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The pseudokinase TRIB1 toggles an intramolecular switch to regulate COP1 nuclear export

Jennifer E. Kung and Natalia Jura

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 8th Jun 2018

Thank you for submitting your manuscript on Trb1 pseudokinase regulation of COP1 nuclear export to The EMBO Journal. I need to apologize for the fact that it has taken significantly longer than usual to get back to you with a decision, owed solely to the fact that one of the three referees who agreed to evaluate your paper has - despite multiple reminders and repeated promises to deliver - still not provided a report. To avoid further loss of time, I have therefore decided to proceed with this manuscript based on the two reports at hand, which I am forwarding to you copied below. As you will see, while both reviewers 1 and 3 consider your study in principle interesting and potentially important, especially referee 1 raises a number of significant concerns that would need to be decisively clarified before publication would appear to be warranted. Most pertinent among those are questions regarding protein functions at endogenous levels of expression, and consideration of COP1 interactions with its other known binding factors.

Should you be able to satisfactorily address these key concerns, as well as the various other major and minor points raised in both reports, we would be interested in considering a revised manuscript further for consideration. I do understand that this may require substantial further time and experimental effort, but nevertheless have to stress that our policy to allow only a single round of major revision will make it important to carefully respond to all points raised at this stage. Therefore, should you have any questions or comments regarding the referee reports or this decision, please do not hesitate to get back to me already during the early stages of your revision. We might further discuss possible extension of the revision period (beyond the regular three months), during which time the publication of any competing work elsewhere would have no negative impact on our final assessment of your own study.

REFEREE REPORTS:

Referee #1:

The manuscript by Jennifer E. Kung and Natalia Jura presents evidence that the pseudokinase Trb1

regulates the localization of the ubiquitin ligase COP1. The authors claim that the mechanism of this regulation lies in the nature of a small site within COP1, which interacts with its WD40 domain in an intramolecular fashion. This interaction serves to expose the NES sequence in COP1 and allow CRM1-mediated nuclear export. The authors claim that Trb1 competes with this site for binding to COP1 WD40, leading to accumulation of COP1 in the nucleus. Although the description of such regulation is quite novel and compelling, the conclusions are mainly based on overexpression experiments, which might not be as significant in a more physiological setting. Another method in addition to confocal microscopy is required to make their conclusions more significant. Moreover, the authors do not acknowledge the fact that in mammals COP1 exists mainly as part of a multi-subunit complex: it binds to DET1, which in turn engages the DDB1-CUL4A-RBX1 core complex that associates with an E2 ubiquitin-conjugating enzyme (Wertz et al. Science 303: 1371-1374; Zhang et al. PNAS 114 (15): 3903-3908). The region implicated in DET1 binding (exon 7 and first 4 aa of exon 4) is in close proximity to the linker region (308-314), depicted by the authors as the "pseudosubstrate latch". It would be necessary considering whether mutations of this region could disrupt DET1 interaction and therefore affect Cop1 localization.

Some major questions and comments that need to addressed are depicted below:

1. How would the localization studies compare to an endogeneous setting. Overexpression of these proteins can considerably lead to different results, especially when cells very tightly regulate the expression levels of Trb1, 2, and 3 according to the biological cell type. I would like to see some of these experiments performed using Abs for endogeneous COP1 in presence or absence of Trb1 or Trb2.

2. Is COP1 protein level altered upon overexpression of Trb1 and/or CEBPa?

3. How are these proteins naturally expressed in COS7 cells (endogeneous levels of all these components: COP1, Trb1, CRM1)?

Major comments:

Fig. 1: Western blot analysis that shows expression of the various players, and whether protein levels change when Trib1 and Cop1 are co-expressed. Do the authors observe degradation of CEBPa when it is co-expressed with both COP1 and Trib1, vs COP1 alone? Is the CUL4A-RBX1 core complex required for degradation of CEBPa? The authors claim that CEBPa expression results in a redistribution of COP1/Trib1 complex, which are now not localized in puncta anymore. What is a possible explanation of such observation?

Fig. 2: The authors show that GFP-Trb1 tail is sufficient to promote COP1 nuclear localization. This is difficult to interpret given that GFP-Trb1 tail alone is localized in both the cytosol and the nucleus. Moreover, the quantification in Fig. 2B shows that GFP-Trb1 tail leads to a higher degree of COP1 nuclear localization compared to GFP-Trb1, which is only localized in the nucleus. The authors should comment on this. It would also be very useful to have a western blot showing expression levels of the different constructs employed in Fig. 1A.

Fig. EV2: The authors attempt to mutate residues in the pseudokinase domain of Trb1 that would compromise its interaction with its substrate CEBPa. I think it is quite a compelling question trying to understand whether substrate binding to Trib1 is important for regulating COP1-Trib1 interaction. In order to clearly address this the authors should mutate or truncate the Trib1 region that has been reported by Murphy et al. Structure 23 (11): 2111-2121 to be crucial for CEBPa binding.

Fig. 3: Include a loading control in the input blot in Fig. 3B. These COP1 mutations not only disrupt binding to Trb1, but also binding of COP1 to a slew of different substrates. This suggests that interaction of COP1 with its substrates triggers nucleocytoplasmic shuttling, rather that specific binding to Trb1. It would be useful to investigate whether the WD40 mutants are able to engage the DDB1-CUL4A-RBX1 core complex. Binding to this complex could depend on the ability of COP1 to engage the substrates, and therefore dictate COP1 localization as a consequence.

Fig. 4: The sequence named by the authors "pseudosubstrate latch" is adjacent to the region implicated to interact with Det1, therefore mutations in this region could affect binding to Det1. The

hypothesis that the "pseudosubstrate latch" is interacting in an intramolecular fashion with WD40 is quite compelling and an important part of this manuscript. For this reason, the fluorescence polarization data is very crucial. This reviewer would be better convinced if the titration was performed in triplicate rather than duplicate, mainly considering the low affinity measured.

Fig. 5: The connection with CRM1 is very interesting. It appears however that any substrate that binds to COP1 WD40 would elicit the same outcome: concentration of COP1 in the nucleus. It would be useful to see how this protein is expressed on COS7 cells at an endogeneous level. Overexpression could also push CRM1 to interact with COP1, although this interaction might not be relevant in a more physiological setting. These experiments should be performed at an endogeneous setting.

Fig. 6: Is the coevolution that is observed between COP1 PSL and Trb1 also seen for the emergence of the COP1 degron sequence in other COP1 substrates? This could imply that the localization effect on COP1 is achieved via engagement by a substrate, rather than specific Trb1 binding.

Minor comments:

Fig. EV1D: Include a loading control in the input blot. Also include molecular weight sizes on western blots.
Page 7 in the text: wrong figure is referenced: Fig. EV1D instead of Fig. EV1E.

Referee #3:

General summary and opinion about the principle significance of the study, its questions and findings

The article by Kung et al reports an interesting study of COP1 regulation, stemming from the initial observation that nuclear-cytoplasmic localisation is regulated by its pseudokinase partner Trb1. From this observation, the authors go on to dissect the mechanism of COP1 nuclear-cytoplasmic regulation via CRM1-mediated transport, and a conserved PSL motif in COP1 that binds its own WD40 domain. Given the important contrasting roles of COP1 as both a tumor suppressor and oncogene in humans and interesting differences vs plant COP1 regulation, this mechanistic study is very interesting and seems like it will be significant to a range of audiences.

In general the manuscript is very clearly written, laid out in a logical manner that is easy to follow, and a pleasure to read. In my opinion, the results are largely consistent with the proposed model. There are some aspects mentioned below that could have been reported or explored in some more depth, but as the manuscript already presents a clear and coherent package I would support publication should they be reasonably addressed.

