nature portfolio

Peer Review File

HURP facilitates spindle assembly by stabilizing microtubules and working synergistically with TPX2

Corresponding Author: Professor Sabine Petry

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In their manuscript, Valdez et al. characterize the contribution of the protein HURP to microtubule nucleation and growth using a Xenopus extract model and immunodepletion of HURP or the addition of recombinant proteins. They first show that HURP is required for microtubule branching in Xenopus extracts and that supraphysiological concentrations of HURP stimulate microtubule nucleation. They then show that TPX2 helps localize HURP to microtubules in vitro, that HURP binding to microtubules stabilizes the lattice, and that while HURP itself does not form condensates in vitro, it associates with and localizes to TPX2 condensates. Finally, they determine the structure of the microtubule-binding domains of HURP bound to microtubules using a cryo-EM approach, which supports their observations. Interestingly, although HURP uses a similar microtubule-binding mechanism to TPX2, their functions appear quite different.

The paper builds on a large body of literature that has elucidated the mechanisms of various microtubule-binding and nucleating proteins, including HURP. Several papers have examined the contribution of HURP and the regulation of its functions, mostly in human cells. However, some have used a Xenopus system, including "Hepatoma up-regulated protein is required for chromatin-induced microtubule assembly independently of TPX2" PMID: 18799614 and "HURP is part of a Ran-dependent complex involved in spindle formation" PMID: 16631581. In contrast to this study, the authors argue that TPX2 and HURP do not interact and function independently.

The main conceptual advance is that HURP is required for microtubule branching, which is convincingly illustrated in Figure 1. A state-of-the-art high-resolution cryo-EM structure of HURP microtubule-binding domains bound to GMPCPPP-stabilized microtubules concludes the manuscript.

Overall, we would support publication in Nature Communications if our concerns were addressed.

General comments:

The figures are generally clear, well-presented and easy to interpret. Similarly, the text is clear and easy to follow.
The manuscript tends to oversell: 'We bring the microtubule field to the same level as the actin field.'
I encourage the authors to engage with the Spire literature and its data quality and quantity.
The self-citation rate and simultaneous ignorance of the microtubule field regarding conceptual contributions and methodological advances are unscholarly.

The authors added recombinant proteins in many experiments, but no gels were shown for their purification. This needs to be corrected in a revised manuscript. We also think that, in general, it would be helpful to have more data points for the experiments than 1 or 2 concentrations to assess dose-dependent relationships.

The major limitation of their experimental system needs to be adequately considered and discussed. For example, the fans are vanadate-induced, and the in vitro data rely entirely on binding to GMPCPPP microtubules. No dynamic reconstitution assays are presented.

The manuscript relies heavily on the author's previous TPX2 manuscript, which did not convince this reviewer. Much of the argumentation in the current manuscript feels like building a house of cards without strengthening the foundation.

The cryo-EM structure needs to be tied into the manuscript. Where are the contact sites, and how does this explain the biochemistry of HURP?

Specific comments:

Introduction lines 70-71: This statement is somewhat vague and misleading, as it has been previously shown that TPX2 and HURP do not directly interact in the same system used by the authors, not in another species. While the interaction is debatable, this should be clarified and commented on in the discussion.

Figure 1A shows effective immunodepletion of HURP from Xenopus extracts. The authors disclose and indicate that the blots were cropped from non-adjacent lanes. The uncropped images were not available for evaluation. Although sufficient, more is needed to increase this reviewer's confidence as this manuscript is used to validate a new antibody.

Figure 2: The authors show the effect of over-endogenous levels of proteins on microtubule nucleation. A Western blot would help to assess the levels of the various proteins present and if concentrations are raised by 1% or by 100%. Although it is stated in the legends that the experiments were performed three times with similar results, there is no averaging of the data in the quantifications or hypothesis testing. Is this an N of 1 for the two experiments shown?

Figure 1C, 2A,C: It needs to be more evident in the figure labelling that the added recombinant proteins are included above the endogenous levels, rather than 125 or 250 nM total protein. Please clarify.

Figure 3, S5: Although the phase boundaries of TPX2 have been previously determined, it would be helpful to repeat that experiment here to verify that it is identical in this system.
The current two-point titration of TPX2 concentration seems rather unconvincing.

Figure 4: For experiments involving microtubule rescue and catastrophe, it would be helpful to show kymographs. The conclusion that HURP binding to microtubules must recruit y-TuRCs or it has been convincingly shown that the two do not interact is simplistic, bordering on flawed given the model the authors provide: 'HURP may serve to stabilize lateral tubulin interactions that assemble on y-TuRC to form the critical nucleus or stabilize the nascent microtubule lattice nucleated by y-TuRC.'

Figure 5: The authors could have used the structure to comment more on their findings from earlier figures and to help tie them together. At the moment, this experimentally strongest part of the manuscript is underutilized.
Which tubulin residues are contacted, and how does that explain stabilization?

The authors argue that HURP simultaneously interacts with four tubulin subunits. Aren't there other MAPs that contact four subunits? Could we learn something from comparing and contrasting?

Discussion
…their microtubule counterparts during mitosis are now discovered to be y-TuRC, XMAP215/chTOG, and HURP…
Why not combine all proteins and reconstitute branched microtubule nucleation outside of extracts?

