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Review COMMONS

The mRNA decapping complex is buffered by nuclear localization

Kiril Tishinov and Anne Spang
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Editor: Maria Carmo-Fonseca

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Submission to Review Commons: 4 September 2020
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Reviewer 1

Evidence, reproducibility and clarity

This manuscript demonstrates a key role for the Dcp2 decapping complex in P body formation that is influenced by nuclear localization of the decapping protein along with ER/mitochondrial associations and interactions with other decapping proteins. Overall I find the story to be very interesting, generally convincing (with one exception noted below) and impactful to the field. I only have a few suggestions to polish the manuscript:
1. Abstract: My understanding is that there remains a debate in the field as to how much mRNA decay (if any) actually occurring in P bodies. Thus I would recommend changing the second sentence in the abstract that states that PBs are the major site of mRNA decay.
2. Fig. 2G-I: These gels should also be presented in a quantified fashion with statistics to provide optimal support for the conclusions drawn. This is particularly important since the data shown with NLS-DCP2 in Fig. 2H looks as though the MET3 mRNA is indeed degrading faster, contrary to the authors' conclusion on page 7.

Significance

The study advances our mechanistic understanding of P body formation, particularly in terms of the impact of DCP2, the role for subcellular localization, and the role of specific protein- protein interactions. It will definitely be of interest to the RNA decay and cell biology communities.

I am an RNA decay researcher who focuses on mammalian systems.

REFEREE'S CROSS-COMMENTING

I concur - I think that all three reviewers see the inherent value and interest of the study and the polishing comments that are suggested will definitely strengthen the support for the conclusions that are drawn.

Reviewer 2

Evidence, reproducibility and clarity

Evaluation of the manuscript "Decapping complex is essential for functional P-body formation and is buffered by nuclear localization" by Kiril Tishinov and Anne Spang.

Summary:

P-bodies are membraneless granules in the cytoplasm of eukaryotic cells that contain proteins of the cellular RNA decay machinery and untranslated mRNAs. While it was initially proposed that P-bodies are sites of cellular mRNA decay, it was later shown that they are not required for general mRNA turnover. The current view is therefore that P-bodies mainly serve as storage sites for translationally repressed mRNA.

For the work presented in this manuscript, the authors generated a yeast strain lacking both edc3 and scd6 (edc3D, scd6DD and analyzed the formation of P-bodies in this strain. While single deletions of edc3 and scd6 had only minor effect, the double mutant showed severe defects in P-body formation and a substantial accumulation of Dcp2 in the nucleus. The authors identify putative nuclear localization signals in Dcp2, indicating that Dcp2 is imported into the nucleus by karyopherin D/D. Nuclear Dcp2 does not participate in mRNA turnover, but is likely stored as inactive pool from which it can be released and exported under certain conditions. Moreover, the authors show that Dcp2 initiates the formation of P-bodies in association with ER membranes and that Dcp2 is essential for P-body assembly. Both, Edc3 and Scd6 can bridge the interaction between Dcp2 and Dhh1, which explains why only in the double edc3D, scd6DDstrain P-body formation is affected

Major comments

a) Are other nuclear-localized proteins also affected in their import in the kap95-aid strain even without adding auxin?
b) What are the levels of Kap95-aid in comparison with wt KAP95? Have the authors tried to inhibit the unintended degradation of Kap95-aid by using the degradation inhibitor "auxinole" (PMID: 31026591)
c) The expression levels of overexpressed Dcp2-GFP (Figure 2D) exhibit some cellular variability. Would it be possible to use some uniform populations for the Northern blots, e.g. through FACS sorting?
d) Would it not be better if the authors looked for global poly(A)+ amounts in the nucleus by fluorescence in situ hybridization rather than for a specific mRNA (MET3) by Northern blotting (Figure 2G-I)?
e) Have the authors tried to mutate the predicted NLS-sequences of Dcp2 to see if these are mediating the nuclear import of Dcp2?
f) Did the authors consider/test if binding of Edc3 and/or Scd6 to Dcp2 masks some of the predicted nuclear localization sequences? This would explain the localization of Dcp2 in the double KO strain.

Minor comments

a) Abstract, second sentence: "Cytoplasmic mRNA is largely turned over in processing bodies (P-bodies)" should be revised, because it is not clear that P-bodies play an important role in mRNA turnover.
b) Not all immunofluorescence data are shown in Figure 1G (wt is missing), while the quantifications in Figure 1H include wt. I suggest that the missing data are shown in a supplementary figure.

Significance

While the first two figures provide very interesting insights into the regulation of P-bodies and Dcp2 by Edc3 and Scd6, the story loses some of its focus in Figure 3 and onwards. Indeed, in some cases the significance of the results is not entirely clear to me and the synthetic- lethal screens provide rather indirect readouts. This is especially true for the ER- and Mitochondria results, which do not really seem to fit into the story. I would even suggest removing Figure 3 and adding other data to the manuscript instead.
Apart from that, it is a solid piece of work with high-quality data that provides interesting insights into P-body biology for interested readers in that field. The function of Dcp2 and its localization for P-body formation in yeast is examined in detail, although some additional experiments would further strengthen the work. However, the story is a bit too specific and of not enough general interest for a very broad readership.