Specific major concerns essential to be addressed to support the conclusions

My main query is related to the intermittent punctate appearance (suggested to occur in ~half of the cells) and how the quantitative data is presented. It should be made clearer how these differences in phenotype are handled. Is the nuclear/cytoplasmic ratio the same if the puncatate/dispersed nuclear populations are analyzed separately or combined? This observation is introduced in the first figure (and supplementary figure) but I am a bit unclear on how it is subsequently handled and this could be clarified. Given the change from punctate to non-punctate upon co-expression of a Tribbles substrate C/EBPa this phenomenon might be interesting in relation to understanding direct vs TRIB1 mediated COP1 substrates, so might be quite relevant for discussion.

The images don't seem to reflect the quantitation in Fig EV5. There seems to be very little difference in the figures, are they representative?

Minor concerns that should be addressed

To improve clarity for those non-expert in looking at fluorescence microscopy (such as myself), the

coloring of the fluorescence images might be made more consistent. It gets quite confusing when the different colors switch around. Pseudo coloring could be applied to (a) make the coloring more consistent and easy to follow, and (b) to increase contrast where necessary (blue on black gives poor contrast relative to green/red, so might be reserved for DAPI if possible)

There is not an input loading control for the Co-IP in Figure 3b. It would be very interesting to see if variation in input GFP-COP1 levels are related to the mutations that cannot bind to the pseudo-substrate latch. An input loading control would be needed to discern if the mutants expressed/turned over at different rates. This would relate PSL binding to activity of COP1 as a Ub ligase, which could add an extra aspect to the mechanism.

Page 7, Reference is made to Fig. EV1D, ->should be EV1E for co-IP?

Fig 2, the labels on the bar chart for the GFP-tail constructs are not consistent with images, and should GFP-tail/GFP-AAAtail, rather than including GFP- Trb1-tail, this could be confusing

Coloring of blue vs light blue bar charts is not very clear and should be modified

The title of supplementary figure EV5 seems inaccurate as "Trb1 inhibits CRM1-mediated nuclear export of COP1." Trb1 doesn't seem involved in these experiments, which are mainly addressing the PSL-CRM1 interplay. Something like the phrase from the text "PSL/WD40 interaction modulates COP1 responsiveness to CRM1" Might be more appropriate.

Additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

Related to the minor concern outlined for 3b loading control, it would be very interesting to see a bit more discussion of COP1 Ub ligase activity (on itself or substrates) related to its localisation/shuttling.

Is there any relevance of ATM mediated phosphorylation of COP1 by ATM (Dornan et al 2006), or differential splicing isoforms of COP1-either of these could be quite significant for the proposed mechanism as they are in the vicinity of the PSL, but don't seem to be mentioned in the discussion.

Everything seems to be carried out in the context of COP1 overexpression, do the controls (Trb1 or C/EBP only) show a similar distribution with and without COP1?

Editorial correspondence 12th Jun 2018

We have now received the attached delayed third review of your manuscript. As you will see, referee 2 is by and large supportive of the study and I will therefore not alter my original decision to consider a (major) revision further for The EMBO Journal. Since the reports contains various well-taken comments and constructive suggestions for the revision, I would nevertheless like to invite you to take the specific points mentioned by this reviewer into account when preparing your revision, and to also discuss them in your resubmission point-by-point response letter.

REFEREE REPORT:

Referee 2:

In this manuscript, Kung and Jura propose a mechanism for Trb1-mediated regulation of nuclear export of the E3 Ligase COP1 in metazoans. Current literature suggests that Tribbles serves as a substrate adaptor to alter the specificity of the E3 ligase COP1 by binding to the b-propeller/WD40 repeat domain of COP1 using a specific COP1-binding sequence motif. This paper suggests that Trb1 stimulates nuclear entry of COP1 by displacing a newly identified COP1 intramolecular autoregulatory sequence (termed the PSL by the authors) that competes with Trb1 for binding to the WD40 propeller domain. This mechanism for promoting nuclear entry is distinct from regulatory mechanisms described in plants. The experimental evidence for the authors' claims relies heavily on

co-localization experiments using overexpressed proteins. If the claims stand up to additional experimental scrutiny (suggested below), then the work will find relevance to a wide audience of scientists including cancer biologists and plant biologists.

Specific comments:

1) The authors rely exclusively on protein overexpression in a single cell type (COS7 cells) for these studies. If the mechanism is generally applicable, then i) forced expression of other Trb proteins (Trb2 or Trb3) should also result in COP1 nuclear import, ii) loss of endogenous Trb proteins should lead to increased cytoplasmic COP1, and iii) the same effects on COP1 localization should occur in a broad range of cell types (including human cells). The authors should test whether or not criteria i) - iii) hold to more rigorously evaluate whether their proposed autoregulatory model is funtionally important at endogenous protein levels and in different cell types. It would also be nice to see an x-ray structure of the complex between the PSL and the COP1 propeller, but not necessary, in my opinion, to include.

2) In Figure 3, the authors show that mutation of the Tribbles binding site on the COP1 propeller results in nuclear localization of COP1. The same result is achieved by overexpression of WT Tribbles. This seemingly inconsistent result is explained by a model (3E,F) in which nuclear export is dependent on binding of the COP1 WD domain to the upstream PSL. If this interaction is disrupted, either by mutation of the binding site itself, deletion of the WD domain entirely, or displacement of the PSL by Tribbles, the authors postulate that the NES is somehow masked and can't be recognized by CRM1. The microscopy data are consistent with this claim, but the diagram in Figure 5D invokes a dimerization event that has not been examined mechanistically. Specifically, it illustrates the NES being masked by dimerization of COP1, but no evidence is provided that masking requires or depends on dimerization through the coiled-coil. If dimerization is part of the mechanism, autoregulation might depend on a domain-swapped interaction between the PSL and the propeller - and it would seem beyond the scope of the manuscript to address this possibility. In any event, it seems - given the current data - that the diagram use to illustrate the masking/unmasking of the NES be simplified by focusing on a single subunit of COP1 and by clarifying that i) the nature of the conformational change masking the NES is unknown, and ii) whether dimerization takes place and/or plays a mechanistic role also remains unresolved.

3) Figure 4A & D- as phosphoserine-regulation was mentioned in the text, is COP1 310 known to be a site of phosphorylation in human cells (e.g. in a large scale phosphoproteomic study, etc.)? Please address in the text. If the site is phosphorylated, it would be interesting to know how serine phosphorylation influences the affinity result of the COP1 propeller for the PSL peptide.

4) If the authors' model for COP1 autoregulation occurs, then both Trb1 and COP1 exhibit autoregulatory interactions that must be overcome to form a complex. Is it possible that the pseudokinase domain engages the PSL or a PSL-adjacent sequence upon complexation? The authors might consider speculating about this possibility (one way or the other).

Minor comments:

1) The "coiled-coil" domain is still a '"putative coiled-coil" domain - no direct evidence that it forms a coiled coil yet exist. The text should be modified accordingly to account for this nuance.

2) p. 12 "Trib 1 is found primarily in the nucleus" please provide a citation

3) In Figure 3B, The authors state "Trib1 (short)" and "Trib1 (long)," presumably to distinguish between exposure times? As shown, the nomenclature is confusing (could be referring to Trib constructs, for example) and could be made clearer.

4) Citations are occasionally overlooked. For example, p. 17 Re: STK40 and COP1 binding: please cite Durzynska et al. Structure 2017.

