. At this point, we find it imperative to report as accurately as possible the effect of deleting N3 on ECT2 function *in vivo* (reduced ability to promote leaf formation), and the effect of deleting it on its molecular properties (reduced association with PABPs). The paper achieves these objectives very well, and there is no need to further explore N8 for the purposes of this story.

In conclusion, we find this reviewer criticism to be unjustified. Inclusion of an analysis of N8 would make the paper unfocused and too long – indeed, we can confidently say that a better understanding of N8 is material for an entire manuscript. Consequently, we have not included further information on N8 in the present manuscript.

Second, the authors rather strongly refute the hypothesis proposed by Song et al that ECTs together with PABs stabilize target transcripts.

We do not really refute the "hypothesis" proposed by (Song *et al.*, 2023) (actually their main conclusion highlighted in the title). That would require clear evidence that it is wrong, and we do not have such evidence. We just note that (Song *et al.*, 2023) do not provide any evidence to support their conclusion. The argument for their conclusion that ECTs act to stabilize mRNA targets via PABP interaction takes the following form:

(1) They demonstrate that ECT2 associates with many proteins, among them PABPs. They also provide evidence that the interaction is likely to be direct.
(2) They then show that a number of mRNA targets of ECT2 turn over more rapidly in *ect2/ect3/ect4* triple knockout mutants than in wild type.
(3) They conclude that the ECT2-PABP interaction stabilizes mRNA targets.

But how can anyone be convinced by this argument? First of all, it is well-known that stress responses are activated in the *ect2 ect3 ect4* triple knockout mutant (Arribas-Hernández *et al*, 2021b), making it hard to link molecular defects in that mutant directly to ECT function. They may be a direct consequence of loss of ECT function, but they may also be an indirect effect of activation of stress responses, as discussed by (Arribas-Hernández *et al.*, 2021b). Second, why is it not the interaction with any of the other proteins found to associate with ECT2 that explains the mRNA stabilization? The answer is that Song et al. have no solid and objective basis to single out PABP among their many ECT2 interactors. It is simple guesswork, based on reported roles of cytoplasmic PABP from other systems. Clearly, what is needed is a mutant of ECT2 that specifically disrupts ECT2-PABP interaction to argue that this interaction is important for anything. Our work provides exactly this information, and goes even

further by showing that increased dosage of PAB4 in the ECT expression domain is sufficient to alleviate growth phenotypes partially.

Instead, they offer an alternative scenario proposing that concentration of PABs is limited in rapidly dividing cells and ECTs serve to recruit PABs to certain transcripts. I must say the study by Song et al is very comprehensive and provides a number of supporting evidence for their model, including the RNA stability data. Model suggested in this paper appears to much less substantiated by presented evidence and more speculative.

We disagree with the reviewer on this point, as explained above. In addition, we actually suggest more than one possible scenario to explain the importance of the ECT2-PABP association. We have now made it even clearer in the discussion that our data does not provide stronger support for one model over the other. We also clarify that in contrast to (Song *et al.*, 2023), we actually provide evidence that the ECT2-PABP association is functionally important, even if we cannot, at this point, say for sure exactly what it does.

In fact, the observation that PAB overexpression suppresses ECT-deficient phenotype supports the "RNA stabilization" hypothesis presented by Song, because Gallie et al (2017) show that PUBs are required for RNA stability.

We agree very much that the observation that PAB4 overexpression in the ECT expression domain does not rule out the model proposed by (Song *et al.*, 2023) and have now explicitly stated so in the manuscript. But nor does it rule out other models that could explain how the ECT-PABP association may be important. Indeed, the key importance of that experiment is not to explain exactly what the ECT-PABP interaction does, but rather to corroborate the conclusion that it is important for ECT function *in vivo*. We have improved the discussion so that this point is now made more clearly and forcefully.

Additional comments

• Figures 1C,F - indicate color code used in charts for different categories of phenotypes

The color codes were in fact given in panel A as frames around the photos corresponding to the different phenotypes. But we agree with the reviewer that this was too subtle a way to show the color coding, and have now improved it so that it is immediately clear what the shades of grey/black mean.

• The authors claim that the N3.2 region is not required for the interaction with RNA. However, the supporting experimental evidence is very weak (Figure 3A). The claim is based on a single IP reaction, there is no quantification of the signal, and there is no control for specificity of binding - e.g. IP reaction from a sample expressing mCherry. Furthermore, mCherry signals in the input and IP fractions see to differ, so it is not clear how the samples compare in terms of loading

We disagree, and note that no other reviewer has raised any criticism on this point. In fact, as far as we can tell, the points raised here are either inaccurate or irrelevant. To break it down:

(1) The figure shows one crosslinking-IP reaction from transgenic lines expressing the following ECT2-mCherry versions:
   (i) $ECT2^{WT}$-mCherry: this is our positive control that we know bind easily detectable quantities of RNA (Arribas-Hernández *et al*, 2021a).
   (ii) $ECT2^{\Delta N3}$-mCherry: this is the sample to be tested.
   (iii) $ECT2^{W464A}$-mCherry: this is the negative control defective in m^6A recognition that associates poorly with RNA.

   Thus, the experiment includes appropriate positive and negative controls. The statement "*there is no control for specificity of binding - e.g. IP reaction from a sample expressing mCherry*" should, consequently, be disregarded.

(2) It is unclear what the reviewer means by the statement "*Furthermore, mCherry signals in the input and IP fractions see to differ, so it is not clear how the samples compare in terms of loading*". The mCherry signals in the three lines in the input are nearly perfectly matched, while a tiny bit more protein has been immunopurified from $ECT2^{W464A}$-mCherry than for the two other lines that are nearly perfectly matched. Perhaps the poor RNA-association of $ECT2^{W464A}$-mCherry makes it easier to capture by immuno-affinity purification? Regardless of the reason, this difference has no bearing on the conclusion.

(3) As for all other results we report, we have repeated the experiment several times. We now provide additional repeats in supplementary information that give exactly the same conclusion, even if the technical quality of them is not quite as good as the one shown in Figure 3A.

(4) The statement "*there is no quantification of the signal*" is true. But what is the number needed for here? Does the reviewer seriously doubt that the ^{32}P-signal obtained by PNK labeling is at least as intense in the $ECT2^{\Delta N3}$-mCherry lane as in the $ECT2^{WT}$-mCherry lane? We explain in the text why it does not make sense to interpret fine differences (for which quantification would be needed) in this experiment. The important observation is that $ECT2^{\Delta N3}$-mCherry behaves roughly like $ECT2^{WT}$-mCherry with regard to gross RNA association, and very differently from the negative control with clearly reduced RNA association. Figure 3A makes that point very clearly, and we have not made any changes as a consequence. We have, however, added an extra supplemental figure with independent repeats of this experiment.

