Cdc48 regulates intranuclear quality control sequestration of the Hsh155 splicing factor in budding yeast

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MS TITLE: Cdc48 regulates intranuclear quality control sequestration of the Hsh155 splicing factor

AUTHORS: Veena Mathew, Arun Kumar, Yangyang Kate Jiang, Kyra West, Annie S Tam, and Peter C Stirling
ARTICLE TYPE: Short Report

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

I have now received comments on your manuscript from two experts. As you will see, both thought that the work was potentially quite interesting and significant but all also raised a number of concerns that must be dealt with before the manuscript can be reconsidered. Please address these issues as thoroughly as possible. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this report, the authors show that the ATPase chaperone Cdc48 is required to control deposition of proteins at the intranuclear protein quality control (INQ) sites. Interestingly, they also show that Cdc48 interacts with splicing factor Hsh155 and regulates its assembly with partner proteins. These new findings build on the authors' previous data showing that Hsh155 localizes to the INQ upon genotoxic stress. They now show that in absence of a functional Cdc48, Hsh155 redistributes to INQ supporting a novel role for Cdc48 in protein deposition at the INQ.

Comments for the author

I think the study is novel and appropriate for presentation as a short report. My only comments are minor. The authors should always include the wild-type control on their figures. per example on figures 2c, 2f, 3b. Also Lots of disease-causing alleles affects/localize to INQ. A couple lines about a potential for Cdc48 modulating these responses could be added.

Reviewer 2

Advance summary and potential significance to field

In the present study, Mathew et al. show that Cdc48 localizes into INQ (intranuclear quality control) structures after inducing genotoxic stress with MMS. Loss of function of Cdc48 in a ts-mutation (temperature-sensitive mutation) yeast cell line increased the Hsh155, a key component of SF3B complex localization to INQ structures. Expressing the wide-type Cdc48 but not the ATPase-deficient Cdc48 prevented MMS-induced Hsh155 foci formation in Cdc48 ts-mutant, suggesting the regulation is depend on Cdc48 ATPase activity. Moreover mutation of the SUMO interaction domain of Cdc48 also failed to suppress MMS-induced Hsh155 foci formation, indicating a potential role of SUMO in regulating Cdc48 mediated Hsh155 foci. Finally, Cdc48 physically interacted with Hsh155 which promoted the SF3B splicing complex formation. Thus, the authors conclude that Cdc48 regulates the deposition of Hsh155 at the INQ and potentially regulates splicing. Overall, the study is intriguing, and the data are convincing and clear. Addressing the following comments would strengthen the manuscript.

Comments for the author

Specific comments.

1. In the study, several statistics results were presented without representative images. Representative images for figures 2C, 3A, 3B, 3C, 4B should be shown.

2. In Figure 1B, the quantification of INQ and CytoQ were not represented, the authors need to label the INQ foci and CytoQ foci separately. Same goes for Figure 1C, quantify Hos-mCherry foci and the Cdc48 foci in the untreated condition.

3. In Figure 1D, the authors should co-stain with PQC marker Hos2 to confirm the mutation disrupts the PQC structures.

4. In Figure 2A bottom panel, the DIC image is out of focus.

5. Does the mutation cdc48-2,-3, or -4 affects cdc48 localization?

6. In Figure 3D. Please show a successful pull-down of Hsh155GFP by western blotting.

7. In Figure 4D, add an IgG control.

8. In Figure S1C it would be helpful to add a schematic presentation of Cdc48-2 and Cdc48-3 constructs used.

First revision

Author response to reviewers' comments

Dear Dr. Carmo-Fonseca,

Thank you for facilitating the positive assessment of our work on Cdc48 and intranuclear quality control sites. We have been able to respond to all of the suggested revisions with new data, revised text and explanations as appropriate. The changes to the manuscript are all highlighted in the manuscript file and I enclose a point-by-point rebuttal below. I also copied this rebuttal into the Response to Reviewers box but I am not sure if the image will show up.

I hope you also feel we have satisfactorily addressed the reviewers comments and improved the manuscript. I look forward to hearing from you.

Best wishes,
Peter Stirling

Point-by-point response to reviewers. Reviewer comments are placed in << >>

Reviewer 1
<<I think the study is novel and appropriate for presentation as a short report. My only comments are minor. >>
We thank the reviewer for this positive assessment of our work.

<<1. The authors should always include the wild-type control on their figures. per example on figures 2c, 2f, 3b.>>
We have now included the wildtype controls in revised versions of Figures 2 and 3. As the number of foci observed in WT for each condition is 0, the bar is invisible.

<<2. Lots of disease-causing alleles affects/localize to INQ. A couple lines about a potential for Cdc48 modulating these responses could be added. >>
We agree that this is an interesting point. The possible consequences of disrupting human nuclear protein quality control in diseases associated with mutations in Cdc48, SF3B1 (human Hsh155) or other INQ resident proteins is unknown but clearly of interest. Little is known about human INQ, although we speculate it relates to nucleoli as quality control sites. Given how little is known we have expanded our discussion of possible human disease connections only slightly in the Context and Perspective section on Page 5. We hope this highlights the potential of the work without getting into extended speculation. The revised text reads:

“There is currently little evidence linking Cdc48 to splicing. Interestingly, a study of motor neuron transcriptome dynamics during iPS differentiation models of human VCP/Cdc48 mutations in amyotrophic lateral sclerosis showed that abnormal intron retention events were increased in the *Cdc48/VCP*-mutated cells (Luisier et al., 2018). Thus, a role for Cdc48 in splicing across species is possible, and we hope that additional research will elucidate if and how Cdc48 may affect splicing. In this regard it is notable that the existence of an orthologous human intranuclear quality control response could involve disease genes including the ALS gene Cdc48/VCP, and Hsh155/SF3B1, which is frequently mutated in various types of cancer (Yoshida and Ogawa, 2014). Given the proximity of INQ to the nucleolus, we speculate that human studies highlighting the nucleolus as a phase-

separated quality control compartment may reflect a conserved phenomenon to the INQ in yeast (Frottin et al., 2019; Tkach et al., 2012). If so, then studies in model organisms should provide insight to nuclear protein quality pathways that could modulate human disease."

Reviewer 2

<<Overall, the study is intriguing, and the data are convincing and clear. Addressing the following comments would strengthen the manuscript. >>

We are really happy that both reviewers saw the potential interest of linking Cdc48 to both INQ and splicing.

<<1. In the study, several statistics results were presented without representative images. Representative images for figures 2C, 3A, 3B, 3C, 4B should be shown.>>

As requested we have now added representative images above Figure 2C, and Figure 3A, 3B and 3C. For Figure 4B we are interested in reporting on the lifetime of total Hsh155 protein pools and the important information is the nuclear intensity not the relative localization of the tandem fluorescent timer fusions between strains. Therefore, in this case we elected to add the requested representative images to the Supplementary Figure S1G, and an associated quantification of protein lifetimes in subcellular structures in Figure S1H. These data are consistent with the simpler measurement we report in the main text to support changes in INQ substrate turnover in *cdc48-4* alleles.

<<2. In Figure 1B, the quantification of INQ and CytoQ were not represented, the authors need to label the INQ foci and CytoQ foci separately. Same goes for Figure 1C, quantify Hos-mCherry foci and the Cdc48 foci in the untreated condition.>>

We thank the reviewer for pointing this out. We now indicate the peri-nuclear INQ foci with a white arrow and the peripheral cytoQ with a yellow arrow in a revised Figure 1. The revised Figure 1C shows the INQ and cytoQ quantification and the untreated condition where no foci are observed. In addition we have also imaged Cdc48-GFP with a histone-mCherry fusion (HTA2-mCh) in order to better delineate INQ from cytoQ without relying on the Hos2 marker. These confirmatory data are shown in Figure S1A.