Dear Dr. Vodermaier,

Thank you for the opportunity to submit a revised version of our manuscript “TRIB1 toggles an intramolecular switch to regulate COP1 nuclear export” (MS# EMBOJ-2018-99708). We would like to thank the reviewers for their positive comments and were encouraged that they consider our study as an important conceptual advancement. We also want to thank the reviewers for taking their time to provide useful contributions to our work. The reviewers’ critical input has been invaluable in helping us to strengthen our conclusions and expand our understanding of the mechanism for regulation of COP1 nuclear localization by Tribbles pseudokinases through the novel regulatory site in COP1 that we discovered and call the pseudosubstrate latch (PSL). We now demonstrate that endogenous COP1 is also subject to the TRIB1-dependent regulatory mechanism that we originally described. We also provide evidence for the role of CRM1 in regulation of COP1 localization at endogenous levels of both proteins. In addition, we now show that TRIB3, another COP1 interacting partner that has a COP1-binding motif analogous to the one present in TRIB1, exerts the same effect on COP1 localization as TRIB1. These results underscore that the mechanism we describe might be more general and apply to a number of COP1 binding partners that have been described to interact with COP1 using the same motif.

We hope that the reviewers find these results to adequately address their concerns and to be a substantial addition to our paper that merits its publication. During the course of the revisions, it was also brought to our attention that abbreviating Tribbles as “Trb” can be confused for telomere repeat-binding factor. We therefore now refer to Tribbles 1 as TRIB1 in the revised manuscript, and the title.

Below we outline responses to specific points of concern raised by the reviewers.

Referee #1:

The manuscript by Jennifer E. Kung and Natalia Jura presents evidence that the pseudokinase *Trb1 regulates the localization of the ubiquitin ligase COP1. The authors claim that the mechanism of this regulation lies in the nature of a small site within COP1, which interacts with its WD40 domain in an intramolecular fashion. This interaction serves to expose the NES sequence in COP1 and allow CRM1-mediated nuclear export. The authors claim that Trb1 competes with this site for binding to COP1 WD40, leading to accumulation of COP1 in the nucleus. Although the description of such regulation is quite novel and compelling, the conclusions are mainly based on overexpression experiments, which might not be as significant in a more physiological setting. Another method in addition to confocal microscopy is required to make their conclusions more significant.*

In response to the Reviewer’s concerns, we have extended our studies to using immunofluorescence-based imaging of endogenous proteins and subcellular fractionation to characterize the effect of TRIB1 on localization of endogenous COP1. We were able to image endogenous COP1 in NIH3T3 cells using the available COP1 antibodies and found that, similar to our exogenous expression studies in COS7 cells, endogenous COP1 partitioned between the cytosol and nucleus in NIH3T3 cells. Likewise, exogenous expression of TRIB1 WT (but not of TRIB1 AAA, which does not bind COP1) increased the ratio of endogenous COP1 in the nucleus compared to the cytoplasm in NIH3T3 cells (Fig EV1G & H). These results are consistent with our experiments in COS7 cells in which COP1 was exogenously expressed (Fig 1A, EV1C, EV1E).

Using subcellular fractionation, we also found that endogenous COP1 can be detected in the nucleus and cytoplasm in multiple cell lines, while being more enriched in the former. Detection of the effect of TRIB1 on COP1 partitioning by subcellular fractionation was not conclusive, however, since we found that TRIB1 was frequently mislocalized to the cytosol when expressed at the level needed for detection in our assay. This mislocalization of TRIB1 in the cytosol led to an increase of cytosolic COP1 levels, possibly because cytosolic TRIB1 was

sequestering newly synthesized COP1 and preventing it from entering the nucleus. In all our imaging experiments, which are conducted under conditions of lower TRIB1 expression, we find TRIB1 to be exclusively localized to the nucleus, consistent with previous studies (Kiss-Toth *et al*, 2006; Soubeyrand *et al*, 2016). Hence, our imaging-based analysis provided a more accurate assessment of the physiological localization of TRIB1, and the single-cell resolution possible in this assay enabled a more controlled and stringent approach for evaluation of relative spatial relationships between COP1 and TRIB1 than a bulk method, such as fractionation.

Moreover, the authors do not acknowledge the fact that in mammals COP1 exists mainly as part of a multi-subunit complex: it binds to DET1, which in turn engages the DDB1-CUL4A-RBX1 core complex that associates with an E2 ubiquitin-conjugating enzyme (Wertz et al. Science 303: 1371-1374; Zhang et al. PNAS 114 (15): 3903-3908).

We thank the reviewer for raising this point. We have revised the text to introduce COP1 as a component of a larger complex and discussed our findings in this context.

*The region implicated in DET1 binding (exon 7 and first 4 aa of exon 4) is in close proximity to the linker region (**308-314**), depicted by the authors as the "pseudosubstrate latch". It would be necessary considering whether mutations of this region could disrupt DET1 interaction and therefore affect Cop1 localization.*

We thank the reviewer for pointing this out. Using co-immunoprecipitation, we found that the COP1 4A mutant (in which the pseudosubstrate latch (PSL) carries mutations that interfere with WD40 domain binding and result in increased nuclear localization of COP1) retains the ability to bind to DET1, although this interaction is weaker than that observed for COP1 WT (Fig EV4D). This suggests that the interaction with DET1 could play a role in regulation of COP1 localization and/or that the PSL/WD40 interaction might be important for DET1 binding. We have included these results in the revised manuscript in the section titled “Identification of an intramolecular WD40 binding site that regulates the subcellular distribution of COP1” and discuss their potential implications in the discussion.

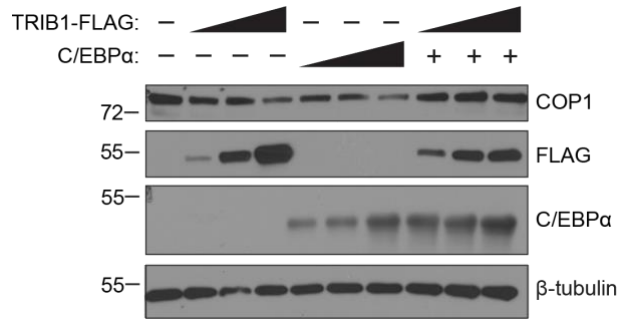
Some major questions and comments that need to addressed are depicted below:

1. How would the localization studies compare to an endogeneous setting. Overexpression of these proteins can considerably lead to different results, especially when cells very tightly regulate the expression levels of Trb1, 2, and 3 according to the biological cell type. I would like to see some of these experiments performed using Abs for endogeneous COP1 in presence or absence of Trb1 or Trb2.

As mentioned above, we examined localization of endogenous COP1 in NIH3T3 cells, which do not express detectable levels of TRIB1 (Appendix Figure S1F). Hence, we can use these cells to investigate a TRIB1 “null” background and ask how introducing exogenous TRIB1 will affect localization of endogenous COP1. As discussed above, using antibodies against endogenous COP1, we visualized the localization of endogenous COP1 in these cells in the presence or absence of exogenously expressed TRIB1 by immunofluorescence (Fig EV1G and H). Consistent with our experiments using overexpressed COP1, we found that exogenous expression of TRIB1 WT, and not TRIB1 AAA, increased nuclear localization of endogenous COP1. These findings further support that the localization of endogenous COP1 is modulated by TRIB1 through mechanisms similar to those controlling localization of exogenously expressed COP1.

2. Is COP1 protein level altered upon overexpression of Trb1 and/or CEBPa?

In our experiments, we do not observe a significant change in the level of COP1 protein levels upon overexpression of TRIB1 and/or C/EBPα (Rebuttal Figure 1).



Rebuttal Figure 1. Effect of TRIB1 and C/EBPα overexpression on endogenous COP1 levels. COS7 cells were transfected with increasing amounts of TRIB1 alone, increasing amounts of C/EBPα alone, and increasing amounts of TRIB1 in the presence of C/EBPα. Lysates were blotted with an anti-COP1 antibody to detect

3. How are these proteins naturally expressed in COS7 cells (endogeneous levels of all these components: COP1, Trb1, CRM1)?

In the revised manuscript, we include a comparison of the endogenous expression levels of COP1, TRIB1, and CRM1 in various cell types by Western blotting (Appendix Fig S1F). Expression of COP1 and CRM1 in COS7 cells is comparable to the levels measured in most of the other cell lines tested, while TRIB1 is not detectable in COS7 cells.