This reviewer's field of expertise: mRNA turnover and processing in mammalian cells

REFEREE'S CROSS-COMMENTING

I find the other reviews balanced and insightful. In essence, we all agree (at least this is my impression) that the work is interesting for a certain smaller readership, but that there is still room for improvement.

Reviewer 3

Evidence, reproducibility and clarity

Summary:

This study provides evidence for a role of P-body factors Scd6 and Edc3 in keeping the yeast decapping enzyme Dcp2 in the cytoplasmic compartment of cells. Concomitant deletion of both genes leads to nuclear localization of Dcp2 in a karyopherin ß Kap95-dependent fashion. Nuclear sequestered Dcp2 does not seem to affect mRNA decay rates, suggesting that Dcp2 is functional in the cytoplasm only. The authors validate and strengthen previous studies suggesting that Dcp2 and functional P-bodies assemble at, or near, the endoplasmatic reticulum (ER). Furthermore, their experiments suggest that Scd6 and/or Edc3 function to bridge Dcp2 and Dhh1, which in turn can facilitate phase separation and P-body assembly and possibly maturation.

Major comments:
1) Generally, most of the presented conclusions are convincing and based on well-conceived and clear experiments, however some conclusions are, in our opinion, based on rather weak and circumstantial evidence. Not all the data is clearly presented and additional experimentation would be required to support conclusions.

2) We find it interesting and convincingly shown that deletion of Scd6 and Edc3 leads to accumulation of Dcp2 (and Dcp1) in the nucleus. Likewise, the kap95-aid strategy elegantly proves that this import receptor is necessary for this localization. The authors should verify the function of the suggested NLS by mutation experiments. It would strengthen the conclusions and enhance the impact of the manuscript if this "nuclear buffer-model" could be validated using an alternative method in a non-Scd6/Edc3-deletion background.

3) The quality of the Dcp2 overexpression experiments presented in figure 2 G-I is of poor quality and quantifications of half-lives should be calculated based on biological triplicate analyses. Also, in figure 2 D, there seem to be cytoplasmic foci in cells expressing NLS-Dcp2 variants - why is this? The expression level of various GFP-Dcp2-versions is very different, based on the presented western blot panel F (e.g. Dcp2 vs. NLS-Dcp2CD), leaving it difficult to firmly compare phenotypes. These issues should at least be discussed when conclusions are made.

4) The evidence from the mitochondria/ER forced localization of Dcp2 (Figure 3) is somewhat confusing and it is unclear what the exact intention is. How does this experimentation contribute with new knowledge? A general concern when comparing e.g. rescue of growth defects by expression of these MITO- and ER-targeted version is expression levels. These should be shown (western blotting) and be very similar to make solid conclusions. In addition, although previous studies have shown that Dcp2 localizes to the ER, the authors should demonstrate that a non-ER-targeted version of Dcp2 co-localizes here (e.g. co-expressing an mCherry version). The co-localization to the ER marker (Figure 3F) is of poor quality and does not clearly suggest ER-localization. These panels should be improved and quantified. The rescue of growth by Dcp2-ER compared to Dcp2-MITO seems quite solid, but this could be due to various effects - e.g. expression levels.

5) In the suggested model, Dcp2 is necessary for PB nucleation at the ER via the recruitment of Scd6 and Edc3, which links to Dhh1 that is important for phase separation of PBs. If Dcp2 nucleates these factors at the ER, then it is hard to reconcile why deletion of Scd6 and Edc3 sends Dcp2 into the nucleus? It would strengthen the conclusions if they could prove some of these interactions biochemically and address the order of events. Also, the expression levels of the various deletion mutants in Figure 4 and fusion constructs in Figure 5 are not shown. Figure 5B is of poor quality and should also be quantified. Is the Edc31-86-Dhh1 fusion nuclear in many cells? Could Dcp2-GFP localization be primarily cytoplasmic due to import competition? Expression levels are important here and this should be controlled.

6) What is the evidence that PB-formation is mainly orchestrated by Dhh1 phase separation? The Parker lab has previously shown that the Edc3 YjeF and Lsm4 prion-like domains promote PB-formation. This is not discussed in detail. Also, depletion of Dcp2 in mammalian cells promote PB formation (number and size), suggesting that decapping-targeted RNAs accumulates here - why this discrepancy between systems? It would be beneficial in general to compare the obtained results to PB-dynamics in mammalian cells.

Minor points:
1) Figure 1 D and E would be better as box-plots.

2) Quantifications of Western and Northern blots plus statistics from triplicate experiments would be beneficial.

3) Nomenclature of Scd6 and Edc3 constucts in Figure 4 B-E could be streamlined.

4) Figure 6 B, color coding is missing.

5) Model figure 7, would be much better with unique factor shapes or factor names written in their respective ovals.

6) Presentation of merged channels could be improved if color schemes were changed from red/green to cyan/magenta.

7) Co-localization analysis of Figure 3 E-F would strengthen the conclusion.

8) Quantification of Dcp2 localization in figure 5 B would be benefitial.

9) Panel labeling of Figure 3 H and Supplementary Figure 3 B-C could be improved.