<<3. In Figure 1D, the authors should co-stain with PQC marker Hos2 to confirm the mutation disrupts the PQC structures. >>

We have added a panel to Supplementary Figure 1B showing quantification of Hos2-mCherry and Cdc48-GFP together in the WT, *btn2*Δ, *hsp42*Δ and *apj1*Δ strains. Hos2 localization confirms published reports that loss of *HSP42* or *BTN2* decreases INQ, while loss of *APJ1* increases INQ. These data also confirm that Cdc48 follows the same trend as we reported in Fig. 1D. Since this is a new set of strains, we have left Figure 1D and now refer to the new confirmatory control data as **Figure S1B**.

<<4. In Figure 2A bottom panel, the DIC image is out of focus.>>

We have now replaced the DIC image in the bottom panel of Figure 2A. As before some cells or buds on cells may appear out of focus. This is not unusual as the cells are actively growing and not fixed onto the slides other than binding to concanvalin A used to pre-treat the slides. The reviewer should rest assured that we do not score cells where the mother cell body is not in focus.

<<5. Does the mutation cdc48-2,-3, or -4 affects cdc48 localization?>>

This is certainly an interesting question and we acknowledge that many mechanistic questions remain around the effects of Cdc48 mutant alleles on INQ. ts-alleles can work in a number of ways including protein destabilization, mis-localization, loss of interactions or dominant negative effects. Our model links Cdc48 function to INQ but does not currently investigate the potential for mis-localization to be a driver of these defects. We have investigated this question in a couple of ways but see no major defects in Cdc48 localization in the ts-alleles.

First we have conducted nuclear and cytoplasmic fractionation experiments which show that the Cdc48 protein is distributed normally to both compartments in WT and ts-alleles (Figure R1, below). Next, we performed immunofluorescence using a native Cdc48 antibody and again observed a punctate distribution of Cdc48 across nuclear (DAPI) and cytoplasmic compartments (Figure R2, below). Thus, to date we have no strong evidence that mis-localization plays an important role in

the effects we observed. Since this is a negative result and does not really speak to the mechanism of loss-of-function, we did not include this data in the revised manuscript. The important point for us is that Cdc48 ts-alleles are well characterized loss-of-function alleles that impact INQ formation and survival after genotoxic stress. We have no evidence that drastic localization changes are the driver of these effects.

Figure R1

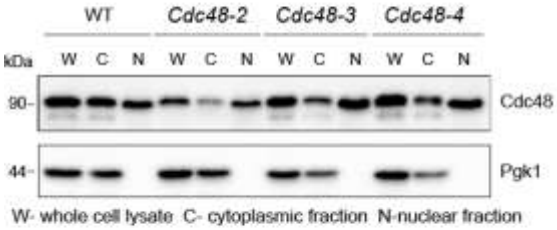
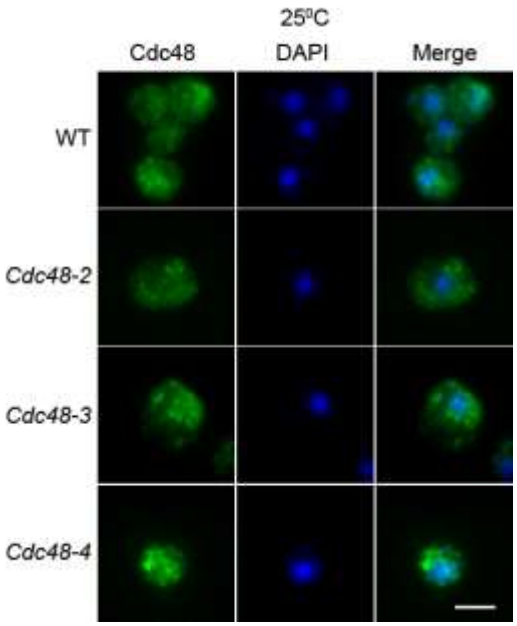