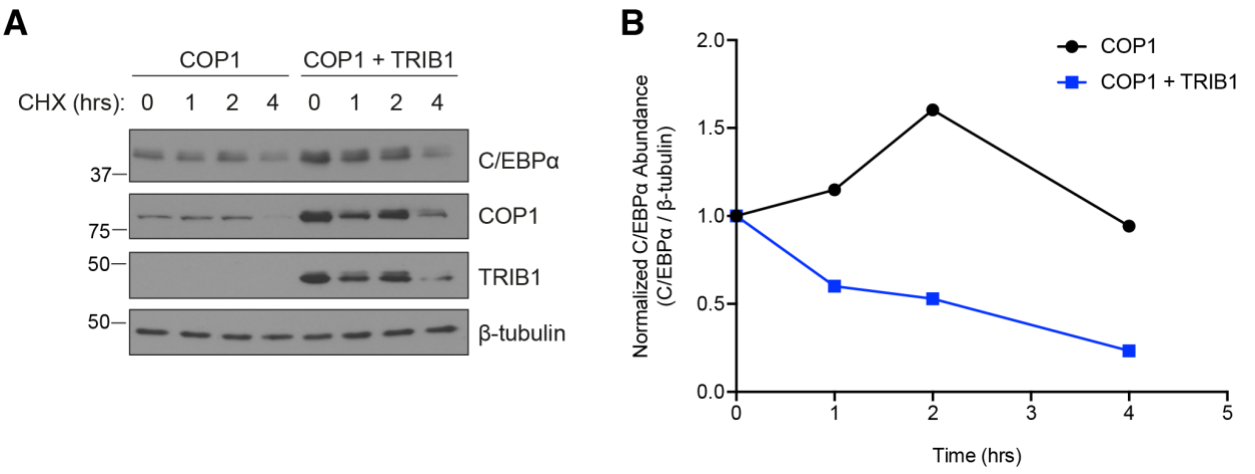
Major comments:

Fig. 1: Western blot analysis that shows expression of the various players, and whether protein levels change when Trib1 and Cop1 are co-expressed.

We have included western blots in Appendix Figure S1 showing the expression levels of each of the constructs used in Figure 1A, as well as for the corresponding figures where experiments were performed in Huh7 and HEK293 cells. We do not observe a significant change in expression levels when TRIB1 and COP1 are co-expressed in any of these cell lines.

Do the authors observe degradation of CEBPa when it is co-expressed with both COP1 and Trib1, vs COP1 alone?

Consistent with previous work showing that COP1 requires the presence of TRIB1 or TRIB2 to promote degradation of C/EBPα (Yoshida *et al*, 2013; Dedhia *et al*, 2010), we have found that co-



Rebuttal Figure 2. Effect of COP1 and TRIB1 vs. COP1 alone on C/EBPα protein stability. (A) COS7 cells were transfected with C/EBPα and either COP1 alone or COP1 and TRIB1. 24 hours post-transfection, cells were treated with 50 µg/mL cycloheximide (CHX) for the indicated times. **(B)** Densitometry analysis of relative C/EBPα protein levels normalized to 0 hr timepoint and β-tubulin levels.

expression of C/EBPα with COP1 and TRIB1 reduces the half-life of C/EBPα compared to when C/EBPα is co-expressed with COP1 alone (Rebuttal Figure 2). This effect could only be measured following cycloheximide treatment, and not under steady state conditions as shown in Rebuttal Figure 1.

Is the CUL4A-RBX1 core complex required for degradation of CEBPa?

It remains unclear whether the CUL4A-RBX1 core complex is necessary for degradation of C/EBPα. It has been shown that the COP1 splice variants known as Δ20 (also known as COP1D) and Δ24, which both lack exon 7, are unable to induce degradation of C/EBPα (Yoshida *et al*, 2013). Since these splice variants are known to be deficient in their ability to interact with DET1 and the DDB1-CUL4A-RBX1 core complex (Wertz *et al*, 2004; Savio *et al*, 2008), these results suggest that the DDB1-CUL4A-RBX1 core complex is important for C/EBPα degradation. This potential role for the DDB1-CUL4A-RBX1 core complex has yet to be confirmed. We would like to respectfully argue that this aspect of COP1 regulation is not a direct focus of our paper and hope that the reviewer agrees that it would be an appropriate topic for a study of its own.

The authors claim that CEBPa expression results in a redistribution of COP1/Trib1 complex, which are now not localized in puncta anymore. What is a possible explanation of such observation?

We considered several potential scenarios to explain these results. It is possible that localization of the COP1/TRIB1 complex to puncta relies on interactions of either of these proteins with unknown factors in PML bodies. C/EBPα could directly compete with binding of these factors. Alternatively, since binding of C/EBPα to TRIB1 has recently been shown to alter the conformation of the TRIB1 pseudokinase domain (Jamieson *et al*, 2018), this conformational change might interfere with the putative interactions that the COP1/TRIB1 complex forms with factors in PML bodies. We have revised the manuscript to include discussion of these possibilities.

Fig. 2: The authors show that GFP-Trb1 tail is sufficient to promote COP1 nuclear localization. This is difficult to interpret given that GFP-Trb1 tail alone is localized in both the cytosol and the nucleus.

Although the GFP-TRIB1 tail construct localizes to both the nucleus and the cytoplasm, it tends to predominantly concentrate in the nucleus. Our model states that COP1 interacts with the nuclear pool of GFP-TRIB1 tail after undergoing nuclear import and is subsequently retained in the nucleus. Thus, we respectfully argue that the ability of the TRIB1 tail construct to promote COP1 nuclear localization the same way as full-length TRIB1 emphasizes that the main function of TRIB1 in regulation of COP1 localization is exerted in the nucleus. We have clarified this point in the revised manuscript.

Moreover, the quantification in Fig. 2B shows that GFP-Trb1 tail leads to a higher degree of COP1 nuclear localization compared to GFP-Trb1, which is only localized in the nucleus. The authors should comment on this.

We attribute this difference to the fact that, in full-length TRIB1, the C-terminal tail binds to the pseudokinase domain and is therefore less accessible to intermolecular interactions with another binding partner, such as COP1. This intramolecular interaction between the pseudokinase domain and the tail has been visualized in the crystal structure of the TRIB1 pseudokinase domain (PDB: 5CEM) (Murphy *et al*, 2015). In the GFP-TRIB1 tail construct, the tail is fully accessible for COP1 binding, and as a result, it is better at promoting nuclear localization of COP1 than full-length TRIB1. We revised the main text to discuss this hypothesis.

It would also be very useful to have a western blot showing expression levels of the different constructs employed in Fig. 1A.

Since the Reviewer already commented on Fig. 1A, we assumed that the Reviewer meant Fig. 2A and addressed their concern accordingly. We have measured the expression levels of all the constructs under the experimental conditions originally described in Fig. 2A and found that GFP-TRIB1 tail and GFP-TRIB1 tail AAA exhibited higher expression than full-length constructs (GFP-TRIB1 or GFP-TRIB1 AAA). We have repeated the experiment under conditions in which all of these constructs were expressed at a similar level (Appendix Fig S1G) and updated the images and quantification in Figures 2A and 2B with the newer data. These new results are consistent with those of our earlier experiments and have not changed our conclusions regarding the effects of the GFP-TRIB1 tail construct on COP1 localization.

Fig. EV2: The authors attempt to mutate residues in the pseudokinase domain of Trb1 that would compromise its interaction with its substrate CEBPa. I think it is quite a compelling question trying to understand whether substrate binding to Trib1 is important for regulating COP1-Trib1 interaction. In order to clearly address this the authors should mutate or truncate the Trib1 region that has been reported by Murphy et al. Structure 23 (11): 2111-2121 to be crucial for CEBPa binding.