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts

Reviewer #3

(Remarks to the Author)
In the manuscript by Valdez et al., entitled "HURP facilitates spindle assembly by stabilizing microtubules and working synergistically with TPX2", the authors use in vitro microtubule reconstitution assays using purified components and Xenopus egg extracts in conjunction with TIRF microscopy and cryo-EM to investigate the role of HURP in spindle microtubule assembly. HURP is a major spindle assembly protein whose mechanistic role in spindle assembly is poorly understood. The authors conclude that HURP stabilizes the growth of nascent microtubules during Ran-GTP-induced branched microtubule nucleation, a key event in spindle formation. Furthermore, they investigate the interplay of HURP with Tpx2, a known mediator of the RanGTP-induced branched microtubule nucleation pathway. The authors report that HURP forms co-condensates and synergizes with Tpx2 to strongly promote microtubule branching. The authors also perform structural studies and report that a minimal fragment of HURP containing a microtubule-binding domain binds to the GMPCPP-stabilized microtubule lattice, straddling protofilaments, thus potentially underlying the ability of HURP to stabilize microtubule growth.

A mechanistic understanding of HURP activity in spindle assembly is warranted and would significantly add to the fields of microtubule dynamics, spindle assembly and cell division. Furthermore, as HURP is a putative oncogene, elucidating HURP's mechanism could have implications for cancer. The authors are equipped with the tools to answer these questions. However, the manuscript in its current form appear very preliminary. The data presented are limited and more experiments and analysis are needed to support the preliminary findings and conclusions.

Major comments

Figure 1:

In Figure 1. the authors deplete HURP from Xenopus egg extracts to show that HURP has a role in branching microtubule nucleation. In Fig 1a, when compared to the mock depletion, the depletion of HURP appears to be robust. However, the loading controls do not appear equal between samples and y-TURC levels appear lower. Indeed, the quantification shows that y-TuRC levels are 20 percent lower than the input. I wonder if the authors could address this, and the implications this has for subsequent experiments looking at the effects of HURP depletion on nucleation (which involves y-TURC). For example, in Figure 1b, the lack of nucleation seen in the HURP-depleted condition could presumably be due to an additive effect of the reduced y-TURC levels and HURP depletion.

Furthermore, if the binding of HURP to Tpx2 and XMAP215 (two other proteins involved in branching nucleation) is RanGTP-dependent, as reported in (ref 28), then this raises an issue with Figure 1b, in which HURP depletion in the extracts is performed in the presence of constitutively active RanGTP. If HURP directly interacts with Tpx2/XMAP215 under these conditions then the addition of the anti-HURP antibody may sequester Tpx2 and XMAP215 activities contributing to the observed loss of nucleation. Could this explain why there are fewer 'fans' in the HURP rescue condition? The authors suggest that fewer 'fans' could be due to rescuing with sub-endogenous (250 nM) levels of HURP. Could the authors not perform rescue experiments with endogenous levels of HURP (~320 nM)? I would suggest performing a titration of HURP protein in the rescue condition encompassing endogenous HURP levels to determine whether the depletion effects are truly specific to HURP.

Additionally, it is imperative to determine the binding relationships between HURP, y-TURC, XMAP215 and Tpx2 with and without RanGTP for example by performing pull-downs with purified proteins and/or size exclusion chromatography.

Notably, SDS-PAGE gels and mass spectrometry analyses of purified HURP protein as well as for all other purified proteins used in the manuscript are not provided, thus protein purity cannot be assessed.

The quantification in Fig 1c should be of at least 3 experimental replicates and statistical significance should be provided to substantiate the claim that the amount of microtubule branching in each 'fan' is the same as the control condition.

Finally, a schematic showing the domain structure of HURP and known/putative binding sites to protein partners would be useful here to set the context for the rest of the manuscript.

Figure 2.

In Figure 2, the authors conclude that HURP induces microtubule nucleation in Xenopus extracts and synergizes with Tpx2. Figures 2a and b show that HURP promotes nucleation of single microtubules but not branches, which differs from Tpx2 activity. However, the two concentrations of HURP tested were both below endogenous levels (125 nM and 250 nM) and the quantification is of a single repeat only. It is possible that branching nucleation could occur at higher HURP concentrations. Therefore, to support this conclusion, a full titration of HURP should be performed as well as a more rigorous quantification of branched versus individual microtubule networks and statistical analysis of multiple experimental replicates.

Do the authors observe the expected activity of HURP as a microtubule bundling protein as previously reported? If not, can they comment on why? The microtubules are somewhat difficult to visualize in the images provided so it is difficult to ascertain whether any bundling is occurring.

Figure 3.

In Figure 3, the authors present data indicating that HURP is recruited to Tpx2 condensates again bringing into question the nature of the interactions between HURP and Tpx2. Is it known which residues/domains in HURP and Tpx2 are responsible for the interaction? Determining whether and how HURP directly binds to TPX2 is central to the proposed mechanism and should be investigated by the authors using standard protein-protein interaction assays.

Figure 4.

In Figure 4, the authors show that HURP facilitates nucleation in vitro from y-TURC using purified proteins and that it does so by stabilizing microtubule growth. However, a more thorough quantification of microtubule dynamics parameters (Fig 4e,f,g) should be performed using standard field-accepted methodology and reporting (i.e. catastrophe and rescue frequencies, effects on shrinkage rate). I would highly recommend performing a titration of HURP activity and quantifying microtubule dynamics parameters across the titration. Representative kymographs should also be provided.