Figure R2



<<6.In Figure 3D. Please show a successful pull-down of Hsh155GFP by western blotting.>>
Figure 3D shows isolation of SUMOylated proteins through Hisx7 tags binding to Nickel beads. We are not actually pulling down GFP in this experiment but simply using it as a tag for western blotting. As you can see the input of Hsh155-GFP is equivalent (lower panel) but that none is pulled down. This indicates that Hsh155 is not detectably SUMOylated in this assay. RFA1, the large subunit of RPA, is used as a positive control and as expected shows MMS-induced SUMOylation. A separate GFP pulldown of Hsh155 is certainly possible but would not relate to the SUMO pulldowns in Figure 3 or the TAP pulldowns in Figure 4.

We have altered to text to try and clarify that the result relates to SUMO pulldowns.

Page 3: “Since we used Hsh155-GFP as a marker for INQ we elected to directly test whether Hsh155 is SUMOylated using an Smt3-Hisx7 purification scheme. While SUMOylation of the Rfa1-GFP control was easily detectable upon MMS treatment, no detectable pulldown of Hsh155-GFP with an Smt3-Hisx7 tag was observed with or without MMS (**Figure 3D**).”

<<7. In Figure 4D, add an IgG control.>>
TAP pulldowns rely upon the interaction of Protein A with immunoglobulin bound beads. Therefore, one of the best controls in our view is to conduct the entire pulldown in an isogenic yeast strain lacking only the TAP fusion. These pulldowns are represented in our “GFP only” lanes. In these lanes, an otherwise identical yeast strains with Hsh155-GFP (4C) or Cdc48-GFP (4D) is used for the pulldown with IgG beads. Any non-specific binding to the beads by these GFP fusions should be evident in these lanes. No non-specific binding is observed in our experiments. Pulldown of GFP-fusion proteins with IgG beads only occurs in strains also expressing the TAP fusions, supporting that there is a physical interaction that can be detected whether Hsh155 or Cdc48 is used as the bait.

We have rewritten our figure legend to better reflect that the ‘GFP only’ controls are really IgG beads and lysate from otherwise isogenic cells without the TAP fusion. In addition, in case we mis-understood the reviewer, we also re-ran the pulldown in Fig. 4D with a true IgG beads-only control

(no lysate) to ensure no contaminant or antibody was interfering with our results. The legend now adds:

"Control (GFP only) lanes are IgG bead IPs from otherwise isogenic cells expressing Hsh155-GFP or Cdc48-GFP without a TAP fusion to account for any non-specific binding. The IgG bead lane is a no lysate control for the beads."

<<8. In Figure S1C it would be helpful to add a schematic presentation of Cdc48-2 and Cdc48-3 constructs used.>>
We have revised **Figure S1E** to show the schematics of the *cdc48-2* and *cdc48-3* alleles. We now cite references which indicate the relevant mutations (Verma et al., 2011; Simoes et al., 2018). As noted in the methods, we did not sequence verify these alleles here. However, cdc48-4 was previously unsequenced and we provide new sequence information here for that allele (also in **Fig. S1E**).

Second decision letter

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AUTHORS: Veena Mathew, Arun Kumar, Yangyang Kate Jiang, Kyra West, Annie S Tam, and Peter C Stirling
ARTICLE TYPE: Short Report

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

In this report, the authors show that the ATPase chaperone Cdc48 is required to control deposition of proteins at the intranuclear protein quality control (INQ) sites. Interestingly, they also show that Cdc48 interacts with splicing factor Hsh155 and regulates its assembly with partner proteins. These new findings build on the authors' previous data showing that Hsh155 localizes to the INQ upon genotoxic stress. They now show that in absence of a functional Cdc48, Hsh155 redistributes to INQ supporting a novel role for Cdc48 in protein deposition at the INQ.

Comments for the author

All my concerns have been addressed. I recommend the manuscript to be accepted for publication.

Reviewer 2

Advance summary and potential significance to field

The authors have addressed all my comments in their revised version.

Comments for the author

The authors have addressed all my comments in their revised version.