We introduced mutations (H168D and F293E) in TRIB1 identified by Jamieson and colleagues (*Sci Signaling* 2018) that lie at the interface of the TRIB1-C/EBPα interaction in the crystal structure of their complex and were shown to disrupt C/EBPα binding (*the mutations identified in Murphy et al. Structure (2015) were in C/EBPα*). We found that neither of these mutations altered the ability of TRIB1 to promote COP1 nuclear localization (Fig EV3D and E). These results indicate that C/EBPα binding to TRIB1 is not necessary for TRIB1 to promote nuclear localization of COP1.

Fig. 3: Include a loading control in the input blot in Fig. 3B.

A loading control is now included for Fig. 3B.

These COP1 mutations not only disrupt binding to Trb1, but also binding of COP1 to a slew of different substrates. This suggests that interaction of COP1 with its substrates triggers nucleocytoplasmic shuttling, rather that specific binding to Trb1.

We agree with the reviewer that this is a possibility and have discussed this point in the original discussion. The potential generality of this mechanism among COP1 substrates is very interesting and, in our future studies, we plan on investigating this further.

It would be useful to investigate whether the WD40 mutants are able to engage the DDB1-CUL4A-RBX1 core complex. Binding to this complex could depend on the ability of COP1 to engage the substrates, and therefore dictate COP1 localization as a consequence.

As brought up earlier, we respectfully ask the reviewer to consider that expanding our studies to include exploration of the role of the DDB4-CUL4A-RBX1 complex is an endeavor that goes beyond the scope of the current study.

Fig. 4: The sequence named by the authors "pseudosubstrate latch" is adjacent to the region implicated to interact with Det1, therefore mutations in this region could affect binding to Det1. The hypothesis that the "pseudosubstrate latch" is interacting in an intramolecular fashion with WD40 is quite compelling and an important part of this manuscript. For this reason, the fluorescence polarization data is very crucial. This reviewer would be better convinced if the titration was performed in triplicate rather than duplicate, mainly considering the low affinity measured.

We apologize that this was not clear in the text, but the fluorescence polarization data in Figure 4D is representative of three independent experiments in which each sample was measured in duplicate. We have edited the methods section to make this clearer.

Fig. 5: The connection with CRM1 is very interesting. It appears however that any substrate that binds to COP1 WD40 would elicit the same outcome: concentration of COP1 in the nucleus.

As discussed above, we agree that this is a possibility and would like to underscore that one of the major implications of our study is that any nuclear substrates of COP1 that bind via the WD40 domain should also promote nuclear localization of COP1. We expanded the discussion of these functional implications of our studies for a broader group of COP1 interacting partners in the revised manuscript.

It would be useful to see how this protein is expressed on COS7 cells at an endogeneous level.

As mentioned above, we have examined endogenous expression of CRM1 in various cell lines and found it to be similar in each of the tested cell lines, including COS7 cells (Appendix Fig S1F).

Overexpression could also push CRM1 to interact with COP1, although this interaction might not be relevant in a more physiological setting. These experiments should be performed at an endogeneous setting.

To assess CRM1-dependent regulation of COP1 nuclear export at the endogenous levels of CRM1 and COP1 expression, we used leptomycin B, a specific inhibitor of CRM1 (Kudo *et al*, 1999). Using subcellular fractionation, we found that treatment of both COS7 and HCT116 cells with the CRM1 inhibitor leptomycin B reduced levels of endogenous COP1 in the cytoplasm (Figure EV6A and B), supporting a role for CRM1 in regulation of COP1 localization in an endogenous setting. These data are consistent with previous work that has demonstrated that inhibition of endogenous CRM1 in COS7 cells with leptomycin B prevented nuclear export of a fragment of COP1 containing the nuclear export signal (Yi *et al*, 2002).

Fig. 6: Is the coevolution that is observed between COP1 PSL and Trb1 also seen for the emergence of the COP1 degron sequence in other COP1 substrates? This could imply that the localization effect on COP1 is achieved via engagement by a substrate, rather than specific Trb1 binding.

This is a great point and we thank the reviewer for bringing it up. We looked at the evolutionary conservation of other known mammalian COP1 substrates: ETS transcription factors and c-Jun (Wertz *et al*, 2004; Vitari *et al*, 2011), and found that, like Tribbles, they are present and possess a COP1 degron in most metazoan species but not in plants. This analysis supports the hypothesis that regulation of COP1 nuclear transport is a broader mechanism adopted by several of its substrates. These analyses are presented in Appendix Table S1.

Minor comments:

Fig. EV1D: Include a loading control in the input blot. Also include molecular weight sizes on western blots.

This figure has been moved to Fig EV1I in the revised manuscript, and a loading control has been included in the revised figure. We added molecular weight ladders to all of the western blots in the figures.

Page 7 in the text: wrong figure is referenced: Fig. EV1D instead of Fig. EV1E.

We thank the reviewer for pointing out this error. This figure is now Fig. EV1I in the revised figures and has been referenced as such in the text.

Referee #2:

In this manuscript, Kung and Jura propose a mechanism for Trb1-mediated regulation of nuclear export of the E3 Ligase COP1 in metazoans. Current literature suggests that Tribbles serves as a substrate adaptor to alter the specificity of the E3 ligase COP1 by binding to the b-propeller/WD40 repeat domain of COP1 using a specific COP1-binding sequence motif. This paper suggests that Trb1 stimulates nuclear entry of COP1 by displacing a newly identified COP1 intramolecular autoregulatory sequence (termed the PSL by the authors) that competes with Trb1 for binding to the WD40 propeller domain. This mechanism for promoting nuclear entry is distinct from regulatory mechanisms described in plants. The experimental evidence for the authors' claims relies heavily on co-localization experiments using overexpressed proteins. If the claims stand up to additional experimental scrutiny (suggested below), then the work will find relevance to a wide audience of scientists including cancer biologists and plant biologists.

Specific comments:

1) The authors rely exclusively on protein overexpression in a single cell type (COS7 cells) for these studies. If the mechanism is generally applicable, then

i) forced expression of other Trb proteins (Trb2 or Trb3) should also result in COP1 nuclear import,

We tested the effect of TRIB2 and TRIB3 on COP1 localization by co-expressing each of these proteins with COP1 in COS7 cells and examining COP1 localization via immunofluorescence (Figure 2C and D). We found that, like TRIB1, TRIB3 expression also increased nuclear localization of COP1, albeit to a lesser extent. TRIB2, however, had no effect. Unlike TRIB1 and TRIB3, which both localize exclusively to the nucleus, TRIB2 lacks a nuclear localization signal (Kiss-Toth *et al*, 2006). Consequently, even though it can be found in the nucleus, a large proportion of TRIB2 resides in the cytoplasm. Thus, we concluded that TRIB2 might be less efficient in blocking COP1 nuclear export due to its lower relative concentration in the nucleus and/or it might engage COP1 while in the cytosol, possibly interfering with COP1 nuclear import. Importantly, a previous study (Xu *et al*, 2014) showed that TRIB2 does in fact increase nuclear localization of endogenous COP1 in a cell line in which TRIB2 is primarily found in the nucleus. This result is therefore consistent with the inherent potential of TRIB2 to modulate the extent of COP1 nuclear localization.