Can the effects of HURP on suppressing microtubule catastrophe be explained by the increased growth rate (rather than lattice stabilization)? Very few rescue events were quantified and more should be analyzed for the interpretation to be meaningful (just 10 shrinkage events were analyzed in the control and 8 in the HURP condition across all replicates).

As the authors have expressed and purified GFP-HURP, it would also be valuable to observe the localization of GFP-HURP on dynamically growing microtubules as well as during branching nucleation.

Figure 5.

In Figure 5, the cryo-EM data presented indicate that HURP binds at the groove between neighboring protofilaments, the authors propose the model that HURP binding could strengthen the lateral interactions between protofilaments. A prediction would then be that HURP could slow down microtubule shrinkage by preventing the peeling apart of protofilaments. Therefore, it would be valuable to report the effects of HURP on dynamic microtubule shrinkage rates.

Discussion

In the discussion the authors speculate that HURP may 'bridge two soluble tubulin dimers or short protofilaments laterally, therefore promoting the formation of early tubulin nucleation intermediates'. However, they show in Fig S6 that HURP does not directly recruit soluble tubulin to pre-existing microtubules. Therefore, this discussion point seems somewhat contradictory. It would be useful to test for direct binding of HURP to soluble tubulin without the presence of pre-existing microtubules using standard protein-protein interaction assays.

Minor comments
- Typo line 973 – should say p-value <0.05?
- In Fig4b, please report errors on the rates.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Overall, the authors have added substantially to an initially very preliminary manuscript, and all our comments have been satisfactorily addressed. We are now completely supportive of publication in Nature Communications. Our only recommendation is that the Western blot and quantitation of expected/calculated protein quantity that is included in the letter below be included in the supplemental information and commented on in the text.

Reviewer #2

(Remarks to the Author)
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Reviewer #3

(Remarks to the Author)
In the revised version of the manuscript, the authors have thoroughly addressed all reviewers' comments and concerns.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Dear Reviewers,

We were delighted to receive the positive responses and recommendations on our manuscript. The review process has helped us improve our work immensely. In this re-submission, we thoroughly address the comments and suggestions made. Notably, we added a set of new experiments that contributed to seven new figure panels and four entirely new supplementary figures. Besides clarifying points, the new experiments very clearly show that there is no direct interaction between HURP and TPX2 or γ-TuRC. In addition, we show that HURP only weakly binds tubulin and prefers binding to a MT. Last, by performing a new dynamic seed assay, we more thoroughly determine that HURP does not affect MT growth, but MT catastrophe, depolymerization and rescue rates. Altogether, these experiments further the manuscript and its conclusions. We thank you in advance for re-examining the paper. We would like to note that the line numbers supplied below in our responses are when track changes are shown.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In their manuscript, Valdez et al. characterize the contribution of the protein HURP to microtubule nucleation and growth using a Xenopus extract model and immunodepletion of HURP or the addition of recombinant proteins. They first show that HURP is required for microtubule branching in Xenopus extracts and that supraphysiological concentrations of HURP stimulate microtubule nucleation. They then show that TPX2 helps localize HURP to microtubules in vitro, that HURP binding to microtubules stabilizes the lattice, and that while HURP itself does not form condensates in vitro, it associates with and localizes to TPX2 condensates. Finally, they determine the structure of the microtubule-binding domains of HURP bound to microtubules using a cryo-EM approach, which supports their observations. Interestingly, although HURP uses a similar microtubule-binding mechanism to TPX2, their functions appear quite different.

The paper builds on a large body of literature that has elucidated the mechanisms of various microtubule-binding and nucleating proteins, including HURP. Several papers have examined the contribution of HURP and the regulation of its functions, mostly in human cells. However, some have used a Xenopus system, including "Hepatoma up-regulated protein is required for chromatin-induced microtubule assembly independently of TPX2" PMID: 18799614 and "HURP is part of a Ran-dependent complex involved in spindle formation" PMID: 16631581. In contrast to this study, the authors argue that TPX2 and HURP do not interact and function independently.

The main conceptual advance is that HURP is required for microtubule branching, which is convincingly illustrated in Figure 1. A state-of-the-art high-resolution cryo-EM structure of HURP microtubule-binding domains bound to GMPCPPP-stabilized microtubules concludes the manuscript.

Overall, we would support publication in Nature Communications if our concerns were addressed.

General comments:

The figures are generally clear, well-presented and easy to interpret. Similarly, the text is clear and easy to follow.
The manuscript tends to oversell: 'We bring the microtubule field to the same level as the actin field.'

I encourage the authors to engage with the Spire literature and its data quality and quantity. The self-citation rate and simultaneous ignorance of the microtubule field regarding conceptual contributions and methodological advances are unscholarly.

We thank the reviewer for pointing this out. That particular sentence is now deleted and the respective paragraph edited (lines 350-353). We also reviewed the manuscript for missing credit and citations.

The authors added recombinant proteins in many experiments, but no gels were shown for their purification. This needs to be corrected in a revised manuscript.

We have now added SyproRuby-stained gels of our recombinant proteins and mass-spec analysis as a new supplement (Supplementary Figure 2). In the same figure, we provide the BSA standard curves that were used in our analysis of the protein concentrations.

We also think that, in general, it would be helpful to have more data points for the experiments than 1 or 2 concentrations to assess dose-dependent relationships.