• The rescue of the leaf growth phenotype in PAB4 overexpression lines appears to be only partial (Figure 5B). The authors should use the same quantitative approach as presented in Figure 1C,F to demonstrate the extent of the rescue.

It is indeed partial as we describe clearly at multiple points in the manuscript (please see above).

It does not make sense to report on complementation frequencies for this experiment inasmuch as it is not a complementation. It is a test for whether or not PAB4 overexpression in the ECT2 expression domain leads to phenotypic rescue of *ect2 ect3 ect4* mutants. For that reason, one has first to isolate lines that actually do overexpress PAB4 in the appropriate pattern, then characterize those lines phenotypically, including by quantitative measurements of leaf size, as we report.

• Were the MS analysis performed in ect2/3/4 mutant background complemented with

a mChrerry tagged construct? I assume so, but it should be clearly stated in the result section.

Yes, but we believe that this information is clearly conveyed. The reviewer can, for example, consult Figure panels 3B-D, 3F-H, 4F. In each case, affinity tags used are clearly indicated, as is the exact nature of the tagged proteins used for comparative IP-MS in each case.

• Do double mutants of PABPs or heterozygous triple mutant generated elsewhere show the leaf primordia development phenotype?

That is a relevant question. We could not obtain germinating seed batches from any of the labs that has published on *pab* double mutants, and our own *pab* double mutants are not ready for analysis at this point. We would have liked to include the results of the analysis, but are not in a position to do so. At the same time, we note that there are many, many such PAB/ECT dosage tests that one could think of. The one we report on – rescue of *te234* phenotypes by increased PAB4 dosage – is more informative on the existence of functional links between them than phenotypic analysis of *pab* mutants, even if the latter is not irrelevant.

• Fig 1H, W464A should be described in the figure legend.
Now added, thank you.

• Correct enzyme/chemical names such as RNase, DNase, MgCl2 instead of Rnase, Dnase, MgCl2. Authors should thoroughly check for such basic mistakes in the material method section.
Thank you, we have done our best to correct such errors.

• In the Abstract, the authors say that IDR of YTH domain protein may mediate phase separation. Is it already known whether this is indeed the case? For example, in cell localization data by Song et al do not show that ECTs form biomolecular condensates.

It is true that (Song *et al.*, 2023) do not show this. Phase separation of ECT2 *in vitro* and *in vivo* in response to osmotic stress was shown by (Arribas-Hernández *et al*, 2018), and cytoplasmic ECT2 bodies induced by heat stress were identified as stress granules by (Scutenaire *et al*, 2018). These findings are properly referenced in the Introduction: "*At the extreme, such a model would be compatible with ECT proteins relying nearly exclusively on their YTH domains for in vivo function, with the IDRs perhaps mainly there to drive sequestration into distinct biophysical phases in response to stress, as shown previously to be the case (Arribas-Hernández et al., 2018; Scutenaire et al, 2018).*"

• The authors extensive cite work from their own lab, but seem to omit some important articles on ECTs from other labs (e.g. DOI: 10.1105/tpc.17.00934).
We actually do cite the paper mentioned in connection with Figure EV5. We cite papers from our own lab on ECTs, because they are important for the current knowledge on ECTs. Papers from other labs are also cited where relevant. For example, the findings of (Scutenaire *et al.*, 2018) on stress granule localization are cited, as is the evidence for ECT2-PAB interaction by (Song *et al.*, 2023). We do not know, however, for what information we should cite the paper mentioned in the main text, simply because most

of its conclusions are now proven not to be accurate. The trichome phenotype of *ect2* mutants is probably correctly described in it, but

- the molecular basis proposed is almost certainly wrong, given that the targets proposed to underlie it are not, as a matter of fact, targets of ECT2,
- the ECT2 “binding site” (URUAY) identified in the study is not even a methylated site *in vivo*,
- the subcellular localization is incorrectly described, even by the authors’ own account in a subsequent publication, and as shown by us previously
- the proposed model for ECT2 function as a regulator of alternative polyadenylation is inconsistent with evidence, again by the authors’ own account in a subsequent publication, and as shown by us previously

We take great care to cite the literature correctly, and we are always happy to be made aware if we have missed something important so that we can correct it. In this case, we do not believe that this is the case.

Referee #3:

The manuscript submitted for review is interesting. Research on the identification of ECTs as m6A readers in plants has been conducted by several research groups including the authors' group for some time. However, the interactors of these reader proteins and the molecular mechanisms behind such RNA modification recognition functions remain unclear. In this study, Tankmar et al demonstrated two short IDR-elements are important for Arabidopsis organogenesis. In addition, authors identified PAB2/4/8 especially interacted with a short IDR element of ECT2. The experiments are well-thought-out and logical and include the necessary controls. Although some results have been reported by a recently published paper (PMID: 37122016), I think this work should still be published after minor corrections.

Minor comments:

1. Some sentences need to be improved: e.g. the N-terminal IDR of ECT2, as well as its N-terminal and C-terminal halves individually, are essential for function in vivo. N-terminal IDR obviously includes two halves.

We do not understand the problem here and think that the sentence successfully conveys what it means to say. As an unbiased test, we tried to send the sentence to ChatGPT to see what it understood the sentence to mean. Here is its answer:

The sentence you provided is discussing the importance of different parts of a protein called ECT2 for its function in a living organism. Here's a breakdown:

1. *"The N-terminal IDR of ECT2": This refers to a specific region or domain at the N-terminus (the beginning) of the ECT2 protein called IDR (Intrinsically Disordered Region).*
2. *"as well as its N-terminal and C-terminal halves individually": This part means that not only the N-terminal IDR but also both the N-terminal half and the C-terminal half of the ECT2 protein are being considered separately.*
3. *"are essential for function in vivo": This is the key part of the sentence. It means that these mentioned regions or domains of the ECT2 protein are crucial for the protein to perform its function inside a living organism (in vivo means within a*

living organism, as opposed to in vitro, which means outside of a living organism in a controlled environment like a test tube).

In summary, the sentence is stating that various parts of the ECT2 protein, including the N-terminal IDR and its N-terminal and C-terminal halves, are all necessary for the protein to function properly within a living organism. Each of these regions plays a role in the protein's function in vivo."

This is the meaning we intend to convey, and we have, consequently, left the sentence unchanged in the revised version.

2. In the study, the authors revealed the important function role of N3.2 and N8 fractions from ECT2 in Arabidopsis. It will interesting to examine the amino acids of these regions from different ECT proteins and/or ECTs from different organisms.