We would also like to respectfully clarify that our study points to inhibition of nuclear export of COP1 by Tribbles rather than promotion of its nuclear import, as eluded to by the Reviewer.

ii) loss of endogenous Trb proteins should lead to increased cytoplasmic COP1,

COS7 cells lack endogenous TRIB1, but express endogenous TRIB2 and TRIB3 (Appendix Figure S1F). As mentioned in our response above, we found that TRIB2 did not modulate COP1 localization in these cells. Thus, we used siRNA to knock down endogenous TRIB3 in COS7 cells and examined localization of endogenous COP1 via immunofluorescence. We also attempted to knock down endogenous TRIB1 and/or TRIB3 in C4-2 and U87MG cells since these cell lines were among the only ones we tested that expressed detectable levels of endogenous TRIB1 (Appendix Figure S1F). In these cells, we used subcellular fractionation to look at changes in COP1 localization. However, in all our experiments we did not see a significant change in COP1 localization upon Tribbles knockdown. As discussed in our response to Reviewer #1's points

regarding Figure 3 and Figure 5, COP1 likely interacts with its other nuclear substrates that contain the COP1-binding motif and likely engage the WD40 domain in a manner similar to how the WD40 domain interacts with Tribbles. Therefore, we suspect that under homeostatic conditions these interactions might largely contribute to keeping COP1 in the nucleus even in the absence of TRIB proteins. At present, we do not know how the interaction between Tribbles and COP1 is regulated. We discuss a few possibilities in our manuscript that involve phosphorylation of COP1. One could envision that the prominence of Tribbles' interactions with COP1 could be significantly influenced by these regulatory mechanisms. Finally, we encountered technical problems with TRIB1 knockdown that prevented us from achieving complete knockdown. The residual levels of TRIB1 remaining could account for the lack of measurable differences in C4-2 and U87MG cells.

iii) the same effects on COP1 localization should occur in a broad range of cell types (including human cells). The authors should test whether or not criteria i) - iii) hold to more rigorously evaluate whether their proposed autoregulatory model is funtionally important at endogenous protein levels and in different cell types.

We have tested whether TRIB1 modulates localization of exogenously expressed COP1 in two additional human cell lines, HEK293 cells and Huh7 hepatocellular carcinoma cells in which TRIB1 has been previously studied (Ye *et al*, 2017; Soubeyrand *et al*, 2015; Dugast *et al*, 2013). Consistent with our observations in COS7 cells, we observed that, in both cases, TRIB1 WT increases COP1 nuclear localization, while TRIB1 AAA (in which the COP1-binding motif has been mutated) has no effect (Fig EV1C-F). As described in the initial paragraph in our response to Reviewer #1, we also examined whether overexpression of TRIB1 could modulate the localization of endogenous COP1 by assessing localization of endogenous COP1 in NIH3T3 cells transfected with an empty vector, TRIB1 WT, or TRIB1 AAA. Consistent with our studies using overexpressed COP1, we found that exogenous expression of TRIB1 WT, but not TRIB1 AAA, leads to an increase in nuclear localization of endogenous COP1 in these cells (Fig EV1G and H).

It would also be nice to see an x-ray structure of the complex between the PSL and the COP1 propeller, but not necessary, in my opinion, to include.

We agree with the reviewer, but due to technical difficulties we encountered while scaling up expression of the recombinant COP1 WD40 domain, we have not succeeded to crystallize this complex as of yet.

2) In Figure 3, the authors show that mutation of the Tribbles binding site on the COP1 propeller results in nuclear localization of COP1. The same result is achieved by overexpression of WT Tribbles. This seemingly inconsistent result is explained by a model (3E,F) in which nuclear export is dependent on binding of the COP1 WD domain to the upstream PSL. If this interaction is disrupted, either by mutation of the binding site itself, deletion of the WD domain entirely, or displacement of the PSL by Tribbles, the authors postulate that the NES is somehow masked and can't be recognized by CRM1. The microscopy data are consistent with this claim, but the diagram in Figure 5D invokes a dimerization event that has not been examined mechanistically. Specifically, it illustrates the NES being masked by dimerization of COP1, but no evidence is provided that masking requires or depends on dimerization through the coiled-coil. If dimerization is part of the mechanism, autoregulation might depend on a domain-swapped interaction between the PSL and the propeller - and it would seem beyond the scope of the manuscript to address this possibility. In any event, it seems - given the current data - that the diagram use to illustrate the masking/unmasking of the NES be simplified by focusing on a single subunit of COP1 and by clarifying that i) the nature of the conformational change masking the NES is unknown, and ii) whether dimerization takes place and/or plays a mechanistic role also remains unresolved.

As suggested by the reviewer, we have simplified Figure 5D to exclude discussion of dimerization and now only consider the model in the context of the COP1 monomer. We have also modified the text to clarify that the conformational change masking the NES is unknown and that it is also unknown whether COP1 indeed forms a dimer and whether dimerization plays a mechanistic role in regulating its localization.

3) Figure 4A & D- as phosphoserine-regulation was mentioned in the text, is COP1 310 known to be a site of phosphorylation in human cells (e.g. in a large scale phosphoproteomic study, etc.)? Please address in the text. If the site is phosphorylated, it would be interesting to know how serine phosphorylation influences the affinity result of the COP1 propeller for the PSL peptide.

So far, COP1 S310 has not been reported to be a site of phosphorylation, which we now mention in the revised text.

4) If the authors' model for COP1 autoregulation occurs, then both Trb1 and COP1 exhibit autoregulatory interactions that must be overcome to form a complex. Is it possible that the pseudokinase domain engages the PSL or a PSL-adjacent sequence upon complexation? The authors might consider speculating about this possibility (one way or the other).

We thank the reviewer for this suggestion. Binding of the PSL to TRIB1 is theoretically possible given the presence of a sufficiently long linker between the PSL region and the WD40 domain, which would enable the PSL to freely interact with TRIB1. We predict this interaction would be rather weak on its own (based on the sequence differences between PSL and TRIB1 tail) but could be additionally stabilized in a complex in which other interactions contribute to binding (such as those between TRIB1 tail and the WD40 domain). We have included discussion of this scenario in the revised manuscript.

Minor comments:

1) The "coiled-coil" domain is still a '"putative coiled-coil" domain - no direct evidence that it forms a coiled coil yet exist. The text should be modified accordingly to account for this nuance.

We have corrected the way we refer to this domain throughout the text.

2) p. 12 "Trib 1 is found primarily in the nucleus" please provide a citation

References have been added to support this statement.

3) In Figure 3B, The authors state "Trib1 (short)" and "Trib1 (long)," presumably to distinguish between exposure times? As shown, the nomenclature is confusing (could be referring to Trib constructs, for example) and could be made clearer.

We have changed the labels on this figure to clarify that we are referring to different exposure times.

4) Citations are occasionally overlooked. For example, p. 17 Re: STK40 and COP1 binding: please cite Durzynska et al. Structure 2017.

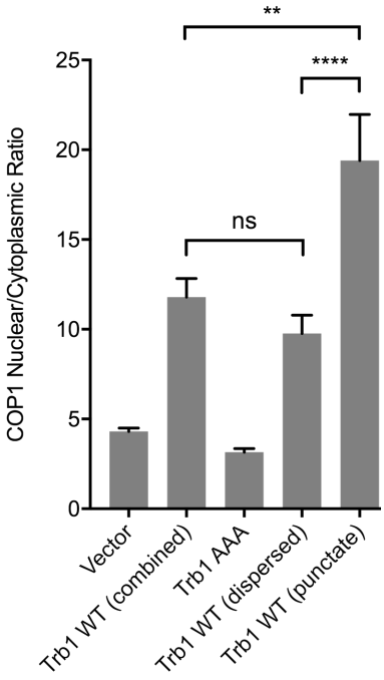
We thank the reviewer for pointing this out and have included this citation in the revised manuscript.

Referee #3:

General summary and opinion about the principle significance of the study, its questions and findings

The article by Kung et al reports an interesting study of COP1 regulation, stemming from the initial observation that nuclear-cytoplasmic localisation is regulated by its pseudokinase partner Trb1. From this observation, the authors go on to dissect the mechanism of COP1 nuclear-cytoplasmic regulation via CRM1-mediated transport, and a conserved PSL motif in COP1 that binds its own WD40 domain. Given the important contrasting roles of COP1 as both a tumor suppressor and oncogene in humans and interesting differences vs plant COP1 regulation, this mechanistic study is very interesting and seems like it will be significant to a range of audiences.