We agree with the reviewer. To remedy this, we performed a titration with five concentrations in Fig.3A to support our findings that TPX2 enriches HURP to the microtubule lattice. While many concentrations are straightforward to test with purified proteins in vitro, the extract experiments in particular are technically challenging with a limited extract lifetime. We believe the claims we make in the paper are substantiated by the results currently provided. Furthermore, we have now removed the mention of dose-dependence in the case where there are only 3 concentrations present (lines 122-123).

The major limitation of their experimental system needs to be adequately considered and discussed. For example, the fans are vanadate-induced, and the in vitro data rely entirely on binding to GMPCPP microtubules.
Based on this comment, we clarified the section on vanadate addition (lines 96-97). In addition, we incorporated the requested discussion of our results that use GMPCPP MTs (lines 311-317).

No dynamic reconstitution assays are presented.

We have clarified in the text (line 182) that the in vitro nucleation assay forming the basis of Figure 4A, B, and Movie 4 contains dynamic microtubules. In addition, to more accurately measure the dynamic parameters of microtubules (i.e., catastrophe and rescue rates and frequency), we have now provided another in vitro assay: microtubule growth and dynamics from stabilized microtubule seeds were measured and new figures and movies added as Figure 4C-G , and Movie 5. The findings from this added assay are presented in lines 208-221, namely that HURP does not affect microtubule growth but catastrophe, depolymerization and rescue rates.

The manuscript relies heavily on the author's previous TPX2 manuscript, which did not convince this reviewer. Much of the argumentation in the current manuscript feels like building a house of cards without strengthening the foundation.

The model in our manuscript utilizes the knowledge that TPX2 forms a phase condensate. We have repeated that experiment, provide it as a new Supplemental Figure 9, and find that it is consistent with our other publications.

We tried to the best of our knowledge to address this concern. However, it was not clear to us which previous TPX2 manuscript the reviewer is referring to. There are a set of manuscripts that focus on the discovery that TPX2 is a phase separating protein and place this phenomenon into the context of TPX2's function within branching microtubule nucleation and spindle assembly (citations 1 to 5 below). Each of these papers has undergone peer review and we find them to be consistent with each other.

1. Structural basis of protein condensation on microtubules underlying branching microtubule nucleation.
   Guo C, Alfaro-Aco R, Zhang C, Russell RW, Petry S, Polenova T.
   Nat Commun. 2023 Jun 21;14(1):3682. doi: 10.1038/s41467-023-39176-z.
2. Acentrosomal spindles assemble from branching microtubule nucleation near chromosomes in Xenopus laevis egg extract.
   Gouveia B, Setru SU, King MR, Hamlin A, Stone HA, Shaevitz JW, Petry S.
   Nat Commun. 2023 Jun 21;14(1):3696. doi: 10.1038/s41467-023-39041-z.
3. A hydrodynamic instability drives protein droplet formation on microtubules to nucleate branches.
   Setru SU, Gouveia B, Alfaro-Aco R, Shaevitz JW, Stone HA, Petry S.
   Nat Phys. 2021 Apr;17(4):493-498. doi: 10.1038/s41567-020-01141-8. Epub 2021 Jan 28.
4. Interaction of spindle assembly factor TPX2 with importins-α/β inhibits protein phase separation.
   Safari MS, King MR, Brangwynne CP, Petry S.
   J Biol Chem. 2021 Sep;297(3):100998. doi: 10.1016/j.jbc.2021.100998. Epub 2021 Jul 21.
5. Phase separation of TPX2 enhances and spatially coordinates microtubule nucleation.
   King MR, Petry S.
   Nat Commun. 2020 Jan 14;11(1):270. doi: 10.1038/s41467-019-14087-0.

The cryo-EM structure needs to be tied into the manuscript. Where are the contact sites, and how does this explain the biochemistry of HURP?

We agree with the reviewer that the structure and biochemistry of HURP could be better connected. We have made a new panel (Fig. 5f) to display the detailed HURP-microtubule interactions. This now includes labeled amino acid residues. Our atomic model revealed that the HURP microtubule interactions are largely electrostatic involving conserved basic residues of HURP and acidic residues of tubulin. We expanded the respective paragraph in the results section. In addition, we expanded the discussion of this topic within the first paragraph of the discussion, which compares HURP binding with other MT binding proteins.

Specific comments:

Introduction lines 70-71: This statement is somewhat vague and misleading, as it has been previously shown that TPX2 and HURP do not directly interact in the same system used by the authors, not in another species. While the interaction is debatable, this should be clarified and commented on in the discussion.

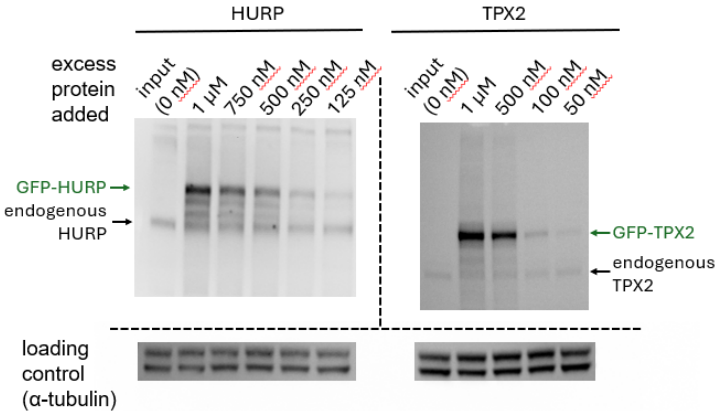
We see the reviewers point that our effort to compile background from multiple papers into one sentence did not work well. We have edited this sentence (lines 70-73) for clarity. We note that an indirect interaction between TPX2 and HURP was seen in human cells (Didaskalou et al, PMID: 37484914) and Xenopus egg extract (Koffa et al, PMID: 16631581). We hope that our motivation for this study is now more clear.

Additionally, our in vitro pull-down that we have newly added (Supplementary Figure 8) supports the finding in Casanova et al (PMID: 18799614), namely that although TPX2 and HURP interact in the presence of the microtubule, they do not seem to directly bind in solution in the Xenopus system. We comment on this in lines 148-158.

Figure 1A shows effective immunodepletion of HURP from Xenopus extracts. The authors disclose and indicate that the blots were cropped from non-adjacent lanes. The uncropped images were not available for evaluation. Although sufficient, more is needed to increase this reviewer's confidence as this manuscript is used to validate a new antibody.

To address this, we now provide the uncropped blot as the new Supplement Figure 1. We have chosen to keep the main figure unchanged to maintain ease and clarity for the reader. Additionally, in the figure provided for the next comment below, we use the same in-house HURP antibody to probe for both the endogenous and recombinant HURP protein.

Figure 2: The authors show the effect of over-endogenous levels of proteins on microtubule nucleation. A Western blot would help to assess the levels of the various proteins present and if concentrations are raised by 1% or by 100%.
Upon this request, we performed a western blot to help determine the levels raised by adding the recombinant protein. We added various concentrations of our recombinant proteins to the Xenopus egg extract, labeled as “excess protein added”, and used densitometry to analyze the fold-increase in comparison to the unperturbed extract (input). We converted fold-increases to concentrations based on the measured concentration of these proteins in the unperturbed extract (determined via mass spectrometry in Wuhr et al PMID: 24954049). When we compare the measured concentrations to the expected concentrations, we find them to be accurate.



Expected total HURP concentration (nM)	Calculated HURP concentration (nM)	Expected total TPX2 concentration (nM)	Calculated TPX2 concentration (nM)
320 (input)	320	50 (input)	50
1320	1727	1050	1157
1070	1180	550	687
820	947	150	137
570	509	100	87
445	465		

Although it is stated in the legends that the experiments were performed three times with similar results, there is no averaging of the data in the quantifications or hypothesis testing. Is this an N of 1 for the two experiments shown?

In the original manuscript, the quantification of microtubule nucleation in extract was shown as an n=1. In the revised manuscript, we have now averaged our data over the three replicates in Fig1C, 2B, and 2D.

Figure 1C, 2A,C: It needs to be more evident in the figure labelling that the added recombinant proteins are included above the endogenous levels, rather than 125 or 250 nM total protein. Please clarify.

We have now clarified this by adding "in excess" to figure panels 2A and 2C.
In Figure 1C, we have clarified that the addition of recombinant protein is an "addback" (or rescue) to HURP-depleted extract, and not an addition above endogenous levels.

Figure 3, S5: Although the phase boundaries of TPX2 have been previously determined, it would be helpful to repeat that experiment here to verify that it is identical in this system.
The current two-point titration of TPX2 concentration seems rather unconvincing.

We have now added a new Supplemental Figure 9A in which we repeat the bulk TPX2 phase assay with an 8-point titration. With this assay, we were able to determine the TPX2 phase boundary to be 47.8 ± 2.8 nM for the TPX2 prep that we used for the revised Figure 3.

Figure 4: For experiments involving microtubule rescue and catastrophe, it would be helpful to show kymographs.
We have now added kymographs as a new panel in Figure 4 (Figure 4C) and a more thorough quantification of microtubule dynamics from the newly performed experiment in which we measured growth from stabilized seeds (new panels Figure 4D-G).

The conclusion that HURP binding to microtubules must recruit γ-TuRCs or it has been convincingly shown that the two do not interact is simplistic, bordering on flawed given the model the authors provide: 'HURP may serve to stabilize lateral tubulin interactions that assemble on γ-TuRC to form the critical nucleus or stabilize the nascent microtubule lattice nucleated by γ-TuRC.'

It seems that our motivation for testing if HURP recruits γ-TuRC to a pre-existing microtubule was not well communicated in our text. Our intention was to understand how HURP is facilitating branching microtubule nucleation. Therefore, we wanted to test whether it does so by acting similarly to augmin or TPX2 (i.e., recruiting γ-TuRC to a pre-existing microtubule). We have edited lines 190-202 and have moved the figure panel for this experiment to the Supplement (now Supplement Fig. 10b and c), as it is not the main focus that we want to convey.

We also revisited whether HURP directly interacts with γ-TuRC by performing an in-vitro pull-down as a new experiment, which is now part of a new figure (Supplement Fig. 10a). In this assay, we see that HURP does not bind to γ-TuRC, further supporting our model that HURP's effect on nucleation comes from binding to the microtubule lattice and not by localizing or binding γ-TuRC.

Figure 5: The authors could have used the structure to comment more on their findings from earlier figures and to help tie them together. At the moment, this experimentally strongest part of the manuscript is underutilized.
Which tubulin residues are contacted, and how does that explain stabilization?

We have made a new panel (Fig. 5f) to display the detailed HURP-microtubule interactions. This now includes labeled amino acid residues. Our atomic model reveals that the HURP microtubule interactions are largely electrostatic involving conserved basic residues of HURP and acidic residues of tubulin. We expanded the respective paragraph in the results section. In addition, we expanded the discussion of this topic within the first paragraph of the discussion, which compares HURP binding with other MT binding proteins.