10) Typing mistake on page 3 line 24 - "of the" is written twice.

11) Typing mistake in figure legend of figure 1 H - "panel H" should be "panel G".

Significance

Generally, the findings presented in this manuscript are interesting. The significance of the manuscript is, in its current form, reasonably high, but the advances in the field are somewhat limited. However, the findings are likely of interest to a relatively specialized audience. The finding that Dcp1/2 could possibly be buffered in the nucleus, as suggested by their nuclear accumulation upon Scd6/Edc3 deletion is novel and a very interesting observation. However, the authors leave it as a phenomenological observation with relatively little characterization. The significance would be improved considerably if these observations could be solidified and shown to play a role for e.g. mRNA decay, the stress response and/or general cell homeostasis.

REFEREE'S CROSS-COMMENTING

I agree with the other reviewer's comments. An Interesting piece of work that could be improved by additional experimentation within the immediate reach of the authors.

Author response to reviewers' comments

We wish to thank the reviewers for the insightful comments, which helped us to improve the manuscript and support our conclusions.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

This manuscript demonstrates a key role for the Dcp2 decapping complex in P body formation that is influenced by nuclear localization of the decapping protein along with ER/mitochondrial associations and interactions with other decapping proteins. Overall I find the story to be very interesting, generally convincing (with one exception noted below) and impactful to the field. I only have a few suggestions to polish the manuscript:

Thank you!

1. Abstract: My understanding is that there remains a debate in the field as to how much mRNA decay (if any) actually occurring in P bodies. Thus I would recommend changing the second sentence in the abstract that states that PBs are the major site of mRNA decay.

We agree with the reviewer that the precise function of P-bodies is currently debated. We changed the abstract accordingly to: Processing bodies (P-bodies) are thought to be sites of mRNA decay and/or storage.

2. Fig. 2G-I: These gels should also be presented in a quantified fashion with statistics to provide optimal support for the conclusions drawn. This is particularly important since the

data shown with NLS-DCP2 in Fig. 2H looks as though the MET3 mRNA is indeed degrading faster, contrary to the authors' conclusion on page 7.

We performed FISH to look at polyA tails in the nucleus. We did not observe a reduction but if at all an accumulation of mRNA in the nucleus in *edc3Δscd6Δ* cells. These data are included into the revised manuscript (Fig. 3C-F). These data from experiments under steady-state and under pulse-chase conditions strongly suggest that nuclear Dcp2 does not promote nuclear mRNA decay in *edc3Δ scd6Δ* cells. (please see also point 3 of Reviewer 3). The Northern blots (incl. quantification) were relegated into Suppl. Material of the revised manuscript (Fig. S3C-E). Thus, we provide two independent strategies to show that nuclear Dcp2 does not promote nuclear mRNA decay.

Reviewer #1 (Significance (Required)):

The study advances our mechanistic understanding of P body formation, particularly in terms of the impact of DCP2, the role for subcellular localization, and the role of specific protein-protein interactions. It will definitely be of interest to the RNA decay and cell biology communities.

Thank you!

I am an RNA decay researcher who focuses on mammalian systems. REFEREE'S CROSS-COMMENTING
I concur - I think that all three reviewers see the inherent value and interest of the study and the polishing comments that are suggested will definitely strengthen the support for the conclusions that are drawn.

Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Evaluation of the manuscript "Decapping complex is essential for functional P-body formation and is buffered by nuclear localization" by Kiril Tishinov and Anne Spang.

Summary

P-bodies are membraneless granules in the cytoplasm of eukaryotic cells that contain proteins of the cellular RNA decay machinery and untranslated mRNAs. While it was initially proposed that P-bodies are sites of cellular mRNA decay, it was later shown that they are not required for general mRNA turnover. The current view is therefore that P-bodies mainly serve as storage sites for translationally repressed mRNA.

Even though we agree with the reviewer that P-bodies also serve as storage sites (we even published a paper about this: Wang et al., elife 2018), we are still open to the longstanding proposed role of P-bodies also as a decay site. Mutations or deletion in/of the exonuclease Xrn1 lead to increased size of P-bodies in yeast, flies and mammalian cells. To reflect both possibilities, we changed the abstract accordingly: Processing bodies (P-bodies) are thought to be sites of mRNA decay and/or storage.

For the work presented in this manuscript, the authors generated a yeast strain lacking both edc3 and scd6 (edc3Δ, scd6Δ) and analyzed the formation of P-bodies in this strain. While single deletions of edc3 and scd6 had only minor effect, the double mutant showed severe defects in P- body formation and a substantial accumulation of Dcp2 in the nucleus. The authors identify putative nuclear localization signals in Dcp2, indicating that Dcp2 is imported into the nucleus by karyopherin α/β. Nuclear Dcp2 does not participate in mRNA turnover, but is likely stored as inactive pool from which it can be released and exported under certain conditions. Moreover, the authors show that Dcp2 initiates the formation of P-bodies in association with ER membranes and that Dcp2 is essential for P-body assembly. Both, Edc3 and Scd6 can bridge the interaction between Dcp2 and Dhh1, which explains why only in the double edc3Δ, scd6Δ strain P-body formation is affected

We would like to point out that we show and reconfirm (Kilchert et al., 2010) that P-body formation is affected in *scd6Δ* cells under Ca^{2+} stress, but not under glucose starvation. This is important because it demonstrate that there is more than one way how P-body formation can be initiated.