In general the manuscript is very clearly written, laid out in a logical manner that is easy to follow, and a pleasure to read. In my opinion, the results are largely consistent with the proposed model. There are some aspects mentioned below that could have been reported or explored in some more depth, but as the manuscript already presents a clear and coherent package I would support publication should they be reasonably addressed.



Rebuttal Figure 3. Comparison of COP1 nuclear/cytoplasmic ratio in cells exhibiting punctate vs. dispersed localization of TRIB1-COP1 complex. Quantification of COP1 nuclear/cytoplasmic ratio for cells analyzed in Figure 1A when cells exhibiting dispersed and punctate localization of TRIB1 and COP1 are combined or when only cells exhibiting dispersed or punctate localization are included.

Specific major concerns essential to be addressed to support the conclusions:

My main query is related to the intermittent punctate appearance (suggested to occur in ~half of the cells) and how the quantitative data is presented. It should be made clearer how these differences in phenotype are handled. Is the nuclear/cytoplasmic ratio the same if the puncatate/dispersed nuclear populations are analyzed separately or combined? This observation is introduced in the first figure (and supplementary figure) but I am a bit unclear on how it is subsequently handled and this could be clarified. Given the change from punctate to non-punctate upon co-expression of a Tribbles substrate C/EBPa this phenomenon might be interesting in relation to understanding direct vs TRIB1 mediated COP1 substrates, so might be quite relevant for discussion.

In the quantifications of the nuclear/cytoplasmic ratio of COP1 signal in the original manuscript, we did not discriminate between cells that have puncta in the nucleus and those that do not, and included both in the quantifications. We apologize that this was not adequately explained and have clarified the way these calculations are described in the revised manuscript. To address the Reviewer's concerns that the pattern of nuclear localization of COP1 might affect the calculations, we have also analyzed these two populations separately and found that cells with a punctate pattern of localization have a nuclear/cytoplasmic ratio that is about 2x higher than cells which have dispersed nuclear localization (Rebuttal Figure 3). Despite this, the difference between the nuclear/cytoplasmic ratio in the combined population and that of only the disperse population is not statistically significant.

The images don't seem to reflect the quantitation in Fig EV5. There seems to be very little difference in the figures, are they representative?

We thank the reviewer for pointing this out and have replaced the images in Fig EV5 with ones that are more representative of the quantitation.

Minor concerns that should be addressed:

To improve clarity for those non-expert in looking at fluorescence microscopy (such as myself), the coloring of the fluorescence images might be made more consistent. It gets quite confusing when the different colors switch around. Pseudo coloring could be applied to (a) make the coloring more consistent and easy to follow, and (b) to increase contrast where necessary (blue on black gives poor contrast relative to green/red, so might be reserved for DAPI if possible)

We have changed the pseudo-coloring in our fluorescence microscopy images in order to be consistent throughout the figures.

There is not an input loading control for the Co-IP in Figure 3b. It would be very interesting to see if variation in input GFP-COP1 levels are related to the mutations that cannot bind to the pseudo-substrate latch. An input loading control would be needed to discern if the mutants expressed/turned over at different rates. This would relate PSL binding to activity of COP1 as a Ub ligase, which could add an extra aspect to the mechanism.

We have added a loading control to Figure 3B, which shows that most of the WD40 domain mutants that do not bind the PSL region, and hence are enriched in the nucleus, seem to be expressed at approximately ~2 fold higher level than wild type COP1 or the control WD40 mutant (E642R), which retains the ability to bind the PSL. This suggests that the mutants that are predominantly nuclear have greater inherent stability, which is consistent with previous studies showing that COP1 degradation is promoted by its translocation to the cytoplasm following phosphorylation by ATM (Dornan *et al*, 2006). Our observations are therefore consistent with a mechanism by which nuclear enrichment of COP1 through interactions with Tribbles would serve not only to concentrate it in the compartment where its substrates are present but also to protect COP1 from degradation. We mention this possibility in the revised manuscript. Our data also indicate that the ΔWD40 mutant does not appear to exhibit enhanced stability relative to wild type COP1 despite being predominantly nuclear, which indicates that this domain might be important for COP1 stability.

Page 7, Reference is made to Fig. EV1D, ->should be EV1E for co-IP?

We thank the reviewer for pointing out this mistake. This figure has been changed to Fig. EV1I in the revised figures and has been referenced as such in the revised manuscript.

Fig 2, the labels on the bar chart for the GFP-tail constructs are not consistent with images, and should GFP-tail/GFP-AAAtail, rather than including GFP- Trb1-tail, this could be confusing

The labels in Figure 2 have been changed to be consistent.

Coloring of blue vs light blue bar charts is not very clear and should be modified

The coloring in these charts has been altered to enhance contrast.

The title of supplementary figure EV5 seems inaccurate as "Trb1 inhibits CRM1-mediated nuclear export of COP1." Trb1 doesn't seem involved in these experiments, which are mainly addressing the PSL-CRM1 interplay. Something like the phrase from the text "PSL/WD40 interaction modulates COP1 responsiveness to CRM1" Might be more appropriate.

We thank the reviewer for pointing this out, and we have changed the title of Figure EV5 to better represent the data.

Additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

Related to the minor concern outlined for 3b loading control, it would be very interesting to see a bit more discussion of COP1 Ub ligase activity (on itself or substrates) related to its localisation/shuttling.

We agree, and we included this discussion in the revised manuscript. As mentioned above when discussing the loading control for Fig 3B, previous studies have suggested a link between COP1 localization and its autodegradation (Dornan *et al*, 2006). Consequently, it is possible that Tribbles can enhance the stability of COP1 and prevent its autodegradation through their ability to promote COP1 nuclear localization. This could serve as another means through which Tribbles are able to enhance COP1-mediated ubiquitination of its substrates.

Is there any relevance of ATM mediated phosphorylation of COP1 by ATM (Dornan et al 2006), or differential splicing isoforms of COP1-either of these could be quite significant for the proposed mechanism as they are in the vicinity of the PSL, but don't seem to be mentioned in the discussion.

These are all very interesting points that we plan to address in our future studies. ATM-dependent phosphorylation could serve as one mechanism to regulate the interaction between the PSL and the WD40 domain since the phosphorylation site recognized by ATM is located in the linker region between the PSL and WD40 domain.

Among the known COP1 splicing isoforms, the two most relevant in the context of the discussed mechanism are Δ20 (also known as COP1D) and Δ24. Both carry short deletions in the C-terminal portion of the coiled coil domain and have been shown to be deficient in DET1 binding (Wertz *et al*, 2004; Savio *et al*, 2008). DET1 is an adaptor protein that recruits the DDB1-CUL4A-RBX1 E3 ubiquitin ligase complex, which is important for ubiquitination of c-Jun and ETS transcription factors (Wertz *et al*, 2004; Vitari *et al*, 2011). As mentioned in our response to Reviewer #1 regarding DET1 binding, we have found that mutation of the PSL region, which is in close proximity to the C-terminus of the coiled coil domain, weakens interaction of COP1 with DET1. Hence, interactions between the PSL and WD40 domain could potentially regulate the extent of DDB1-CUL4A-RBX1-dependent ubiquitination of these transcription factors.

We have revised our manuscript to include discussion of the potential relevance of ATM-mediated phosphorylation of COP1, as well as the splicing isoforms mentioned and the implications of our finding that mutation of the PSL weakens DET1 binding.

Everything seems to be carried out in the context of COP1 overexpression, do the controls (Trb1 or C/EBP only) show a similar distribution with and without COP1?

Yes, both TRIB1 and C/EBPα localize predominantly to the nucleus in the absence of COP1. Images of these controls have been added (Figure EV2C).