The authors argue that HURP simultaneously interacts with four tubulin subunits. Aren't there other MAPs that contact four subunits? Could we learn something from comparing and contrasting?

This is an excellent point. We have made a new supplemental Figure S14 to compare the MT binding modes among HURP, TPX2, EB3, DCX and CAMSAP1, which all bind between protofilaments. We also expanded the text in the Discussion to relate the MAP binding sites/modes to their effects on parameters for MT dynamics (growth, shrinkage, catastrophe and rescue).

Discussion
…their microtubule counterparts during mitosis are now discovered to be y-TuRC, XMAP215/chTOG, and HURP…
Why not combine all proteins and reconstitute branched microtubule nucleation outside of extracts?
That is a great suggestion. Our published reconstitution from 2020 was with augmin, TPX2 and g-TuRC. When XMAP215 is added to this assay, MTs form too fast and the MTs grow into each other, preventing clear observations. In order to add all of the components, including XMAP215 and HURP, we have to develop a modified assay, which also takes into account the binding hierarchy. This will be subject of future work and a future manuscript.

Reviewer #2 (Remarks to the Author):

In the manuscript by Valdez et al., entitled "HURP facilitates spindle assembly by stabilizing microtubules and working synergistically with TPX2", the authors use in vitro microtubule reconstitution assays using purified components and Xenopus egg extracts in conjunction with TIRF microscopy and cryo-EM to investigate the role of HURP in spindle microtubule assembly. HURP is a major spindle assembly protein whose mechanistic role in spindle assembly is poorly understood. The authors conclude that HURP stabilizes the growth of nascent microtubules during Ran-GTP-induced branched microtubule nucleation, a key event in spindle formation. Furthermore, they investigate the interplay of HURP with Tpx2, a known mediator of the RanGTP-induced branched microtubule nucleation pathway. The authors report that HURP forms co-condensates and synergizes with Tpx2 to strongly promote microtubule branching. The authors also perform structural studies and report that a minimal fragment of HURP containing a microtubule-binding domain binds to the GMPCPP-stabilized microtubule lattice, straddling protofilaments, thus potentially underlying the ability of HURP to stabilize microtubule growth.

A mechanistic understanding of HURP activity in spindle assembly is warranted and would significantly add to the fields of microtubule dynamics, spindle assembly and cell division. Furthermore, as HURP is a putative oncogene, elucidating HURP's mechanism could have implications for cancer. The authors are equipped with the tools to answer these questions. However, the manuscript in its current form appear very preliminary. The data presented are limited and more experiments and analysis are needed to support the preliminary findings and conclusions.

Major comments

Figure 1:

In Figure 1. the authors deplete HURP from Xenopus egg extracts to show that HURP has a role in branching microtubule nucleation. In Fig 1a, when compared to the mock depletion, the depletion of HURP appears to be robust. However, the loading controls do not appear equal between samples and y-TURC levels appear lower. Indeed, the quantification shows that y-TuRC levels are 20 percent lower than the input. I wonder if the authors could address this, and the implications this has for subsequent experiments looking at the effects of HURP depletion on nucleation (which involves y-TURC). For example, in Figure 1b, the lack of nucleation seen in the HURP-depleted condition could presumably be due to an additive effect of the reduced y-TURC levels and HURP depletion.

For quantification of the western blots in this experiment, each band intensity was normalized to the respective loading control. Therefore, we are confident that any difference in loading of the samples is accounted for.

Although we see a 20% depletion of y-tubulin in the HURP-depleted extract, this depletion matches that of the mock-depleted extract (Figure 1a), and the mock depletion still forms very nice branched microtubule networks (see Figure 1b). Since both samples are similarly depleted of y-TuRC, we believe that we can confidently state that the difference in branching microtubule nucleation between the two conditions is not due to reduced y-TuRC levels.

Furthermore, if the binding of HURP to Tpx2 and XMAP215 (two other proteins involved in branching nucleation) is RanGTP-dependent, as reported in (ref 28), then this raises an issue with Figure 1b, in which HURP depletion in the extracts is performed in the presence of constitutively active RanGTP. If HURP directly interacts with Tpx2/XMAP215 under these conditions then the addition of the anti-HURP antibody may sequester Tpx2 and XMAP215 activities contributing to the observed loss of nucleation. Could this explain why there are fewer 'fans' in the HURP rescue condition?

We thank the reviewer for bringing up this possible point of confusion. Our depletions are done using unperturbed extract, with no RanGTP added. We only add constitutively active RanGTP to the extracts after depletion has already been done, to induce branching microtubule nucleation in our assays. We have edited line 88 for clarity regarding this point.

Additionally, we have clarified in our background lines 71-72 that HURP has not been previously shown to directly interact with either TPX2 or XMAP215 (Casanova et al PMID: 18799614 and Koffa et al PMID: 16631581), and the nature of the interactions forming the complex in Koffa et al have not been characterized.

If significant depletion of other known factors (i.e., y-TuRC, augmin, XMAP215, TPX2) was occurring in the HURP-depleted extract, it would have been observed in our quantification in Fig1a.

The authors suggest that fewer 'fans' could be due to rescuing with sub-endogenous (250 nM) levels of HURP. Could the authors not perform rescue experiments with endogenous levels of HURP (~320 nM)? I would suggest performing a titration of HURP protein in the rescue condition encompassing endogenous HURP levels to determine whether the depletion effects are truly specific to HURP.

When we performed this assay, we had believed that the protein concentration was double what we reported (500nM). After assessing our purified protein further via densitometry, we corrected the reported concentration (250nM).

The experiments using depleted extracts are particularly challenging, given that the quality of the extract must not only be high to start, but must also endure the manipulations of the depleting process. In general, add-back experiments are even more challenging and the resulting observation always look a little bit different than the starting condition (PMID: 29695792 and PMID: 23415226).

Nonetheless, we do not believe the number of fans lends uncertainty to our statement that "HURP is necessary for branching microtubule nucleation", given that we are able to observe branching microtubule nucleation when we add back purified HURP protein alone. Furthermore, the nucleation dynamics of these structures is fully rescued. Therefore, we have now edited the text in lines 107-116 to better reflect the result that we found.
We concede that it is possible there is depletion of an unknown factor, but it is not required to rescue branching microtubule nucleation and the dynamics of these structures. In this manuscript, we focus on characterizing the role of HURP and its synergy with TPX2.

Additionally, it is imperative to determine the binding relationships between HURP, y-TURC, XMAP215 and Tpx2 with and without RanGTP for example by performing pull-downs with purified proteins and/or size exclusion chromatography.

To address this point, we have newly performed in vitro pull-down experiments of HURP with TPX2 and y-TuRC. We find that HURP does not directly bind to TPX2 or y-TuRC (new Supplementary Figures 8a and 10a) in solution. These finding are now discussed in lines 148-151 and lines 191-193.

Notably, SDS-PAGE gels and mass spectrometry analyses of purified HURP protein as well as for all other purified proteins used in the manuscript are not provided, thus protein purity cannot be assessed.

We have now added SyproRuby-stained gels and mass-spec analysis of our HURP and TPX2 proteins as a new supplement (Supplementary Figure 2). In the same figure, we provide the BSA standard curve that was used in our determination of the protein concentration of the full-length construct.

The quantification in Fig 1c should be of at least 3 experimental replicates and statistical significance should be provided to substantiate the claim that the amount of microtubule branching in each 'fan' is the same as the control condition.

We have replaced Fig1C with the averaged the results from three replicates, showing the standard error mean cloud for each plot and determining statistical significance between the conditions.

Finally, a schematic showing the domain structure of HURP and known/putative binding sites to protein partners would be useful here to set the context for the rest of the manuscript.

That is a helpful suggestion. We have added the requested schematic to Supplementary Figure 12. Since there are no known/putative binding sites with any of the proteins mentioned in the relevant background (i.e., XMAP215, augmin, TPX2, etc), we are not including it in the main figure.
We provide secondary structure elements and the known/putative microtubule binding domains in the sequence alignment of Supplementary Figure 12b.

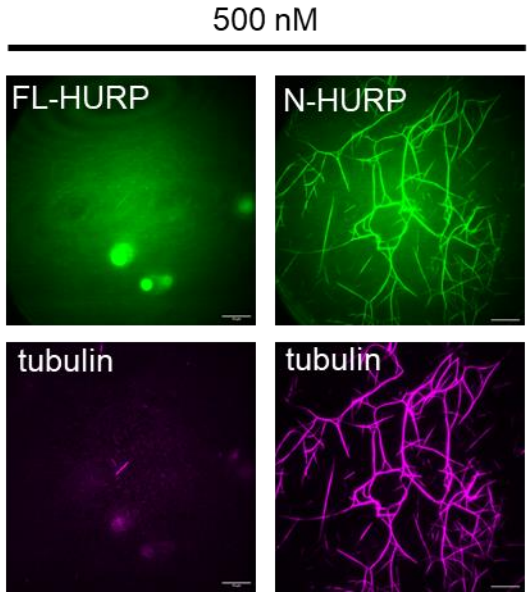
Figure 2.
In Figure 2, the authors conclude that HURP induces microtubule nucleation in Xenopus extracts and synergizes with Tpx2. Figures 2a and b show that HURP promotes nucleation of single microtubules but

not branches, which differs from Tpx2 activity. However, the two concentrations of HURP tested were both below endogenous levels (125 nM and 250 nM) and the quantification is of a single repeat only. It is possible that branching nucleation could occur at higher HURP concentrations. Therefore, to support this conclusion, a full titration of HURP should be performed as well as a more rigorous quantification of branched versus individual microtubule networks and statistical analysis of multiple experimental replicates.

We thank the reviewer for this comment. These experiments were not done in HURP-depleted extract. Therefore, the HURP concentrations (125 nM and 250 nM) are not indicating the total amount of HURP present in the extract, but rather the amount of protein added above the endogenous concentration. We have now added "in excess" to figure panels 2A and 2C. Additionally, we have averaged the data in panels 2B and 2D and provide statistical analysis.

Do the authors observe the expected activity of HURP as a microtubule bundling protein as previously reported? If not, can they comment on why? The microtubules are somewhat difficult to visualize in the images provided so it is difficult to ascertain whether any bundling is occurring.

We tested this in an in vitro MT bundling assay using our purified protein. Our full-length construct did not bundle microtubules at a concentration of 500 nM. However, an N-terminal construct of HURP does heavily bundle microtubules at this concentration (see below). Based on this finding and previous literature on HURP regulation, we believe that this is an interesting avenue for future structure function studies. It provides an excellent basis for a future manuscript and is beyond the scope of this current study of HURP.