Major comments

a) Are other nuclear-localized proteins also affected in their import in the kap95-aid strain even without adding auxin?

We did not test other proteins for nuclear localization, but we assume this is to be the case as Kap95 is required for the import of more than 90% of NLS-containing proteins. We expressed SV40 NLS-3xGFP and determined the nuclear/cytoplasmic ratio in wild-type and the kap95-aid strain. In the kap95-aid strain less GFP was imported into the nucleus. These data are included into the revised manuscript (Fig. S2E and F). Our data are consistent with the notion that due to the lower Kap95 cellular concentration (see also point below), Kap95-dependent nuclear transport is less efficient.

b) What are the levels of Kap95-aid in comparison with wt KAP95? Have the authors tried to inhibit the unintended degradation of Kap95-aid by using the degradation inhibitor "auxinole" (PMID: 31026591)

We have not tried to inhibit the unintended degradation, as this was beyond the point of the experiment. We sought a way to reduce Kap95 levels/function. This was achieved by adding the AID tag. Under these conditions, nuclear import of Dcp2 was blocked. Whether the AID tag impairs Kap95 function or whether it leads to premature degradation, is not really important in this context. Nevertheless, we determined the levels of Kap95 and the AID tagged version.

Addition of the AID tag reduced Kap95 protein levels. These data are included into the revised manuscript (Fig. S2C and D).

c) The expression levels of overexpressed Dcp2-GFP (Figure 2D) exhibit some cellular variability. Would it be possible to use some uniform populations for the Northern blots, e.g. through FACS sorting?

This is an interesting suggestion, but unfortunately not feasible. Understandably, our FACS facility does not want to have any live yeast in their area, and therefore the experiment cannot be performed. However, the FISH experiment you suggest below will take care of this point.

d) Would it not be better if the authors looked for global poly(A)+ amounts in the nucleus by fluorescence in situ hybridization rather than for a specific mRNA (MET3) by Northern blotting (Figure 2G-I)?

We thank the reviewer for the suggestion. In fact, we had already done the experiment and observed an increase in fluorescence intensity in the nucleus. We did not initially include these data in the manuscript because we felt the Northern blots would provide more direct evidence. We included the FISH data into the revised manuscript (Fig. 3C-F). The Northerns (including the quantifications) were relegated to Supplementary Material (Revised manuscript, Fig. S3C-E).
Thus, we provide two independent experimental lines to show that nuclear Dcp2 does not induce nuclear mRNA decay.

e) Have the authors tried to mutate the predicted NLS-sequences of Dcp2 to see if these are mediating the nuclear import of Dcp2?

We mutated the predicted NLS-sequences and determined how these mutations affect Dcp2 localization and protein stability as well as cellular homeostasis. When we repeated the bioinformatic analyses, we found an additional, rather cryptic NLS that would be in the RNA

binding site. Of the four potential NLSs, two of them when mutated, interfered with Dcp2 nuclear localization, and the effect on nuclear localization was additive when both NLSs were mutated. These data indicate that mRNA binding to Dcp2 might interfere with nuclear localization and that this be part on how optimal cytoplasmic Dcp2 concentration is sensed; the other part might be through Scd6 and Edc3. These new data are included in the revised version of the manuscript (Fig. 2B-D and S2A and B).

f) Did the authors consider/test if binding of Edc3 and/or Scd6 to Dcp2 masks some of the predicted nuclear localization sequences? This would explain the localization of Dcp2 in the double KO strain.

We checked the available structures in pdb. From there it is not clear whether Edc3 and Scd6 would mask the potential NLS in Dcp2. During these analyses, however, we found that the RNA binding site could potentially also serve as a cryptic NLS. We tested this possibility, and indeed the RNA binding site in Dcp2 contains an NLS, indicating the RNA binding state of Dcp2 contributes to the nuclear-cytoplasmic distribution. (see also point above)

Minor comments

a) Abstract, second sentence: "Cytoplasmic mRNA is largely turned over in processing bodies (P-bodies)" should be revised, because it is not clear that P-bodies play an important role in mRNA turnover.

We agree with the reviewer that the precise function of P-bodies is currently debated. We changed the abstract accordingly to: Processing bodies (P-bodies) are thought to be sites of mRNA decay and/or storage.

b) Not all immunofluorescence data are shown in Figure 1G (wt is missing), while the quantifications in Figure 1H include wt. I suggest that the missing data are shown in a supplementary figure.

We apologize for the omission. The data will be included in the revised manuscript (Fig. S1A).

Reviewer #2 (Significance (Required)):

While the first two figures provide very interesting insights into the regulation of P-bodies and Dcp2 by Edc3 and Scd6, the story loses some of its focus in Figure 3 and onwards. Indeed, in some cases the significance of the results is not entirely clear to me and the synthetic-lethal screens provide rather indirect readouts. This is especially true for the ER- and Mitochondria results, which do not really seem to fit into the story. I would even suggest removing Figure 3 and adding other data to the manuscript instead.

We are sorry that our attempts to reveal mechanistic insights in P-body regulation and assembly appear to be less appealing to this reviewer. Localization does matter, and this is clearly also true for P-bodies. When we restrict P-body formation to the outside of mitochondria, these P- bodies cannot function properly and the cells fail to cope with stress appropriately. This effect is exacerbated when ER-mitochondrial contact sites are eliminated. If functional P-body formation could occur anywhere in the cytoplasm, tethering the P-bodies to the cytoplasmic face of mitochondria should not affect their function and clearly cells would not care about ER- mitochondrial contact sites. We maintain that this an important and novel finding and therefore we did not remove these data, we rather expanded them: We used the $Dcp2^{ER}$ construct in conjunction with Dcp2 to provide independent data on the Dcp2 buffering in the nucleus.

Apart from that, it is a solid piece of work with high-quality data that provides interesting insights into P-body biology for interested readers in that field. The function of Dcp2 and its localization for P-body formation in yeast is examined in detail, although some additional experiments would further strengthen the work. However, the story is a bit too specific and of not enough general interest for a very broad readership.

In this manuscript, we provide the first functional account on buffering of the essential decapping complex in the nucleus (Revised manuscript, Fig. 3G and H). This is a novel regulatory pathway. We show that this Dcp1/2 can be released under stress into the cytoplasm to cope with the increasing demand of decapping activity (Revised manuscript, Fig. 3I-K). This mechanism provides the basis of a stress response within 5 min after the insult. This response is essential for the cell to cope with stress. This buffering mechanism is most likely conserved from yeast to mammals because one of the NLSs that we identified in Dcp2 is also present in human Dcp2.
With the experiments and analyses performed during the revisions, we provide more insights into the mechanism of nuclear buffering and took the manuscript to the next level. We think that we uncovered a novel regulatory mechanism required for mRNA homeostasis and stress response. These finding should be interesting for a broad readership.

This reviewer's field of expertise: mRNA turnover and processing in mammalian cells REFEREE'S CROSS-COMMENTING

I find the other reviews balanced and insightful. In essence, we all agree (at least this is my impression) that the work is interesting for a certain smaller readership, but that there is still room for improvement.

Naturally, we disagree that our work is only of interest to a certain smaller readership. We uncover a novel mechanism which is essential for stress response and cellular RNA homeostasis.

Reviewer #3 (Evidence, reproducibility and clarity (Required)):

Summary:

This study provides evidence for a role of P-body factors Scd6 and Edc3 in keeping the yeast decapping enzyme Dcp2 in the cytoplasmic compartment of cells. Concomitant deletion of both genes leads to nuclear localization of Dcp2 in a karyopherin ß Kap95-dependent fashion. Nuclear sequestered Dcp2 does not seem to affect mRNA decay rates, suggesting that Dcp2 is functional in the cytoplasm only. The authors validate and strengthen previous studies suggesting that Dcp2 and functional P-bodies assemble at, or near, the endoplasmatic reticulum (ER). Furthermore, their experiments suggest that Scd6 and/or Edc3 function to bridge Dcp2 and Dhh1, which in turn can facilitate phase separation and P-body assembly and possibly maturation.

Major comments:

1) Generally, most of the presented conclusions are convincing and based on well-conceived and clear experiments, however some conclusions are, in our opinion, based on rather weak and circumstantial evidence. Not all the data is clearly presented and additional experimentation would be required to support conclusions.

Thank you for the general assessment that most of our data are convincing and based on well-conceived experiments. We performed additional experiments which strongly support our previous conclusions.

2) We find it interesting and convincingly shown that deletion of Scd6 and Edc3 leads to accumulation of Dcp2 (and Dcp1) in the nucleus. Likewise, the kap95-aid strategy elegantly proves that this import receptor is necessary for this localization. The authors should verify the function of the suggested NLS by mutation experiments. It would strengthen the conclusions and enhance the impact of the manuscript if this "nuclear buffer-model" could be validated using an alternative method in a non-Scd6/Edc3-deletion background.

We mutated the predicted NLSs in Dcp2 and analyze the localization and protein stability of these mutants as well as determine their contribution to cellular homeostasis/viability/stress

responsiveness (Revised manuscript, Fig. 2B-D and S2A and B). This would nicely complement the data on NLS-Dcp2. Moreover, we will also mutate a potential NLS, present in the RNA binding site (see also response to Reviewer 2 point f).
We reasoned that if the nuclear Dcp2 pool buffers the cytoplasmic pool, we should be able to drive the nuclear-stored Dcp2 into the cytoplasm. To test this, we treated cells that express NLS- Dcp2-GFP with Verrucarin A, a peptidyl-transferase inhibitor, to block translation initiation and increase the amount of non-translating mRNA, hence the demand for Dcp2 in the cytoplasm. Our data indicate that even though Dcp2 is efficiently retained in the nucleus by the strong SV40 NLS under normal growth conditions, under Verrucarin A treatment, NLS-Dcp2 relocates partially to the cytoplasm (Revised manuscript, Fig. 3I-K). Moreover, when we treat the *edc3Δ scd6Δ* strain, in which Dcp2-GFP is nuclear, such redistribution into the cytoplasm does not occur. Therefore, with this line of experiments we provide an independent way to demonstrate Dcp2 buffering in the nucleus and strengthen our conclusion that Dcp2 is retained in the cytoplasm by Scd6 and Edc6.

3) The quality of the Dcp2 overexpression experiments presented in figure 2 G-I is of poor quality and quantifications of half-lives should be calculated based on biological triplicate analyses. Also, in figure 2 D, there seem to be cytoplasmic foci in cells expressing NLS-Dcp2 variants - why is this? The expression level of various GFP-Dcp2-versions is very different, based on the presented western blot panel F (e.g. Dcp2 vs. NLS-Dcp2CD), leaving it difficult to firmly compare phenotypes. These issues should at least be discussed when conclusions are made.

We would like to stress that all experiments reported in this manuscript were independently performed at least three times. We never publish preliminary observations.

We have included a polyA mRNA FISH experiment to look at the nuclear mRNA degradation (Revised manuscript, Fig. 3C-F), which shows no excess mRNA degradation when Dcp2 is enriched in the nucleus either in the *edc3*Δ *scd6*Δ background or by an overexpression of NLS-Dcp2. The Northern (including the quantifications) have been relocated to Supplementary Material (Revised manuscript, Fig. S3C-E). Thus, we provide two independent experimental lines to show that nuclear Dcp2 does not induce nuclear mRNA decay.

The reviewer is right, that in figure 2D cytoplasmic foci can be observed. The reason for this is that this strain expresses endogenously Dcp2-GFP and the plasmid borne NLS-Dcp2-GFP. This also applies to the panels E and F. We wanted to test in these experiments whether NLS-Dcp2 constructs would also drive the import of endogenous Dcp2 (i.e. in a complex). This appeared not to be the case.

We have provided the quantifications of the Western blot analysis in Fig 2F. The overexpression levels are about 2-3 fold for the catalytically dead versions and 4-5 fold for WT and the NLS-WT constructs (Revised manuscript, Fig. S3A and B).

4) The evidence from the mitochondria/ER forced localization of Dcp2 (Figure 3) is somewhat confusing and it is unclear what the exact intention is. How does this experimentation contribute with new knowledge? A general concern when comparing e.g. rescue of growth defects by expression of these MITO- and ER-targeted version is expression levels. These should be shown (western blotting) and be very similar to make solid conclusions. In addition, although previous studies have shown that Dcp2 localizes to the ER, the authors should demonstrate that a non-ER-targeted version of Dcp2 co-localizes here (e.g. co-expressing an mCherry version). The co-localization to the ER marker (Figure 3F) is of poor quality and does not clearly suggest ER-localization. These panels should be improved and quantified. The rescue of growth by Dcp2-ER compared to Dcp2-MITO seems quite solid, but this could be due to various effects -e.g. expression levels.

We were apparently not clear enough in presenting our rational for these experiments. The novelty of these findings is that P-bodies need to be localized at the ER to be functional- if not the cells die under stress! Even though PB-like structures or pseudo PBs can be formed in the cytoplasm (freely diffusing as is most widely thought) or as we did, formed them on the cytoplasmic face of mitochondria, these structures are not functional. Therefore, **location matters**!!. P-bodies do not form randomly in the cytoplasm but at distinct/specific sites on

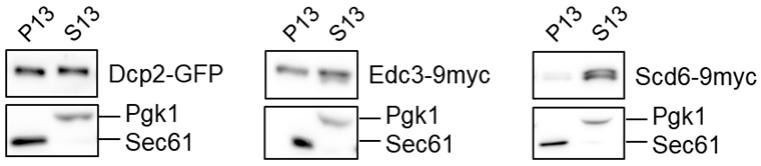
the endoplasmic reticulum. This has not been appreciated at all in the field and is a novel and important finding

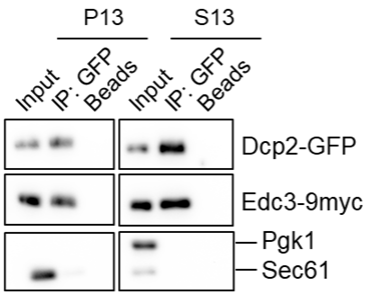
We co-expressed the Dcp2-GFP^{ER} and Dcp2-mCherry. While we did not observe much intermixing of the Dcp2 pools, this experimental setup provided unexpectedly additional confirmation of the nuclear Dcp2 as buffer and indicated that cytoplasmic Dcp2 levels are tightly controlled. Excess Dcp2-mCherry was transported into the nucleus. Moreover, we observed three expression patterns: co-expression of both Dcp2 variants to about the same level, or expression of one variant at the expense of the other. These data suggest that besides the nuclear buffering, additional mechanism(s) exist to control the levels of Dcp2 in the cytoplasm, and that the cytoplasmic Dcp2 levels are tightly controlled. These data have been added to the manuscript (Fig. 3 G and H).
We quantified the expression levels of Dcp2^{ER} and Dcp2^{MITO} in *edc3Δ scd6Δ* cells and Dcp2^{MITO} in the *edc3Δ scd6Δ mmm1Δ* strain and did not find any significant differences. These data are included in the revised manuscript (Fig. S4C and D, H and I). Therefore, we can conclude that the rescue is not due to different expression levels.

5) In the suggested model, Dcp2 is necessary for PB nucleation at the ER via the recruitment of Scd6 and Edc3, which links to Dhh1 that is important for phase separation of PBs. If Dcp2 nucleates these factors at the ER, then it is hard to reconcile why deletion of Scd6 and Edc3 sends Dcp2 into the nucleus? It would strengthen the conclusions if they could prove some of these interactions biochemically and address the order of events. Also, the expression levels of the various deletion mutants in Figure 4 and fusion constructs in Figure 5 are not shown. Figure 5B is of poor quality and should also be quantified. Is the Edc3$^{1-86}$-Dhh1 fusion nuclear in many cells? Could Dcp2-GFP localization be primarily cytoplasmic due to import competition? Expression levels are important here and this should be controlled.

We performed the requested Western blots. Our data show comparable levels of the different constructs. We would also like to point out that we use the Dhh1 promoter for the constructs in Figure 5, and not a heterologous promoter. Thus, we can exclude that there is import competition. The data on the expression levels and the quantification thereof, as well as the quantification of Fig. 5B are included in the revised manuscript (Fig. 5C, S5A and B, S6A and B).

Our biochemical data suggests that Dcp2 outside the nucleus is distributed between the cytoplasm and ER membranes, and is bound to either Scd6 or Edc3, which prevents its nuclear import. Moreover, Edc3 can be co-immunoprecipitated with Dcp2-GFP from soluble and membrane fractions. Please see the data below that we have appended for your information.





The Scd6-Dcp2 interaction was weaker in our hands, but there is structural and biochemical data on this interaction (Fromm et al., EMBO J. 2012). Excess Dcp2 will go to the nucleus and some of it might be degraded. The data from the co-expression experiment of Dcp2-GFP^{ER} and Dcp2-mCherry suggest a tight control of Dcp2 levels. Since our biochemical analyses did not provide any information beyond that what was already published, we did not include these data in the manuscript.

To determine the order of events for P-body assembly at the ER, we anchored truncated versions of Edc3 and Scd6 insuring either only Dcp2 binding or interaction with both Dcp2 and Dhh1 at the ER in an *Δedc3* strain and tested whether Dcp2 can still be recruited to form PBs. As predicted by our model, either Edc3 or Scd6 on the ER was sufficient to recruit Dcp2 (Revised manuscript, Fig. S5C and D). These data are also consistent with published data from the Weis lab who showed slow FRAP recovery rates for Edc3 and Dcp1/2 (Mugler, Hondele et al eLife, 2016)

How Dcp2 is recruited to the ER is indeed not entirely clear. One possibility that we will explore is coincidence detection. We have shown previously that Dcp2 -as well as Scd6- are associated with polysomes at the ER (Weidner et al. 2014).

6) What is the evidence that PB-formation is mainly orchestrated by Dhh1 phase separation? The Parker lab has previously shown that the Edc3 YjeF and Lsm4 prion-like domains promote PB-formation. This is not discussed in detail. Also, depletion of Dcp2 in mammalian cells promote PB formation (number and size), suggesting that decapping-targeted RNAs accumulates here - why this discrepancy between systems? It would be beneficial in general to compare the obtained results to PB-dynamics in mammalian cells.

The Weis lab has shown recently in two very exciting papers how Dhh1 drives phase separation and P-body formation. Moreover, we have shown previously that there are at least two different pathways that can drive P-body formation (Kilchert et al. 2010), which converge on Dhh1. In the original version of the manuscript, we already discussed the pathway for P-body formation put forward by Roy Parker et al. We think that Edc3 is the common denominator of at least the two pathways that are driving phase separation. As indicated by the fact that a number of P-body components contain unstructured regions and that these regions have the propensity to form low affinity contacts, they all can contribute in some way to the phase separation. The data on Dhh1 are just very compelling and nothing to a similar extent has been shown for the Lsm4 prion domain. We also mention now the paper by Decker et al (JCB 2007) on Edc3 and Lsm4 in the discussion; we did cite the paper in the previous version throughout the manuscript.

Minor points:

1) Figure 1 D and E would be better as box-plots.

We respectfully disagree that box plots would better. In the case of the *edc3Δscd6Δ* strain, almost 100% of cells do not form any PBs and therefore would be on the 0 line, i.e. x-axis, which would make our finding less clear.

2) Quantifications of Western and Northern blots plus statistics from triplicate experiments would be beneficial.

We finished the quantifications of the Western blots and did not observe any surprises in the expression levels. As for the Northerns, we relegated them to Suppl. Material (incl. quantifications) and provide now FISH experiments to show that Dcp2 in the nucleus does not drive mRNA decay.

3) Nomenclature of Scd6 and Edc3 constucts in Figure 4 B-E could be streamlined.

Done

4) Figure 6 B, color coding is missing.

Done

5) Model figure 7, would be much better with unique factor shapes or factor names written in their respective ovals.

While we think writing the names into the shapes is not useful (too small, hard to read), we changed the shapes.

6) Presentation of merged channels could be improved if color schemes were changed from red/green to cyan/magenta.

We will change the merged channels to cyan/magenta to make the figure also accessible to the color blind. We apologize for the negligence.

7) Co-localization analysis of Figure 3 E-F would strengthen the conclusion.

We improved the imaging and proved new pictures.

8) Quantification of Dcp2 localization in figure 5 B would be benefitial.

Done

9) Panel labeling of Figure 3 H and Supplementary Figure 3 B-C could be improved.

Done

10) Typing mistake on page 3 line 24 - "of the" is written twice. corrected
11) Typing mistake in figure legend of figure 1 H - "panel H" should be "panel G". corrected

Reviewer #3 (Significance (Required)):

Generally, the findings presented in this manuscript are interesting. The significance of the manuscript is, in its current form, reasonably high, but the advances in the field are somewhat limited. However, the findings are likely of interest to a relatively specialized audience. The finding that Dcp1/2 could possibly be buffered in the nucleus, as suggested by their nuclear accumulation upon Scd6/Edc3 deletion is novel and a very interesting observation. However, the authors leave it as a phenomenological observation with relatively little characterization. The significance would be improved considerably if these observations could be solidified and shown to play a role for e.g. mRNA decay, the stress response and/or general cell homeostasis.

We took our analysis to the next level by the identification of two NLSs in Dcp2 and by providing a potential mechanism in that the RNA binding to Dcp2 will mask one of the NLSs ensuring that more Dcp2 stays in the cytoplasm. When no RNA is bound, the NLS is recognized and sends Dcp2 into the nucleus. We assume that the second NLS could be masked by the interaction with Edc3 or Scd6. Moreover, we provide independent evidence for Dcp2 buffering in the nucleus by the expressing a membrane anchored and cytoplasmic Dcp2. Under these conditions, excess cytoplasmic Dcp2 was transferred into the nucleus. Moreover, we show that the nuclear localized Dcp2 serves as a readily releasable pool because under stress conditions promoting translational attenuation, Dcp2 sequestered in the nucleus either by a strong independent NLS or in the *edc3Δ scd6Δ* mutant was released into the cytoplasm. Finally, we show that an mRNA, which is decayed in P-bodies under glucose starvation is stabilized when Dcp2 is depleted. The stabilization was reversed to decay when we expressed NLS^{SV40}-Dcp2-GFP. These data demonstrate the biological significance in addition to the inability of cells to cope with stress, when Dcp2 was mislocalized, which was already part of the previous version of the manuscript. We show that Dcp2 cytoplasmic localization is not sufficient, it has to be on the endoplasmic reticulum to perform its essential function under stress. Recruiting Dcp2 just

on the cytoplasmic face of mitochondria is not sufficient to efficiently cope with and respond to stress, demonstrating that P-body formation on the ER is key to survive stress.

REFEREE'S CROSS-COMMENTING

I agree with the other reviewer's comments. An Interesting piece of work that could be improved by additional experimentation within the immediate reach of the authors.

Original submission

First decision letter

MS ID#: JOCES/2021/259156

MS TITLE: The mRNA decapping complex is buffered by nuclear localization

AUTHORS: Kiril Tishinov and Anne Spang
ARTICLE TYPE: Research Article

Thank you for sending your manuscript to Journal of Cell Science through Review Commons

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This manuscript demonstrates a key role for the Dcp2 decapping complex in P body formation that is influenced by nuclear localization of the decapping protein along with ER/mitochondrial associations and interactions with other decapping proteins. It experimentally establishes the fact that Dcp2 protein localization matters biologically

Comments for the author

The authors have done an excellent and thorough job in responding to the previous round of review. I find the manuscript to be improved and convincing.

Reviewer 2

Advance summary and potential significance to field

P-bodies are membrane less assemblies in the cytoplasm of eukaryotic cells that contain proteins of the cellular RNA decay machinery, as well as untranslated mRNAs. In this work, the authors generated yeast strains lacking the P-body components edc3 and scd6. Single deletions of edc3 and scd6 had only minor effects, but the double mutant showed severe defects in P-body formation and a substantial accumulation of Dcp2 in the nucleus, mediated by import via Kap95. The authors suggest that nuclear Dcp2 does not participate in mRNA turnover, but is stored as inactive pool, from which it can be released and exported under certain conditions, e.g. stress. Moreover, the authors show that Dcp2 initiates the formation of P-bodies in association with ER membranes and that Dcp2 is essential for P-body assembly.

Overall, the data are convincing and of high quality, the story is novel and will be interesting to colleagues working in the field of decapping, P-bodies and mRNA turnover in yeast.

Comments for the author

I had already reviewed this manuscript for Review Commons. So, this time I focus less on the general significance and quality of the work, but more on how the manuscript has been revised since then. Back then, I had listed a number of critical points, many of which have been addressed in the version submitted to JCS. I think the work has been significantly improved and now a well-rounded story is presented. I highly appreciate the work of the two authors and recommend that the manuscript is accepted for publication in its present form.