We have now done so by alignment of YTHDF domain proteins from many species. The results are very interesting and have been included in Figure 4, as described in the response to reviewer 1. It was a very good suggestion to do this work more seriously, and we thank the reviewer for pushing us to do it.

We do not include a similar analysis of N8 here. As we state in the paper, the properties of this region will be communicated in detail in a separate report. The present paper provides the reader with the full results of the IDR deletion screen, but restricts its focus on molecular characterization to N3 to maintain focus and clarity. We stand by this choice, and hence, do not include further experimentation or sequence analysis on N8 in the present manuscript. Indeed, with all of the additions in this report, it is challenging enough to keep the focus on N3 within the word limits allowed for a Report.

3. Employing the IP-MS, the authors identified that three PAMPs proteins interact with the N3.2 region of ECT2. Please provide more direct experimental evidence to show such interaction.

We have conducted four types of experiments to satisfy this requirement:

(1) We did directed yeast two-hybrid assays based on reconstitution of nuclear Gal4 activity as a transcription factor when its DNA binding and transcription activation domains are brought together by protein-protein interaction. In contrast to the work of (Song *et al.*, 2023), we found the expression of *Arabidopsis thaliana* PABPs in the yeast nucleus to be toxic to the yeast. Consequently, the growth tests on selective media used as a read-out for interaction were not reliable in the case of PABPs in our hands. We do not report on these negative results in the paper, but want the reviewer to be aware that we did try this experiment.

(2) We changed two-hybrid system to a bacterial system based on reconstitution of adenylate cyclase activity by protein-protein interaction. The interaction tests using this system provide evidence that the N3.2 region ECT2 is sufficient to mediate interaction with PAB8. This is reported in Figures 4 and EV3A.

(3) We purified GST-PAB8 and an N-terminal IDR fragment of ECT2 containing N3.2 and showed specific, albeit low-affinity, interaction with GST-PAB8 *in vitro*. This is reported in Figure 4H.

(4) We used purified GST-PAB8 as an affinity matrix and showed that it retains $ECT2^{WT}$-mCherry much more efficiently than $ECT2^{\Delta N3.2.2}$-mCherry from total lysates. This is reported in Figure 4G.

Cross-comments from referee 1:
Concerning Rev2 P1: The interaction between ECT and PABP has been validated by several quantitative proteomics and I believe that these data are highly convincing. Doing co-IP, as suggested by Rev2, could be informative and should not take a long time (less than a month). In contrast, I am not a big fan of yeast two hybrid assay, since these experiments are prone to artifact and will require more time to be set up. Concerning the N8 region, doing a sequence analysis as suggested by rev3 could be a good idea. I will not go for more experiments on this particular region.

Concerning Rev2 P2: The authors should easily answer to this point by toning-down their discussion.

Regarding the Rev2 P1, are you aware of the attached correspondence recently published in Nature Plants concerning the incongruity of using Co-IP to validate quantitative proteomics? https://doi.org/10.1038/s41477-022-01314-8

Cross-comments from referee 3:
I totally agree with Referee 2's concerns. This manuscript seems not incomplete. Especially, the first concern from Referee 2, which I also raised, was that authors should provide more evidence supporting the interaction between ECT2 and PABs, e.g. Y2B, Co-IP, and Pulldown..

We believe that this point of criticism has been sufficiently addressed with the additional biochemical and genetic tests for direct interaction included in the revised version.

REFERENCES

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Arribas-Hernández L, Rennie S, Köster T, Porcelli C, Lewinski M, Staiger D, Andersson R, Brodersen P (2021a) Principles of mRNA targeting via the Arabidopsis m6A-binding protein ECT2. *eLife* 10: e72375

Arribas-Hernández L, Rennie S, Schon M, Porcelli C, Enugutti B, Andersson R, Nodine MD, Brodersen P (2021b) The YTHDF proteins ECT2 and ECT3 bind largely overlapping target sets and influence target mRNA abundance, not alternative polyadenylation. *eLife* 10: e72377

Roy G, De Crescenzo G, Khaleghpour K, Kahvejian A, O'Connor-McCourt M, Sonenberg N (2002) Paip1 Interacts with Poly(A) Binding Protein through Two Independent Binding Motifs. *Molecular and cellular biology* 22: 3769-3782

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Dear Peter,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees. As you will see, referee 2 still has a few more comments that I would like you to address and incorporate before we can proceed with the official acceptance of your manuscript. Please also submit a point-by-point response to the last comments.

A few editorial requests will also need to be addressed:

- Please remove the author credits from the ms file. All credits need to be entered in our online submission system.

- Please correct "Data set" to "Dataset" for your 6 Dataset files and the callouts in the ms.

- The APPENDIX FILE needs a table of content with page numbers on the title page.

- Please place the References before the figure legends.

- The figure files have titles/legends, please remove.

- Please address these comments from our data editors on the figure legends:

1. Please note that in figures 1c, f; 2c, g; 4b, d; EV1c there is a mismatch between the annotated p values in the figure legend and the annotated p values in the figure file that should be corrected.
2. Please indicate the statistical test used for data analysis in the legends of figures 3b-c; f-h; 4f; EV5c.
3. Please note that the measure of centre for the error bars needs to be defined in the legend of figure 2k.
4. Please note that information related to n is missing in the legend of figures 3b-c, f-h; 4f; 5d
5. Please note that the box plots need to be defined in terms of minima, maxima, centre, bounds of box and whiskers, and percentile in the legend of figure 5d.

Regarding the title:
"A YTHDF-PABP axis is required for m6A-mediated organogenesis in plants"
Is it indeed clear, that organogenesis is m6A-mediated? If not, it would be better to remove that part of the title. Also, instead of "axis" what about saying "YTHDF-PABP interaction"?

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the final manuscript.

I look forward to seeing a final version of your manuscript as soon as possible.

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors have adequately addressed my comments, primarily by presenting a thorough analysis of the PAB-interacting sequence of ECT2.I am therefore very satisfied with the current content of the paper.

Regarding Rev3 comments: I believe that the points concerning the sequence content of the N3.2 region and the experimental validation of the ECT2-N3.2/PAB interaction have been satisfactory addressed by the authors.

Referee #2:

The authors disregarded many of my suggestions and criticism, and extensively argue for their case. However, the revised manuscript and the author´s response only partially addresses my concerns. While in some cases their response clarified the point, response to some other points is not convincingly addressed; in some cases the authors resorted to semantic arguments rather than getting to the merit of the problem.

1) My initial and foremost concern pertained to validating the ECT-PAB interaction, a critical element of this study. This query arose due to the central role of this interaction in the paper. The initial submission only included IP-mass spectrometry data without additional confirmation of this interaction. It is customary for any reputable journal to ask supplementary methods for validating IP-mass spectrometry data, particularly when it forms the cornerstone of the research. I suggested options like IP-western or yeast two-hybrid as examples, but there are many other independent assays that could have been considered to fortify the study's foundation with multiple lines of evidence establishing the role of identified regions in ECT-PAB interaction. Therefore, I am really surprised that his was even matter of discussion in the response to my concerns. I appreciate that authors provide additional evidence for ECT2-PAB8 interaction, it strengthens the key point of the study. Nevertheless, I still have one question: why the authors conclude that that N3.2 regions is necessary for PAB binding when Fig 4F shows that ECT2 delta 3.2.2 mutant still retains capacity to bind to GST-PAB8? What is the evidence for this claim?

2) While I acknowledge the authors' decision to present N8 as a separate narrative, I find their outright rejection of the reviewers' summary as inaccurate, unwarranted and excessively forceful. The insistence on highlighting the minute distinction between synergism and additive response appears superfluous, even with the accompanying figure, as it remains challenging to discern definitively whether it leans towards additivity or synergism. I could readily engage in a counter-argument, but I deem it unnecessary, given the understanding of the potential separate publication of the N8 story.

3) The authors still refute the hypothesis by Song et al. with language " we propose a rather less bold interpretation of the evidence at hand". I can only recapitulate that this language is uncalled for. It implies that Song et al. made bold interpretation without any evidence. Fact is Song et al did provide ample evidence from different fronts to support their hypothesis. To mention some

a. They used three independent protein interaction assay to show ECT protein from complex by direct interaction with each other. They further confirmed with formaldyhyde crosslinke RNA immunoprecipitation assay that this interaction enhances m6A binding capacity
b. They further showed that ECT proteins are required for seed germination and also post seed germination under ABA treatment.
c. They next generated RNA-seq on ECT mutant, used previously public FA-CLIP data on ECT2 and generated RNA stability data to claim that ECT is required for stabilisation. Along with it, they thoroughly analsysed previously published sequencing data to support their claim
d. They did IP-mass spectrometry data to obtain interaction partner with ECT2. They obtain many porteins among them they chose PABs because PABs in other system was shown to perform RNA stabilization function similar to their current data on ECTs. This seem very reasonable target to test with previous history of same function.
e. They chose two independent assays to prove this interaction, and a third independent assay was performed to show that PrLD domains of ECT2 interact with PAB2. I must highlight here that after IP-MASS spectrometry they used three independent assay to prove the interaction. So in total 4 independent assays to show direct interaction.
f. They re-analysed previously published CLIP data on PAB2 and PAB4 along with mRNA stability data to reconfirm whether PABs are indeed invoved in mRNA stabilization in Arabidopsis.
g. Further, they showed that PABs and ECT2 are bound to similar targets. I must point out that CLIP data on PAB and ECTs are generated from two different labs. Its very difficult to get any meaningful correlation on data generated at two different time let alone two different labs. Even then, they got more then 50% overlap with target of PABs and ECTs which in my opinion is outstanding.
h. Not only ECTs and PAB share the same target, their binding positions are also similar, suggesting that ECTs and PAB together stabilizes m6A-containing targets.
i. They use this knowledge to further link it to the biological function (ABA response) and they generated a battery of experiments such as FA-RIP-qPCR, m6A-qpcr, FA-CLIP,mRNA stability assay on genes involved in ABA response to strengthen their claim.
The point is Song et al. did provide multiple independent assays, analyzed data from other labs to make their claim about stabilizing role of ECT and PAB along with its biological significance. The only thing they didn't do is breaking the interaction between ECT-PAB.

Is it really justifiable to refute their hypothesis with such strong language? Especially when the authors in this paper didn't provide direct counter-evidence? The authors have ECT-PAB mutants which don't interact; they could perform an RNA stability assay on a few RNA targets if they feel that Song et al. hypothesis is incorrect. Until that point, I strongly suggest removing the language "rather less bold interpretation of the evidence at hand" and replacing it with " We suggest an alternative hypothesis"

4) The authors strongly claim that " N3.2 is not required for RNA binding in vivo" and reject my criticism of the experimental data because this point was not criticized by the other reviewers. I must say that this is really peculiar way to argue around this point. They made an attempt to address this point experimentally, which I appreciate. However, this experiment did not addressed my

criticism and I am still of the opinion that the presented RNA pull down assay is inconclusive and does not support the claim.

a) The new supplementary figure shows that the pattern of RNA pull-down is completely different than in the main figure raising a question what is actually being detected there. Authors did not include any negative control (e.g. pull down with wild type extract) to show what is background binding of RNA to the beads. With no negative control in the supplement figure, it is hard to distinguish whether pull-down RNA is from non-specific binding or whether it comes specifically with ECT protein.
b) The difference of a supplementary figure from the main body experiment suggests that the reproducibility of this method is low in the author`s hands. In fact, even the different samples in the main figure show different pattern; some bands are completely lost, and others are newly obtained. I do not want to imply that the quality of author´s work is low, this may be really difficult experiment. Nevertheless, regardless of the reasons, the concern regarding what is being detected in these pull downs is real. Considering the variation between the experiments, the call whether the difference between ECT2 no-binder and other ECT variants represents fluke or meaningful result is open.
c) Minor technical comment, it is unclear from the text or figure what serves as normalization factor for the input? Is it total amount of protein, or is the input normalized to the amount of ECT-mCherry by western blot? I didn't find this information anywhere in the text.

5) Regarding information about IP-Mass data on tagged lines in the mutant background: I asked this question because this information is not available in the paper. What is available in the said panel regards Tag construct, but it is not clear whether this line is generated in the mutant background or in wild type. Please mention it in legend/method/Panel

6) Does the comment "That is relevant" imply that other comments are irrelevant?

7) Thank you for providing the explanation in regards to not citing the Plant Cell paper: on the first look, this paper seems to link plant development (trichome morphology) with ECT2 and to be highly relevant to this study. It was surprising that it was not mentioned in the introduction where morphological defects are described. Frankly, I do not have sufficient insights to judge the legitimacy of that study. It is up to the authors to consider whether all merits of the paper are inaccurate or only some aspects and act accordingly and fairly. Often it happens that earlier studies are less accurate and are corrected by later studies, but this decision goes to the authors.

RESPONSE TO REVIEWER COMMENTS ON REVISION1

We thank reviewers 1 and 2 for having taken the time to evaluate the revised version of our manuscript. We are sorry that reviewer 2 appears to have taken so much offense by our arguments. This was not intended.

Unfortunately, the largely unfounded criticism of the UV-CLIP experiment to evaluate gross ECT2-RNA interaction, and the lengthy defense of conclusions by Song et al. (2023) that are not supported by direct evidence, require a firm response that we deliver below.

Referee #1:

The authors have adequately addressed my comments, primarily by presenting a thorough analysis of the PAB-interacting sequence of ECT2.I am therefore very satisfied with the current content of the paper.

Regarding Rev3 comments: I believe that the points concerning the sequence content of the N3.2 region and the experimental validation of the ECT2-N3.2/PAB interaction have been satisfactory addressed by the authors.

We again thank the reviewers (1 & 3) for pushing us to do a more thorough sequence analysis of the part of the IDR of ECT2 that is necessary for most of the PABP interaction. The realizations that

(1) the element is specifically lost in those ECT proteins that cannot substitute ECT2 function in leaf primordia
(2) the element is conserved not only in plant YTHDF proteins, but also in animal YTHDFs

substantially increase the impact of the paper in our view.

The request for evidence to probe whether the interaction is direct (reviewers 2 and 3) also pushed us to get results that clearly improve the paper, and that provides us with tools for our future work.
All in all, we are very happy with the improvements in the revised version and thank all three reviewers for their efforts in this regard. We are also very happy that reviewer 1 finds these improvements to address the comments raised on the original version in a satisfactory way.

Referee #2:

The authors disregarded many of my suggestions and criticism, and extensively argue for their case. However, the revised manuscript and the author´s response only partially addresses my concerns. While in some cases their response clarified the point, response to some other points is not convincingly addressed; in some cases the authors resorted to semantic arguments rather than getting to the merit of the problem.

1) My initial and foremost concern pertained to validating the ECT-PAB interaction, a critical element of this study. This query arose due to the central role of this interaction in the paper. The initial submission only included IP-mass spectrometry data without additional confirmation of this interaction. It is customary for any reputable journal to ask supplementary methods for validating IP-mass spectrometry data, particularly when it forms the cornerstone of the research. I suggested options like IP-western or yeast two-hybrid as examples, but there are many other independent assays that could have been considered to fortify the study's foundation with multiple lines of evidence establishing the role of identified regions in ECT-PAB interaction. Therefore, I am really surprised that his was even matter of discussion in the response to my concerns. I appreciate that authors provide additional

evidence for ECT2-PAB8 interaction, it strengthens the key point of the study. Nevertheless, I still have one question: why the authors conclude that that N3.2 regions is necessary for PAB binding when Fig 4F shows that ECT2 delta 3.2.2 mutant still retains capacity to bind to GST-PAB8? What is the evidence for this claim?

We are not going to repeat the arguments for why it makes little sense to "validate" IP-MS experiments by IP-western analysis, as advocated by the reviewer. The reviewer is referred to the previous response to reviewers and to the reference provided by Reviewer 1.

We agree with the value of conducting experiments to assess whether the ECT2-PABP interaction is direct or not. The revised version contains ample evidence that the N3.2 region mediates direct interaction between ECT2 and PABPs (PAB8 chosen as an example here): it is necessary for full interaction *in vivo* as demonstrated by the series of IP-MS assays already reported in the original version, it is necessary for full interaction with GST-PAB8 in the binding assay measuring retention of ECT2 in total lysates by a GST-PAB8 affinity column, a fragment that contains N3.2 is sufficient for binding to GST-PAB8 in vitro, and N3.2 is sufficient for a positive two-hybrid interaction with PAB8 in bacteria.

We do not mean to say that deletion of N3.2 completely abrogates PABP interaction. That would obviously be absurd given that PAB2, PAB4 and PAB8 are still identified in ECT2$^{\Delta N3}$, ECT2$^{\Delta N3.2}$ and ECT2$^{\Delta N3.2.2}$ IP-MS experiments, albeit in significantly lower abundance than in experiments with the wild type (full length) ECT2. In fact, we specifically comment on that:

"Because the C-terminal half of the IDR does not include N3.2, the observations by (Song et al., 2023) raise the possibility that regions of ECT2 other than N3.2 may also be implicated in PABP binding, perhaps akin to the interaction between mammalian Paip1 and PABP mediated by distinct PAM1 and PAM2 motifs (Roy et al, 2002). Such a scenario would be consistent with our IP-MS and GST-PAB8 binding data that ***show significant reduction, but not abolishment, of ECT2-PABP interaction upon deletion of N3.2 and/or N3.2.2****."*

Thus, there does not appear to be any disagreement between the reviewer and the interpretation of our experiments, as expressed in the paper. To avoid confusion, we have, however, amended one of the subheadings so that it now reads
"*N3.2 is required for full interaction with the cytoplasmic PABPs, PAB2/4/8"*
to emphasize that the requirement for N3.2 is not absolute.

2) While I acknowledge the authors' decision to present N8 as a separate narrative, I find their outright rejection of the reviewers' summary as inaccurate, unwarranted and excessively forceful. The insistence on highlighting the minute distinction between synergism and additive response appears superfluous, even with the accompanying figure, as it remains challenging to discern definitively whether it leans towards additivity or synergism. I could readily engage in a counter-argument, but I deem it unnecessary, given the understanding of the potential separate publication of the N8 story.
We agree with the reviewer that the data obtained with the double deletion mutant does not rule out that the N3 and N8 elements could act together in some way. At this point, the results lean towards independent action, but we agree that we cannot conclude so with certainty and we have, therefore, amended the text accordingly, so that it now reads:
"We also combined ΔN3.2 and ΔN8 (Fig EV1B) and observed a largely additive effect of the two deletions on complementation frequency (Fig EV1C), perhaps implying that independent mechanisms of action underlie their requirement for function. We conclude that the deletion analysis identified two potentially independently acting elements whose removal from the N-terminal IDR of ECT2 results in partial loss of function."

However, the reviewer used an assertion that the elements act synergistically to argue that we should include more information on N8 in this manuscript, and that the study would be “incomplete” without it. If anything, the available evidence points towards independent action, and we very much disagree that the study requires more information on N8 to appear complete. It is, in our view, neither “inaccurate, unwarrantred nor excessively forceful” to reject this piece of reviewing that simply asks for more information without linking that request to conclusions drawn in the paper. We are satisfied that the reviewer now agrees that getting into N8 in more detail is not appropriate for the present manuscript.

3) The authors still refute the hypothesis by Song et al. with language " we propose a rather less bold interpretation of the evidence at hand". I can only recapitulate that this language is uncalled for. It implies that Song et al. made bold interpretation without any evidence.
This sentence simply means that we propose a more cautious interpretation, and there is nothing impolite in its formulation. We have shown it to two native English speakers with a knowledge of molecular biology, and they both agree that there is nothing impolite, offensive or “uncalled for” about the language used. We have also consulted with a completely objective party to be sure. ChatGPT says the following:

”In the sentence you provided, the author is discussing a scientific study or research findings related to the "ECT-PABP function" at mRNA targets. Let's break down the sentence to understand it better:

1. *"While our data clearly does not disprove the model for ECT-PABP function at all mRNA targets suggested by (Song et al., 2023)": This part of the sentence acknowledges that the author's data doesn't contradict or prove wrong a model proposed by Song and their colleagues in 2023 regarding the function of ECT-PABP at mRNA targets. In other words, the author's research doesn't provide evidence against the model proposed by Song and their team.*
2. *"we propose a rather less bold interpretation of the evidence at hand": This part of the sentence indicates that the author is suggesting an interpretation of the available evidence that is less assertive or less daring than the interpretation provided by Song and their team. The word "bold" in this context implies that the original interpretation by Song et al. might have been more speculative, ambitious, or confident. The author is proposing an interpretation that is more cautious, modest, or restrained.*

So, in summary, the sentence means that the author's research doesn't contradict the model proposed by Song et al., but they are offering a more conservative or less assertive interpretation of the evidence they've collected. They are suggesting a less daring or less confident explanation based on the available data.”

Asked directly whether the sentence is impolite or offensive, the answer is

”The sentence you provided does not appear to be impolite or offensive on its own. It seems to be a standard scientific or academic statement expressing a difference in interpretation or findings between the author and Song et al. The use of the phrase "less bold interpretation" is a way to express a more cautious or modest perspective on the evidence without being inherently impolite. Scientific discourse often involves the respectful exchange of differing interpretations and perspectives, and it's common for researchers to propose alternative explanations or interpretations without necessarily being impolite or offensive.”

We prefer the original sentence and really cannot see anything wrong with it. However, since the reviewer may not be alone in her or his interpretation, and it is not our intention that anyone be offended by it, we have changed it to “*we propose a rather more cautious interpretation of the evidence at hand*”.

Fact is Song et al did provide ample evidence from different fronts to support their hypothesis.
The problem with all of the evidence cited below is that it does not address the relevant question, namely whether the ECT2-PABP interaction is important for anything *in vivo*. To pronounce oneself on this matter, mutants that specifically disrupt the interaction are required. Song et al. do not have such mutants. Instead, they base their mutational analysis entirely on total knockouts to produce, at the very best, circumstantial evidence. Although that is the essence of the problem, we go through the arguments presented by the reviewer point by point below.

a. They used three independent protein interaction assay to show ECT protein from complex by direct interaction with each other.
We agree that they provide evidence that ECT proteins associate with each other, perhaps via direct interactions as indicated by the yeast two-hybrid assays. This finding is, however, not relevant for the conclusions and interpretations for which our criticism arose: whether the ECT-PABP interaction is important *in vivo*. Furthermore, the proposition of ECT2-ECT3-ECT4 acting as a complex in the way proposed by Song et al is questionable given the genetic redundancy between ECT2 and ECT3, but this is not relevant for the present paper.

They further confirmed with formaldyhyde crosslinke RNA immunoprecipitation assay that this interaction enhances m6A binding capacity
As noted above, ECT-ECT interactions are really off-topic here, the question in focus is ECT-PABP interaction. Suffice it to say that evidence that ECT-ECT interaction enhances m6A binding capacity (or indeed is important for anything) would require mutants that specifically disrupt this interaction. Such mutants were not isolated by Song et al.

b. They further showed that ECT proteins are required for seed germination and also post seed germination under ABA treatment.
Yes, that result is clear, but it is irrelevant for evaluating the importance of ECT2-PABP interaction in vivo. It is just another phenotype of *ect2 ect3 ect4* knockout mutants; it may involve PABP, or it may not. It is not possible to conclude that based on the evidence presented.

c. They next generated RNA-seq on ECT mutant, used previously public FA-CLIP data on ECT2 and generated RNA stability data to claim that ECT is required for stabilisation. Along with it, they thoroughly analsysed previously published sequencing data to support their claim
As for the previous argument, nothing in this analysis allows conclusions on the importance of ECT2-PABP interactions to be drawn, as the experiments are conducted with *ect2 ect3 ect4* knockout mutants, obviously impaired in ECT2/3/4-PABP interaction, but also impaired in all other molecular activities of ECT2/3/4, including interactions with the ~100 other proteins enriched in ECT2 immunoaffinity purifications.

d. They did IP-mass spectrometry data to obtain interaction partner with ECT2. They obtain many porteins among them they chose PABs because PABs in other system was shown to perform RNA stabilization function similar to their current data on ECTs. This seem very reasonable target to test with previous history of same function.
We agree that PABs are reasonable candidates to test, but there is no valid experimental test of the importance of the interaction in study. Instead, a large mosaic of results is presented, but none of it allows conclusions on the molecular consequences or the biological relevance of the ECT-PABP interaction to be drawn.

e. They chose two independent assays to prove this interaction, and a third independent assay was performed to show that PrLD domains of ECT2 interact with PAB2. I must

highlight here that after IP-MASS spectrometry they used three independent assay to prove the interaction. So in total 4 independent assays to show direct interaction.
Yes, we agree. Song et al. show that there is interaction, and therefore we properly cite their article at multiple instances for this finding. We also put it properly into context with our findings, for example with the following paragraph:
" *a recent report also found an association between ECT2 and PAB2 to be mediated by a 400-aa N-terminal IDR fragment of ECT2 (Song et al, 2023). Although two 200-aa fragments corresponding to N- and C-terminal halves of the IDR were sufficient for PAB2 interaction in qualitative in vitro binding assays, the region necessary for interaction was not more accurately defined (Song et al., 2023), precluding conclusive experiments on the importance of the ECT2-PAB2 interaction to be conducted. Nonetheless, because the C-terminal half of the IDR does not include N3.2, the observations by (Song et al., 2023) raise the possibility that regions of ECT2 other than N3.2 may also be implicated in PABP binding, perhaps akin to the interaction between mammalian Paip1 and PABP mediated by distinct PAM1 and PAM2 motifs (Roy et al, 2002). Such a scenario would be consistent with our IP-MS and GST-PAB8 binding data that show significant reduction, but not abolishment, of ECT2-PABP interaction upon deletion of N3.2 and/or N3.2.2."*

But again, neither this experiment nor the ones described above investigate the importance of the interaction. That is the key of this discussion. Of note, we knew of this interaction 4 years ago. The difficulty is to prove its existence, but rather its *in vivo* importance.

f. They re-analysed previously published CLIP data on PAB2 and PAB4 along with mRNA stability data to reconfirm whether PABs are indeed invoved in mRNA stabilization in Arabidopsis.
Apart from the fact that the validity of this analysis is questionable, it still does not address the heart of the matter. Even if one concedes that PABs impact mRNA stability, this still does not address what the ECT-PABP interaction may be doing. It is, at best, circumstantial evidence.

g. Further, they showed that PABs and ECT2 are bound to similar targets. I must point out that CLIP data on PAB and ECTs are generated from two different labs. Its very difficult to get any meaningful correlation on data generated at two different time let alone two different labs. Even then, they got more then 50% overlap with target of PABs and ECTs which in my opinion is outstanding.
Is it really? We do not find this result to be outstanding in any way. It really is rather trivial. It is, after all, a general feature of eukaryotic mRNAs to have a poly(A) tail and hence to be bound by PABs. We would not expect anything less, and do not find this analysis to be meaningful in any way.

h. Not only ECTs and PAB share the same target, their binding positions are also similar, suggesting that ECTs and PAB together stabilizes m6A-containing targets.
No, it does not suggest this. m^6A is located in 3'-UTRs, and ECT2 binds to 3'-UTRs, as clearly shown by Simpson's lab Nanopore/miCLIP data and our ECT2-iCLIP data. PABs bind to the poly(A) tails that follows the 3'-UTR in all mRNAs. 3'-UTRs are generally quite short in *Arabidopsis thaliana*. The two proteins must bind close to one another for these reasons.

i. They use this knowledge to further link it to the biological function (ABA response) and they generated a battery of experiments such as FA-RIP-qPCR, m6A-qpcr, FA-CLIP,mRNA stability assay on genes involved in ABA response to strengthen their claim.
The point is Song et al. did provide multiple independent assays, analyzed data from other labs to make their claim about stabilizing role of ECT and PAB along with its biological significance. The only thing they didn't do is breaking the interaction between ECT-PAB.
None of what is presented in the Song et al 2023 allows conclusions neither on the molecular consequences nor the biological significance of the ECT-PABP interaction to be

drawn. We think that this should be abundantly clear after this lengthy discussion.

Is it really justifiable to refute their hypothesis with such strong language?
We would like to summarize our arguments by answering to this question

(1) The language is not strong. It asserts in a polite and correct way what the facts are and what we think about them.
(2) Song et al. do not put forward the model that the ECT-PABP interaction is important for stabilizing m6A-containing transcripts as a *hypothesis.* It is their main conclusion. If in any doubt, let's recapitulate what the title is:
'm6A readers ECT2/ECT3/ECT4 enhance mRNA stability through direct recruitment of the poly(A) binding proteins in Arabidopsis'

Especially when the authors in this paper didn't provide direct counter-evidence? The authors have ECT-PAB mutants which don't interact; they could perform an RNA stability assay on a few RNA targets if they feel that Song et al. hypothesis is incorrect.
We do not say that the Song et al. *hypothesis* is incorrect, we say that their *conclusions* cannot be drawn based on the experimental evidence presented, and explain why this is so. It is an important element of scientific progress to point out where beliefs that seem to be shared by more members of the scientific community than the original authors are not sufficiently supported by evidence.

Until that point, I strongly suggest removing the language "rather less bold interpretation of the evidence at hand" and replacing it with " We suggest an alternative hypothesis"
We cannot do that, because the interpretations by Song et al. are presented as conclusions supported by their results, not hypotheses. In fact, we used the phrasing 'less bold interpretation' as a much more polite way of saying 'unfounded conclusions'. We are willing to change it to 'more caution interpretation', but we cannot refer to their interpretations as hypotheses because they themselves do not qualify them as such.

4) The authors strongly claim that " N3.2 is not required for RNA binding in vivo" and reject my criticism of the experimental data because this point was not criticized by the other reviewers. I must say that this is really peculiar way to argue around this point. They made an attempt to address this point experimentally, which I appreciate. However, this experiment did not addressed my criticism and I am still of the opinion that the presented RNA pull down assay is inconclusive and does not support the claim.
The comment on the absence of criticism from other reviewers was just a remark to strengthen our disagreement with this reviewer's opinion, but the arguments for the disagreement were abundantly detailed in our previous answer (after 'to break it down:'). We believe that, perhaps, the problem here may be an incomplete understanding of the peculiarities of this type of experiment with a protein whose IDR has a strong tendency to rapidly truncate at specific points (seen by us and other groups), even more so in the CLIP buffer (thoroughly detailed by us in Arribas-Hernández et al., eLife 2021). The extent of this degradation – that increases enormously over time and has inevitable variability from one experiment to another - profoundly affects the band-pattern, and so do the deletions introduced in our transgene. Indeed, please remember that the resulting autoradiograms show 32P label incorporated into covalently linked ECT2-mCherry-RNA complexes. Thus, the resulting patterns of bands follow the size of the ECT2 variant (and its potential truncation products during IP) studied. Hence, the protein-RNA complexes detected with an ECT2 deletion mutant run more quickly in the gel than the complexes detected ECT2 wild type, exactly as it appears from Figure 3A.
We try to clarify the key points that might be the source of misunderstanding below.

a) The new supplementary figure shows that the pattern of RNA pull-down is completely different than in the main figure raising a question what is actually being detected there. Authors did not include any negative control (e.g. pull down with wild type extract) to show

what is background binding of RNA to the beads. With no negative control in the supplement figure, it is hard to distinguish whether pull-down RNA is from non-specific binding or whether it comes specifically with ECT protein.
The supplemental figure provides yet another example that the same amount of signal is obtained when using ECT2-mCherry WT or ΔN3. **To make the result more robust**, instead of repeating the same, we used separately three independent lines for each type of transgene, to account for secondary mutations or any other line-specific artifacts. Unfortunately, the gels that one must use to do that experiment do not admit more samples, and hence we had to sacrifice the negative control for this line-by-line repeat of the experiment. We judged that the line-by-line check was more important than the negative control, because we have repeated this type of experiment with ECT2 uncountable times, and the negative control with either crosslinked Col-0 WT tissue or not-crosslinked ECT2WT-mCherry tissue is always completely clean (see Arribas-Hernández et al., 2021 eLife, this point is abundantly illustrated in the supplementary figures). Likewise, the ECT2W464A-mCherry control is always very clearly less intense than wild type.

b) The difference of a supplementary figure from the main body experiment suggests that the reproducibility of this method is low in the author`s hands. In fact, even the different samples in the main figure show different pattern; some bands are completely lost, and others are newly obtained.
I do not want to imply that the quality of author´s work is low, this may be really difficult experiment. Nevertheless, regardless of the reasons, the concern regarding what is being detected in these pull downs is real. Considering the variation between the experiments, the call whether the difference between ECT2 no-binder and other ECT variants represents fluke or meaningful result is open.
We strongly disagree with these comments, and believe that the difficulty of the reviewer in understanding the legitimate source of variability between samples and experiments may require a more detailed explanation from our side:

ECT2 degrades very rapidly, in a reproducible manner, in the CLIP buffer. The reason is a high sensitivity to the proteases present in the lysate, in these conditions, of what seems to be specific points in the IDR (Arribas-Hernández et al., 2021). Of note, we use high concentrations of two different protease inhibitors and low incubation times to minimize the extent of this problem, but it proved impossible to avoid altogether. According to the band pattern in the full length (WT) protein, some protease-sensitive sites are at the N-terminal part of the IDR. Because the IDR seems to contact mRNA and protect it from PNK-labelling in vitro (Arribas-Hernández et al., 2021), these truncations greatly affect the pattern of labelled mRNA detected, as this results in a smear of RNPs (ECT2 with mRNAs of different sizes) that run upwards from the height of the protein **and protein truncations**. The more degradation occurs, the more the smears from the different fragments overlap with each other. Taking that into consideration:

- The difference between the band pattern in input and IP in Fig 3A is due to more degradation in the IP (due to incubation time with beads), than in the input (taken from the lysate prior bead-incubation), but the overall pattern is the same. Of note, difference in the sizes relative to the ladder is most likely due to the different PAGE buffers for each gel.
- The difference in the band-pattern between WT and ΔN3 in Fig 3A is due to a different truncation pattern caused by the deletion (protease-sensitive points may be inside the deleted fragment).
- The difference between main and EV figure is simply due to the fact that, unfortunately, we had more degradation in the EV experiment. However, because we are not concluding anything about the band-pattern, but rather about the intensity of the bands (indicative of amount of mRNA), we can conclude that there is no apparent difference in mRNA binding between WT and ΔN3.

c) Minor technical comment, it is unclear from the text or figure what serves as normalization factor for the input? Is it total amount of protein, or is the input normalized to the amount of ECT-mCherry by western blot? I didn't find this information anywhere in the text.
The input is just a sanity check to see that we immunopurified an amount of ECT2-mCherry that is proportional to what is in the tissue.
The only important comparison for our conclusion, that ΔN3 does not have a defect in RNA-binding, is the amount of mRNA (autoradiogram) relative to the amount of protein immunopurified (IP-WB) in the mutant compared to WT. The different band pattern between both (due to the different pattern of truncations caused by the deletion) makes the comparison by quantification difficult, but it is clear that, if anything, ΔN3 has more signal (= pulls down more RNA) than WT. For the only band of the same size (at 50 kDa), the intensity is roughly equal.

Finally, please notice that we have always been very cautious in our interpretation of this experiment. From the originally submitted version of the manuscript and throughout the evaluation process, we have used the following guardedly phrased statement:

"Because ECT2-RNA complexes with or without the N-terminal IDR generated by proteolysis during immunopurification are labelled with different efficiency, presumably due to different accessibility of 5'-ends as a consequence of IDR-RNA contacts (Arribas-Hernández et al., 2021a), we think it unwise to interpret this result more deeply than to conclude that ECT2$^{\Delta N3.2}$ retains RNA-binding capacity. We also note that this experiment does not provide insight into the identities of the co-purified mRNAs, and it remains possible that N3.2 is implicated in mRNA target selection."

Nonetheless, to be sure to avoid confusion, we have changed the subheading of the paragraph describing this experiment so that it now reads:

"ECT2$^{\Delta N3.2}$ has no obvious defect in RNA binding in vivo"

We think that this subheading correctly summarizes the evidence we present.

5) Regarding information about IP-Mass data on tagged lines in the mutant background: I asked this question because this information is not available in the paper. What is available in the said panel regards Tag construct, but it is not clear whether this line is generated in the mutant background or in wild type. Please mention it in legend/method/Panel
We apologize for not having included this information explicitly earlier, and thank the reviewer for bringing it to our attention. Now it is explicitly included in the Methods. Our oversight was probably due to the fact that all transgenic ECT2-mCherry lines reported here derive from the functional screen in the *ect2 ect3 ect4* mutant background.

6) Does the comment "That is relevant" imply that other comments are irrelevant?
No, It does not. Indeed, we undertook substantial efforts to Improve the work In response to other good comments made, most notably the request for experimentation to test whether the ECT2-PABP interaction is direct. What we meant is that it is a very good question (we should have written 'that is a very relevant question'. We are sorry if the reviewer understood our response to imply that all other comments were irrelevant. This was not intended.

7) Thank you for providing the explanation in regards to not citing the Plant Cell paper: on the first look, this paper seems to link plant development (trichome morphology) with ECT2 and to be highly relevant to this study. It was surprising that it was not mentioned in the introduction where morphological defects are described. Frankly, I do not have sufficient

insights to judge the legitimacy of that study. It is up to the authors to consider whether all merits of the paper are inaccurate or only some aspects and act accordingly and fairly. Often it happens that earlier studies are less accurate and are corrected by later studies, but this decision goes to the authors.

We do not examine the trichome morphology phenotype in this manuscript, we concentrate fully on leaf formation on which the study by Wei et al. (Wei *et al*, 2018) provides no insight. Indeed, what we mention in the introduction regarding the role of ECTs in development is:

"*ECT2-4 are necessary for the rapid division of these cells, such that organogenesis is delayed due to slow growth in ect2-1 ect3-1 ect4-2 (henceforth abbreviated te234) mutants (Arribas-Hernández et al, 2018; Arribas-Hernandez et al, 2020).*"

Wei et al. do not study ECT3, ECT4, cell division or delayed organogenesis in their article. Please notice that this paper is otherwise cited in the appropriate context where some of the data generated in the study is inspected (Figure EV5).

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Prof. Peter Brodersen
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Dear Peter,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

I shortened your summary a little bit, as our word limit is 36. I think 39 will be fine too:

An interaction between m6A-binding YTHDF proteins and cytoplasmic poly(A)-binding proteins promotes leaf formation. It is mediated by a short motif conserved across plant and animal YTHDF proteins, suggesting an effector function of m6A readers shared between animals and plants.

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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
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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 χ2 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Data Set EV4

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Yes	Materials and Methods
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Materials and Methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)

If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	**Yes**	Figures 1,2,5, Appendix Figure S1
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	**Yes**	Described for the protein sequence alignments underlying the results reported in Figure 4J, Figure EV3B and Figure EV4
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	**Yes**	Materials and Methods, Figures 1-5, Figure EV1

Sample definition and in-laboratory replication	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	**Yes**	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	Figure legends

Ethics

Ethics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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	
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	

Data Availability

Data availability	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	**Yes**	Data availability section
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	**Yes**	Figure EV5