Scalebar = 10 microns

Figure 3.
In Figure 3, the authors present data indicating that HURP is recruited to Tpx2 condensates again bringing into question the nature of the interactions between HURP and Tpx2. Is it known which residues/domains in HURP and Tpx2 are responsible for the interaction? Determining whether and how

HURP directly binds to TPX2 is central to the proposed mechanism and should be investigated by the authors using standard protein-protein interaction assays.

There are no interaction domains/residues identified between HURP and TPX2. A previous paper (Casanova et al PMID: 18799614) has shown that although TPX2 and HURP interact on the microtubule, they do not directly bind to one another in solution. We newly performed an in vitro pull-down and confirmed this (Supplementary Figure 8a). Therefore, we believe that the interaction between these two proteins is dependent on the co-condensation of HURP in the TPX2 droplets on the microtubule. We note this in lines 148-158.

Figure 4.
In Figure 4, the authors show that HURP facilitates nucleation in vitro from γ-TURC using purified proteins and that it does so by stabilizing microtubule growth. However, a more thorough quantification of microtubule dynamics parameters (Fig 4e,f,g) should be performed using standard field-accepted methodology and reporting (i.e. catastrophe and rescue frequencies, effects on shrinkage rate). I would highly recommend performing a titration of HURP activity and quantifying microtubule dynamics parameters across the titration. Representative kymographs should also be provided.

We thank the reviewer for this comment. To more accurately measure the dynamic parameters of microtubules (i.e., catastrophe and rescue rates and frequency), we have now provided another in vitro dynamics assay in the form of growth from stabilized microtubule seeds (Figure 4C-G). These findings are discussed in lines 209-222

Can the effects of HURP on suppressing microtubule catastrophe be explained by the increased growth rate (rather than lattice stabilization)? Very few rescue events were quantified and more should be analyzed for the interpretation to be meaningful (just 10 shrinkage events were analyzed in the control and 8 in the HURP condition across all replicates).

We agree that our previous assay limited our ability to determine microtubule dynamics in our conditions. In our new quantification using the standard assay, we see that HURP does not significantly affect the growth rate of microtubules (Figure 4D). However, there was a significant decrease in catastrophe rate, shrinkage rate, and an increase in rescue frequency (Figure 4E-G). In this new quantification, 68 rescue events were quantified for the buffer condition, 51 events for the 25 nM HURP, and 26 events for 50 nM HURP.

As the authors have expressed and purified GFP-HURP, it would also be valuable to observe the localization of GFP-HURP on dynamically growing microtubules as well as during branching nucleation.

We now provide kymographs in Figure 4C of HURP localization on dynamically growing microtubules. In our assays, we don't clearly see a difference in binding to the GDP vs GMPCPP lattice. This is in contrast to previously published work (Castrogiovanni et al, https://doi.org/10.1038/s41467-022-32421-x), in which they conducted various experiments to show HURP has a preference for the GDP-lattice. It is possible that we see this discrepancy due to differences in HURP concentration, however the publication mentioned does not state their concentrations used for their lattice-preference experiments. We note the implications of HURP's lattice preference in our model in the discussion (lines 309-315)
Additionally, we attempted to visualize HURP localization during branching microtubule nucleation, but it is challenging to visualize localization of HURP on single microtubules given the high microtubule density in the fan structures.

Figure 5.
In Figure 5, the cryo-EM data presented indicate that HURP binds at the groove between neighboring protofilaments, the authors propose the model that HURP binding could strengthen the lateral interactions between protofilaments. A prediction would then be that HURP could slow down microtubule shrinkage by preventing the peeling apart of protofilaments. Therefore, it would be valuable to report the effects of HURP on dynamic microtubule shrinkage rates.

We agree with the reviewer and have now added quantification of the shrinkage rates in our results (Figure 4E). As predicted, we do is see the shrinkage rate of the microtubules decreases when HURP is present. These results are now noted in lines 213-216.

Discussion
In the discussion the authors speculate that HURP may 'bridge two soluble tubulin dimers or short protofilaments laterally, therefore promoting the formation of early tubulin nucleation intermediates'. However, they show in Fig S6 that HURP does not directly recruit soluble tubulin to pre-existing microtubules. Therefore, this discussion point seems somewhat contradictory. It would be useful to test for direct binding of HURP to soluble tubulin without the presence of pre-existing microtubules using standard protein-protein interaction assays.

We thank the reviewer for this excellent suggestion. We directly tested the binding of tubulin in a pull-down assay (Supplemental Figure 11A). Indeed, HURP only weakly binds to soluble tubulin. As observed in the prior assay, soluble tubulin is not recruited to the pre-existing MT, indicating that HURP prefers binding to the MT lattice over soluble tubulin. This new result is now incorporated in lines 202-206. Moreover, we edited the discussion in the first paragraph to remove the original speculation and better reflect our findings (lines 290-300). All results, including the newly performed dynamics assay and tubulin binding assay, confirm that HURP binds to the formed MT lattice and stabilizes this state.

Minor comments
- Typo line 973 – should say p-value <0.05?

We did not find the text being referred to on line 973 (line 973 appears to be in the References section) and are unsure of where this possible error is. We have looked at all mentions of p-values to ensure they are correct. In doing so, we have corrected the notation for not significant p-values (p-value>0.05) to be "ns" rather than "n.d." in line 918

- In Fig4b, please report errors on the rates.
Errors are now reported in Fig4b and in the main text (lines 187 and 188)