References

Dedhia PH, Keeshan K, Uljon S, Xu L, Vega ME, Shestova O, Zaks-Zilberman M, Romany C, Blacklow SC & Pear WS (2010) Differential ability of Tribbles family members to promote

degradation of C/EBPalpha and induce acute myelogenous leukemia. *Blood* **116:** 1321–1328

Dornan D, Shimizu H, Mah A, Dudhela T, Eby M, O'Rourke K, Seshagiri S & Dixit VM (2006) ATM engages autodegradation of the E3 ubiquitin ligase COP1 after DNA damage. *Science* **313:** 1122–1126

Dugast E, Kiss-Toth E, Docherty L, Danger R, Chesneau M, Pichard V, Judor JP, Pettre S, Conchon S, Soulillou JP, Brouard S & Ashton-Chess J (2013) Identification of Tribbles-1 as a Novel Binding Partner of Foxp3 in Regulatory T Cells. *Journal of Biological Chemistry* **288:** 10051–10060

Jamieson SA, Ruan Z, Burgess AE, Curry JR, McMillan HD, Brewster JL, Dunbier AK, Axtman AD, Kannan N & Mace PD (2018) Substrate binding allosterically relieves autoinhibition of the pseudokinase TRIB1. *Science Signaling* **11:** eaau0597

Kiss-Toth E, Wyllie DH, Holland K, Marsden L, Jozsa V, Oxley KM, Polgar T, Qwarnstrom EE & Dower SK (2006) Functional mapping and identification of novel regulators for the Toll/Interleukin-1 signalling network by transcription expression cloning. *Cellular Signalling* **18:** 202–214

Kudo N, Matsumori N, Taoka H, Fujiwara D, Schreiner EP, Wolff B, Yoshida M & Horinouchi S (1999) Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *Proceedings of the National Academy of Sciences* **96:** 9112–9117

Murphy JM, Nakatani Y, Jamieson SA, Dai W, Lucet IS & Mace PD (2015) Molecular Mechanism of CCAAT-Enhancer Binding Protein Recruitment by the TRIB1 Pseudokinase. *Structure* **23:** 2111–2121

Savio MG, Rotondo G, Maglie S, Rossetti G, Bender JR & Pardi R (2008) COP1D, an alternatively spliced constitutive photomorphogenic-1 (COP1) product, stabilizes UV stress-induced c-Jun through inhibition of full-length COP1. *Oncogene* **27:** 2401–2411

Soubeyrand S, Martinuk A, Lau P & McPherson R (2016) TRIB1 Is Regulated Post-Transcriptionally by Proteasomal and Non-Proteasomal Pathways. *PLoS ONE* **11:** e0152346

Soubeyrand S, Martinuk A, Naing T, Lau P & McPherson R (2015) Role of Tribbles Pseudokinase 1 (TRIB1) in human hepatocyte metabolism. *Biochim. Biophys. Acta*

Vitari AC, Leong KG, Newton K, Yee C, O'Rourke K, Liu J, Phu L, Vij R, Ferrando R, Couto SS, Mohan S, Pandita A, Hongo J-A, Arnott D, Wertz IE, Gao W-Q, French DM & Dixit VM (2011) COP1 is a tumour suppressor that causes degradation of ETS transcription factors. *Nature* **474:** 403–406

Wertz IE, O'Rourke KM, Zhang Z, Dornan D, Arnott D, Deshaies RJ & Dixit VM (2004) Human De-etiolated-1 regulates c-Jun by assembling a CUL4A ubiquitin ligase. *Science* **303:** 1371–1374

Xu S, Tong M, Huang J, Zhang Y, Qiao Y, Weng W, Liu W, Wang J & Sun F (2014) TRIB2 inhibits Wnt/β-Catenin/TCF4 signaling through its associated ubiquitin E3 ligases, β-TrCP, COP1 and Smurf1, in liver cancer cells. *FEBS Letters* **588:** 4334–4341

Ye Y, Wang G, Wang G, Zhuang J, He S, Song Y, Ni J, Xia W & Wang J (2017) The Oncogenic Role of Tribbles 1 in Hepatocellular Carcinoma Is Mediated by a Feedback Loop Involving microRNA-23a and p53. *Front Physiol* **8:** 789

Yi C, Wang H, Wei N & Deng XW (2002) An initial biochemical and cell biological characterization of the mammalian homologue of a central plant developmental switch, COP1. *BMC Cell Biol.* **3:** 30

Yoshida A, Kato J-Y, Nakamae I & Yoneda-Kato N (2013) COP1 targets C/EBPα for degradation and induces acute myeloid leukemia via Trib1. *Blood* **122:** 1750–1760

Acceptance 20th Dec 2018

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by all three original reviewers, whose comments are copied below. As you will see, all of them consider the study significantly improved in response to their original comments. Although referee 2 is still missing validation whether TRIB1 depletion alone would be sufficient to trigger COP1 export, we appreciate that there may be additional factors/determinants of COP1 localization and feel that the described new COP1 autoregulatory mechanism will be of interest nevertheless. We shall therefore be happy to accept your manuscript for publication in The EMBO Journal!

REFEREE REPORTS:

Referee #1:

The revised manuscript by Jennifer E. Kung and Natalia Jura has addressed the main concerns raised in the original submission. The authors present compelling evidence that COP1 nuclear localization is aided by interaction with TRIB1, which disrupts an intramolecular interaction within COP masking the NES. Furthermore, the authors clarify in the revised version of the manuscript that other potential COP1-WD40 interacting partners, such as TRIB1, TRIB2, STK40, ETS transcription factors could perform a similar task, underscoring the hypothesis that regulation of COP1 localization via interaction with its WD40 domain is a broader mechanism adopted by several of its substrates. Although, the most employed experimental system to gather evidence is confocal microscopy in an overexpression scenario, the authors have now attempted to use cellular fractionation and immuno-staining of endogenous proteins, claiming that they obtain similar results.
Analysis of the potential role for the DDB1-CUL4A-RBX1 core complex in COP1-TRIB1 regulation axis might indeed be beyond the scope of this manuscript.

Referee #2:

First, apologies to the authors for equating nuclear import with inhibition of nuclear export in the original review (though both processes, of course, result in nuclear accumulation).
Second, the authors have most certainly made a thorough, good faith effort to address reviewer criticisms. Nevertheless, the most relevant prediction of the model still has not been adequately tested because of incomplete knockdown. Ultimately, whether the appeal of the model overcomes any reservations about the failure to demonstrate COP1 export upon Trb depletion is a decision I defer to the journal editor. It would be nice to see, however, whether knockout of endogenous Trb does indeed result in increased COP1 export - the key prediction of the model. Otherwise, if loss of Trb doesn't affect the distribution of COP1 in cells, then is the observed effect of Trb <I>overexpression</I>, which is all we see in the manuscript, functionally important at endogenous levels?

Referee #3:

The authors have done a very nice job in addressing outstanding concerns and I am happy to recommend for publication.

Re. Novelty and significance justification: COP1 is important for transcriptional regulation in both plants and animals. This work uncovers new COP1 regulatory mechanism, which could be relevant to both cancer biology (re. COP1-TRIB1/C/EBP) and evolution of the COP1 system (relative to plants).

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Natalia Jura
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2018-99708R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple 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.

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

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes for imaging experiments were chosen to minimize variability between experiments based on experience and are consistent with or larger than what is typically used in the field.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For imaging experiments, cells were excluded from analysis if they contained saturated pixels or in cases where TRIB1 was mislocalized (present in both the nucleus and cytoplasm) rather than being primariy nuclear, as has been reported in previous studies.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Cells were randomly selected for imaging analysis.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Yes, we used the standard error of the mean, as specified in the figure legends.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies used are commercially available and are listed in the Materials and Methods section along with their manufacturer and catalog number.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Sources for cell lines used are listed in the Materials and Methods section. All cell lines were tested regularly for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA