A teamwork promotion of formins-mediated actin nucleation by Bud6 and Aip5 in Saccharomyces cerevisiae

Ying Xie, Feng Zhou, Qianqian Ma, Lanyuan Lu, and Yansong Miao

Corresponding author(s): Yansong Miao, yansongm@ntu.edu.sg

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RE: Manuscript #E21-06-0285
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Dear authors:

Two expert reviewers find the subject of your work appropriate for MBoC, but both have substantive reservations about the design and interpretation of the experiments. They offer you sound advice about additional experiments and revision of the text. IF you are willing to do this extra work, I will ask these reviewers to evaluate the revised manuscript.

Sincerely,

Thomas Pollard
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Miao,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

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Eric Baker
Journal Production Manager
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Reviewer #1 (Remarks to the Author):

See attached.

Reviewer #2 (Remarks to the Author):

Review on the manuscript "A teamwork promotion of formins-mediated actin nucleations by Bud6 and Aip5 in Saccharomyces cerevisiae" by Ying Xie et al.
Xie and colleagues report on the interplay between the yeast formins, and the nucleation promoting factors Bud6 and Aip5 in the formation of actin filaments.
The authors provide evidence that the simultaneous interaction of Bud6 and Aip5 with the yeast formin Bni1 has a synergistic effect on the complex's ability to nucleate actin filaments. A synergistic effect of the two NPFs was not measurable for the Bnr1-Bud6-Aip5 complex.
A Monte Carlo simulation predicts a structure of the Bni1-Bud6-Aip5 as well as the Bnr1-Bud6-Aip5 complex.
By focusing on the combined biochemical activity of two different nucleation promoting factors the authors provide a more realistic picture of how actin nucleation might be achieved in the cell. The manuscript is therefore a valuable contribution to the field that merits publication.
Below I summarize my critique and suggestions that might help to strengthen the conclusions of the paper.

1.) The authors work with fragments of Bud6, Bni1, Bnr1 and Aip5 to postulate a network of factors that influence actin nucleation. Aip5 and Bud6 interact physically with each other. With Aip5 (1110-1234) and Bud6 (550-789) two fragments of the proteins were chosen in this work that lack the binding site between the two proteins (Bud6 1-144); Aip5 (1000-1125) (Glomb et al., 2019). This is a very unfortunate choice as the physical interaction between the two actin nucleation factors might change the properties of the trimeric Bni1-Aip5-Bud6 complex. An experiment that measures actin filament formation in the presence of Aip5 (1000-1234) and the full length Bud6 would resolve some of the open questions. If this is not feasible, the authors might at least give room for a fair discussion of the limitations of their current measurements.
In that respect, the statement of the authors in the discussion: "Although Aip5C does not interact with Bud6C, whether the full-length Aip5 and Bud6 would collaborate to promote formin function has yet to know, because the intrinsically disordered regions of Aip5 and Bud6 could drive macromolecular assembly at heterogeneous states under different cellular conditions (8,36)" is at least misleading and difficult to understand. The two proteins are known to form a complex in vivo.

2.) The authors perform a simulation to predict the structure of the trimeric Bni1-Aip5-Bud6 and the Bnr1-Aip5-Bud6 complexes. The predictions are very specific. Testing these predictions would strengthen the proposed models of the simulations. I highly recommend to use the outcome of the simulation to construct minimal mutants of the fragments that are predicted to not interact and to test their properties with the biochemical assays.

3.) Minor points:

3.1 Page 2 line 39: in the presence of profilin (Pfy1)....

3.2 Page 2 last line: reminiscent instead of reminiscence.

3.3 Page 3 line 25: further activated instead of has further activated.

3.4 Page 3 line 48: stoichiometry

3.5 Figures 1 and 2 A,B: Are we looking at the averages of more than one
measurement for each condition? Please indicate.

3.6 Fig 2 F "Anosotropy"; Fig 1E "Anistropy" .
3.7 Page 7 Discussion: "By having long IDR, Aip5 accumulates at the bud tip for cell polarization...." This statement is misleading as it imposes that phase separation might drive Aip5 into the bud tip. However, it was shown that a direct interaction between Spa2 and Aip5 localizes Aip5 at the bud tip.
3.8 Page 7 Discussion: "During cell signal transductions under physiological and pathological conditions, IDR creates diverse states of macromolecular assembly in spatiotemporally-regulated manners, which could provide tunable activities of the functional complex, such as NF-NFPs." Here and in other parts of the discussion (see point 1) the accumulation of ill-defined terms makes it very difficult to follow the argument of the authors. Please be more precise.

3.9 Page 8: "where 3 μM yeast profilin was added as an indication". ??

Reviewer 1

In this manuscript the authors characterize the cooperation between two budding yeast formin nucleation promotion factors (NPFs), Bud6 and Aip5, in enhancing actin nucleation by the two yeast formins Bni1 and Bnr1. This work builds on the authors' recent publication characterizing Aip5 as a nucleation factor (NF) and an NPF for the formin Bni1, and another paper of theirs showing that *Candida albicans* homologues of Bni1, Bud6, and Aip5 synergize in promoting nucleation. In the present study, they provide evidence for a similar synergy between budding yeast Aip5 and Bud6 in enhancing Bni1-mediated actin nucleation. Further, they characterize the effects of Aip5, alone and together with Bud6, on the formin Bnr1. At the protein concentrations used in their assays, Aip5's NPF activities on Bnr1 are still unclear, as is the question of whether or not Bud6 and Aip5 cooperate to activate Bnr1 (see comments). The authors also largely ignore previous work showing that full-length Bud6 and Aip5 interact (Glomb et. al 2019), and instead characterize the activities of C-terminal fragments of Bud6 and Aip5 that lack their interacting regions. This is relevant because deletion of the Bud6-binding region of Aip5 results in fewer actin cables in vivo (Glomb et. al 2019), suggesting that Aip5-Bud6 interactions may be important for actin nucleation in vivo. In my assessment, for this work to make a meaningful and lasting contribution to our understanding of formin regulation, a few additional experiments are needed (see below), as are modifications to the text (and a model) clarifying the mechanism by which Aip5 collaborates with Bud6 to differentially regulate Bni1 and Bnr1.

We thank the comments and suggestions raised by the Reviewer to improve our manuscript. We have addressed the Reviewer's comments in the following sections. We also want to respectively argue that doing biochemical experiments with minimum components is a standard approach to dissect complex interactions and functional connections between members within the macromolecular complex. A minimum component reconstitution system enables a clear dissection of inter- and intramolecular interactions of the macromolecular complex members. Such an experimental system does not conflict with the experiments using a longer version (or the full-length) of protein components but instead contributes the fundamental principle by a simplified and direct relationship to understand the association between individual components towards an understanding of the sophisticated interactions within the protein complex, and knowing the synergistic or antagonistic effects between members.

Minor comments

1) **Comment**: The sentence in the abstract, "*By examining the functions and interactions of NPFs and NFs via biochemistry, genetics, and mathematic modeling approaches, we found that two NPFs, Aip5 and Bud6, showed joint teamwork effort with Bni1 and Bnr1, respectively, via the C-terminal intrinsically disordered region (IDR) of formin, in which two NPFs displayed synergistic stimulation on Bni1mediated actin nucleation,"* is unclear. It is unclear how Aip5 and Bud6 show "joint teamwork effort" with both formins yet only synergistically enhanced Bni1-mediated (and not Bnr1-mediated) actin nucleation.
   Suggestion: The authors should clarify what "joint teamwork effort" means.
   Joint teamwork effort means the tri-protein complex function for formin-mediated actin nucleation by intermolecular interactions among one formin and two NPFs. We have now clarified this on page 1.

2) **Comment:** In the Results the authors state, "*We previously characterized NFs and NPFs in vivo to understand their physiological- relevant range of concentrations and stoichiometry at their concentrated sites. In addition to our previously measured in vivo Aip5 and Bni1 concentration at the bud tip, Aip5 (~120 nM) and Bni1 (~80 nM) (24).*" However, reference 24 in the reference list is: Li, J., Henty-Ridilla, J. L., Staiger, B. H., Day, B., and Staiger, C. J. (2015) Capping protein

integrates multiple MAMP signaling pathways to modulate actin dynamics during plant innate immunity. Nat Commun 6, 7206. This does not appear to be the correct citation. Additionally, how information about the cellular concentrations of these proteins influenced the design of their in vitro biochemical assays or model for how the tripartite protein complexes Bud6-Aip5-(Bni1/Bnr1) function is not made clear.

Suggestion 1: Check that the correct paper is referenced, Xie et. al 2019, reference #8.

We apologize for the wrong citation, which has been corrected now.

Suggestion 2: In the results, clarify the motivation for quantifying cellular concentrations of Aip5 and Bnr1, and expand on how these concentrations are related to the in vitro assays.

Quantifying cellular protein concentration could provide a relative concentration between two NPFs, Aip5 and Bud6, and yeast formin protein with physiology relevance. Such protein concentration would inform the design of in vitro assays for two types of formin-NPFs complexes, Bni1-Aip5-Bud6 and Bnr1-Aip5-Bud6, which facilitate the interpretation of the activity comparison between two types of NF-NPFs complex. We added these clarifications now on page 4.

3) **Comment**: In the Results describing Figure 2, the authors state, "*Since Aip5C and Bud6C do not have evident interaction...*" While this statement is correct, additional clarification will help the reader because full-length Aip5 and Bud6 have been shown to interact.

Suggestion: Acknowledge that full-length Aip5 and Bud6 interact and that the relevant interacting domains are missing in the C-terminal fragments used in the present study. It has been previously shown that Aip5 residues 1000-1131 are required and sufficient to interact with Bud6, and a C-terminal fragment of Aip5 (1000-1234) is pulled down by an N-terminal fragment of Bud6 (1-141) (Glomb et. al 2019).

Thanks for the suggestion. We have now added the sentence to describe the previously identified interaction regions in Glomb et. al 2019 between Aip5 (1000-1234) and Bud6 N-terminus (1-141), before introducing the Aip5C and Bud6C in this report (on Page 4). We also added the clarification in the corresponding results part (on Page 4) to elaborate the reason why we use minimum regions of two NPFs without their intermolecular interaction to reconstitute their simultaneous interactions with a formin as a dissection strategy towards a complete understanding the complex interactions of all full-length proteins in the future.

4) **Comment:** The Results section describing Figure 2F is unclear. The authors state, "*Interestingly, a higher Bni1FH1C: Aip5C stoichiometry at 1:1 (60 nM each) demonstrated a further enhanced interaction in response to the increasing concentration of Bud6C, such as 60 nM Bud6C: Bni1FH1C: Aip5C at the stoichiometry of 1:1:1. The above results indicate that a sufficient Bni1 creates enough binding sites to host both Aip5 and Bud6, thereby creating a better synergy of two NPFs*." The authors' model for how "*sufficient Bni1 creates enough binding sites"* for both NPFs is unclear. It is also unclear how they are inferring the stoichiometry of the tri-protein complex, and what they are concluding about this.

Suggestion 1: Clarify what is being measured in the assay (also see major comment #4 below). This assay does not directly yield information about the stoichiometry of a putative tripartite complex involving both NPFs and each formin.

This experiment was not designed to conclude a physiology-relevant in vivo stoichiometry or a most optimized stoichiometry in vitro for Bud6C:Bni1FH1C:Aip5C complex, but rather indicate that Bud6C: Bni1FH1C: Aip5C are able to reach a complex assembly with a stoichiometry of 1:1:1. In addition, to

clarify the confusion caused by the wording, we have now changed a few words in the original sentence "The above results indicate that a Bni1 sufficient creates enough binding sites to host both Aip5 and Bud6, thereby creating a better synergy of two NPFs." to " The above results suggest that Bni1C have enough binding sites to host both Aip5C and Bud6C, thereby creating a joint effort of two NPFs towards a formin protein" on Page 4.

Suggestion 2: Clarify in the text that Bni1 and Bnr1 form dimers (Moseley et al. 2003 DOI: 10.1091/mbc.e03-08-0621, Garabedian 2018 DOI: 10.1083/jcb.201803164, Xu et al. 2004 DOI: 10.1016/S0092-8674(04)00210-7), as do Aip5 (DOI: 10.1038/s41467-019-13125-1) and Bud6 (Tu et al. 2012 DOI: 10.1073/pnas.1203035109). When referring to a binding stichometry of 1:1, do you mean one dimer binds to one dimer? Please clarify.

Thanks for the excellent point. Indeed, as the Reviewer pointed out, the three truncating versions of NF and NPFs are all at a dimeric state. We have now added such clarification with the suggested references on page 4.

5) **Comment:** Anisotropy binding curves in Figure 1E, 2F, and 4A appear sigmoidal suggesting the Hill slope differs from 1, potentially indicating cooperativity. The shape of the curves and the implications of this are not adequately addressed in the text.
Suggestion: In the Results for each figure, provide the Hill coefficient for each curve, and explain the implications, i.e., regarding potential positive or negative cooperativity.

Thanks for raising this very interesting point. Indeed, most of the Hill Coefficient from the curve showed higher than 1 (see below), suggesting positive cooperativity.
In Fig. 1E: Bnr1FH1C: h=2.2 (positive cooperativity); Bnr1C:h=1.5 (positive cooperativity)
In Fig. 2F: Bni1: 30nM h=1.5 (positive cooperativity); 60nM h=1.5 (positive cooperativity); Bnr1:30nM h=6.4 (positive cooperativity); 60nM h=3.0 (positive cooperativity)
In Fig. 4A: Bnr1-LC: h=2.043 (positive cooperativity)

Such results are potentially derived from the nature of intrinsically disordered regions of the formin C-terminus. IDRs have highly variable binding affinities for different inter and intramolecular interactions and exhibit a broad range of binding modes with biomolecules, depending on the length and amino acid composition. The detailed dissection towards this point requires future systematical studies of the complex assembly. To avoid over speculation and overstatement, we have now discussed such nonlinearities of their interactions briefly in the discussion on pages 7-8. This is undoubtedly an important area for future study of macromolecular complex assembly using full-length proteins via biochemical assays or multi-scale modeling approaches.

6) **Comment:** In the Results, the sentence, "*The triple mutants bnr1Δ aip5Δ bud6Δ demonstrated the greatest extent of genetic sickness in the presence of LatA, suggesting the teamwork function of two NPFs in regulating Bnr1-mediated actin turnover for cell growth*," is unclear. Bni1 is the only remaining formin in *bnr1*Δ cells. Therefore, synthetic growth defects observed in *bnr1*Δ cells upon deletion of *AIP5* and *BUD6* indicate that Aip5 and Bud6 are required for Bni1-mediated actin assembly.
Suggestion: Clarify text and explain results in light of the points above.

Triple mutant bnr1Δ aip5Δ bud6Δ has a disruption of both Bnr-NPFs and Bni1-NPFs complexes by missing both NPFs. Such disruption of both NF-NPFs complexes and genetic sickness do suggest the importance of keeping the trip-protein complex intact and functional. We can not completely differentiate which complex is more important than the other one for such reason. We have now

elaborated on these defects in both complexes to support our original claim on page 6 to avoid potentially misleading descriptions.

7) **Comment:** The paper would be strengthened by including a working molecular model, based on work here and in previous studies, for how Aip5 and Bud6 cooperate to enhance actin nucleation by Bni1 and Bnr1.
Suggestion: Show the model in a new figure.

We have now shown a model in a new Figure 6.

Major Comments

1. **Comment**: The authors do not provide strong evidence that Aip5 works *with* Bnr1 to enhance Bnr1mediated actin assembly activity. In the text describing Figure 1, the authors state "*Interestingly, we observed that Aip5C is capable of promoting the actin nucleation of both Bni1FH1C and Bnr1FH1C, in an Aip5 dose-dependent manner (Figs. 1A, B)*" and "*In the presence of 20 nM Aip5C, Bni1FH1C- and Bnr1FH1C-initiated actin nucleation were significantly enhanced by ~1.73-fold and ~1.69-fold, respectively (Figs. 1F, G)*." It is unclear if the enhanced nucleation is due to Aip5 working synergistically with the formin, or instead due to additive effects of Aip5-mediated actin nucleation (~2-fold above control) combined with formin nucleation. This is especially true for Bnr1, which is characterized interacting with Aip5 for the first time here and which on its own is a far more potent nucleator than Bni1 (Moseley and Goode 2005 DOI: 10.1074/jbc.M503094200).
Suggestion 1: Titrate Aip5 and Bnr1 in TIRF assays to identify concentrations of the proteins that show minimal nucleation activity on their own, allowing a stronger test of enhanced nucleation in the presence of both proteins. In their 2019 publication (DOI: 10.1038/s41467-019-13125-1), the authors used a lower concentration of Bni1FH1C (5 nm) in TIRF assays, where Bni1 alone and Aip5C alone only modestly enhanced nucleation compared to control (~5-fold and 2-fold, respectively). However, the simultaneous addition of both Bni1FH1C and Aip5 resulted in a profound ~17-fold increase in nucleation. The concentrations of Bnr1 and Aip5 used here make it harder to determine whether there is synergy, or merely additive effects.

Here we want to clarify that the experiments were designed to compare the overall activity of NF-NPFs pairs for Bni1 and Bnr1, respectively, by using fixed concentrations of two NPFs. And we also have purposely optimized the nucleation activity of both formins to a similar nucleation level that allows us to reasonably compare how two NPFs work together to promote the formins at a similar nucleation activity. We agree with the Reviewer that the synergistic effect is not obvious for Bnr1 compared to Bni1 with formin at a similar activity. Here, we have now clarified this point, rationalized our experimental design better, and carefully compared the difference between two sets of NF-NPFs to avoid potential overclaim on page 4.

Suggestion 2: Use a Bnr1 construct lacking its C-terminal portion that interacts with Aip5, and demonstrate that nucleation is no longer enhanced by addition of Aip5. The authors used this technique previously to show that Aip5 works with Bni1 by binding to the Bni1 C-terminus; a Bni1 construct lacking this segment was blind to further addition of Aip5 (DOI: 10.1038/s41467-019-13125-1).

We thank the Reviewer for appreciating our previously published work that used Bni1-FH2 without C-terminal IDR as additional validation approaches to demonstrate the importance of specific interactions between Bni1-C and Aip5-C. In this report, we have also shown the specific interactions between Bnr1C

and Aip5-C in Figure 1E. In addition, our simulation work also additionally demonstrated the complex interaction mode at the C-terminus. Thereby, with sufficient evidence of Bnr1-C and Aip5-C interaction, we directly used the more functional relevant Bnr1-FH1COOH version to study the complex interaction and function with NPFs. And this additional experiment involves a series of experiments, including making new constructs and performing a new set of experiments to support a point already demonstrated by other assays. Currently, by having a rather difficult time in Singapore due to the pandemic and graduations of the first author, we would appreciate any consideration and understanding.

2. **Comment:** The authors do not provide strong evidence that Bud6 and Aip5 cooperate to regulate Bnr1. In the results section describing Figure 2, the authors state "*However, the overall promotion rate of two NFPs on Bnr1FH1C (~2.1fold) is slightly lower than the multiplied rates (~ 3.5) of Bud6C (~1.83) and Aip5C (~1.69), indicating an* ***additive effect*** *instead of a synergy of two NPFs on Bnr1 (Figs. 1F, G and Figs. 2C, D)*." The authors also state " *Compared to Bni1FH1C-mediated actin nucleation in the presence of Bud6C, additional Aip5C resulted in a ~3.4-fold increase in the generation of actin seeds, whereas Aip5C enhanced the Bnr1FH1C-Bud6C-mediated actin nucleation by ~1.13-fold (Figs. 2C, D).*" I agree with the authors' conclusion that the addition of Aip5 has an additive effect on Bnr1-Bud6 actin nucleation. However, this additive effect could be due to Aip5 enhancing nucleation independently of Bnr1-Bud6 given that Aip5 enhances nucleation ~2-fold over control.

   Suggestion 1: Given that Bnr1 is 10-15 fold more potent than Bni1 in nucleation (Moseley and Goode 2005 DOI:10.1074/jbc.M503094200), the authors may need to use a lower concentration of Bnr1 to see synergistic effects of these two NPFs. They should repeat the pyrene assays using a lower concentration of Bnr1, and titrate the effects of Aip5 and Bud6 until they reach maximal enhancement of Bnr1-mediated nucleation.
   Suggestion 2: The authors should also change the language in the manuscript to make it clear that while Aip5 and Bud6 synergistically enhance Bni1-mediated actin nucleation, they may only show additive effects on Bnr1-mediated actin nucleation.

   In the same experiments with the same condition, we have calculated the polymerization rate and described the results with the following sentence "…Bnr1FH1C (~2.1fold) is slightly lower than the multiplied rates (~ 3.5) of Bud6C (~1.83) and Aip5C (~1.69), indicating an additive effect…..". I believe an increase from 1.69 to 2.1 is considered an actual increase. The experiments were designed to use the comparable nucleation activities between two formins that allow us to describe the differential NPFs effects on two formins. We would not be surprised to imagine that if we titrate much concentration of formin, in the combination of more additional concentrations of NPFs for each formin condition, which will create a different actin assembly curve. In theory, such a design makes a comprehensive map for every single situation. But in reality, it can also easily increase the experimental load by more than 5 folds. And the comparison between the triple mutants bni1Δ aip5Δ bud6Δ (lethal, only Bnr1) with the double mutants, bni1Δ aip5Δ (with Bnr1 and Bud6), bni1Δ bud6Δ (with Bnr1 and Aip5), also supported that the importance of having all three components of Bnr1, Aip5, and Bud6. So we decided to take suggestion two from the Reviewer by clarifying this point with a better background of the experimental design and a clear description of the difference between to NF-NPFs pairs, either being synergistic or additive. The revised information is now on page 4.

3. **Comment:** As noted above, the authors largely ignore that Bud6 and Aip5 interact and do not discuss how this interaction may influence cooperation between the two NPFs. Aip5 residues 1000-1131 are necessary and sufficient to interact with Bud6, and a C-terminal fragment of Aip5 (1000-1234) interacts

with an N-terminal fragment of Bud6 (1-141). Furthermore, Bud6-Aip5 interaction promotes the production of actin cables in vivo (Glomb et. al 2019 DOI:10.1242/bio.044024). However, in this publication, the authors use C-terminal fragments of Bud6 and Aip5 in their in vitro experiments that lack these binding domains.

Suggestion 1: Previously (DOI: 10.1038/s41467-019-13125-1), the authors purified and characterized full-length Aip5. Why not test if full-length Aip5 and Bud6 (or fragments of these proteins that interact) synergize? If the authors are unable to purify sufficient quantities for these experiments, explain this in the Results.

Suggestion 2: The authors need to acknowledge that while the fragments they use are sufficient to bind and activate formins in vitro, Bud6-Aip5 interactions likely contribute to the mechanism in vivo.

We apologize for not discussing well the interactions between Aip5 and Bud6, although our experimental setup did not involve these interactions. Please also see our above reply to Minor Comment 3, which is similar to this Major one.

1. We have explained the reason for carrying these minimum-reconstitution experiments on formin by using the functional domains of NPFs without their intermolecular interactions. Please also see the reply in Minor Comment 3.
2. The expression of all full-length proteins of Bni1, Aip5, and Bud6 is technically challenging. We are still working on it using different expression systems, including insect cells, yeast cells, or even mammalian cells. It is the final goal to study macromolecular assembly. However, the technical challenges in purifying enough soluble proteins for biochemistry work is still a significant hurdle, which would not be solved easily. As the Reviewer suggested, we have described such challenges on page 4 now.
3. We have now also added a discussion to acknowledge the additional interaction between two NPFs (Glomb et. al 2019 DOI:10.1242/bio.044024) might further contribute to the regulation of formin activities with a higher-order assembly on pages 2,4,5,8 now.

Suggestion 3: The authors showed that *Candida albicans* Aip5 and Bud6 interact through their Ctermini (DOI: 10.1074/jbc.RA120.013890). Sequences of scAip5C and caAip5C (and scBud6C and caBud6C) should be aligned, to see if residues predicted to mediate Aip5C-Bud6C interactions are conserved, and this should be reported in the text. It is possible that weak interactions between scAip5C and scBud6C, not detected in solution, become relevant in the context of a tri-protein complex where Aip5 and Bud6 may be brought into close proximity, scaffolded by the formin. This possibility should be raised in the Results describing Figure 2F.

First, we have shown the alignment of the Aip5C and Bud6C in the supplementary Figure 3 in the published JBC (DOI: 10.1074/jbc.RA120.013890). Only 58% of residues of Aip5 and 41% for Bud6 are identical between two fungal species. This is a fascinating point that raises a more hypothesis for our future basic research studies. These sequence variations during evolution might be a reason to have

different intermolecular interactions with evolutionary unconserved IDR of formin C-terminus. How these evolutionary diversity offers different regulatory mechanisms for other yeast and filamentous fungi are striking and worth future studies to understand the regulatory role of evolutionary selection for the final macromolecular assembly of NF-NFPs complex among fungi.

Second, although we can not exclude the possibility of weak interaction between Aip5C and Bud6C, we can not claim this in the results of Fig. S2, where we did not detect clear interactions. Furthermore, such speculation of weak interactions between Aip5C and Bud6C would likely be less important than the interactions reported in Glomb et. al 2019 (DOI:10.1242/bio.044024). To emphasize the interaction between Aip5 and Bud6 again, we have added the discussion that describes more regulatory potentials in formin function by having a higher-level assembly of NF-NPFs on page 7, citing Glomb et. al 2019 (DOI:10.1242/bio.044024).

4. **Comment:** The authors only use one approach to characterize interactions in the Bud6-Aip5-Formin triprotein complex, and the limitations of this approach leave it unclear how each protein influences complex formation. In Figure 2F, the authors use anisotropy to quantify how increasing concentrations of Bud6C alters the rotational diffusion of Aip5C-A488 in the presence of a constant concentration of either formin (Bni1 or Bnr1). They state, "*Since Aip5C and Bud6C do not have evident interaction, we next added increasing concentrations of Bud6C to the pre-mixed sample of Alex488 labeled Aip5C and Bni1FH1C, in which the changes in anisotropic value would reflect the interaction changes between Aip5 and Bni1FH1C*." How can the authors conclude that a change in anisotropic value reflects altered interactions between Aip5C and the formin, rather than changes in anisotropy due to Bud6C joining the complex of Aip5C + formin, thus changing the rotational diffusion of the entire complex?
Suggestion: In their 2019 publication (DOI: 10.1038/s41467-019-13125-1), the authors used anisotropy to quantify the affinity of Bni1FH1C for Aip5C by using a fixed concentration of labeled Aip5C and adding increasing concentrations of Bni1FH1C. By this approach, they measured a Kd of 16 nM. To determine if Bud6 enhances the affinity between Aip5C and Bni1FH1C, the authors should repeat this experiment with a fixed high concentration of Bud6 in molar excess of the other two proteins (Aip5 and the formin). This should also be done for Bnr1FH1-C. If the presence of Bud6 in the assay alters the Kd, then they can conclude that Bud6 influences the affinity of Aip5C and either formin. This same approach can be used to determine if Aip5C alters the interaction between Bud6 and either formin by using Alex488 labeled Bud6C with a molar excess of Aip5 and increasing concentrations of either formin.

First, "…., thus changing the rotational diffusion of the entire complex…." The question Reviewer 1 raised is rather challenging to address, which we felt is out of the scope of this report. Such precision description requires titration experiments with NMR-based structural determination that would best characterize if it has to be fully addressed. We currently do not have such capacity to perform such a level of structural studies and draw such specific conclusions.

Second, the purpose of the experiment here is to understand the cooperation between two NFPs on one formin. This is slightly different from our 2019 published NC paper, where we only have one NF and one NFP. We tried to understand the interaction between two proteins, which can be done by fixing either one and adding an increasing concentration of the other. Here, to know how one NPF will affect another NPF function of an existing NFP-NF pair, we have to add an increasing concentration of NPF instead of a formin on its own. Sorry for any potential confusion; we hope our explanation here elaborates better on the difference between the two types of purpose and experimental designs.

Nevertheless, in vivo, all three proteins work together at the same time. And our simulation works here were performed with three proteins together simultaneously without having an order of timing.

5. **Comment:** It is unclear why the authors have not taken advantage of point mutations available to more rigorously test the in vivo role of Aip5-Bud6 NPF synergy. Previous studies show that *aip5Δ* cells are sensitive to latrunculin, and reveal synthetic defects in cell growth and actin cable organization between *aip5Δ* and *bud6Δ* mutants (Glomb et. al 2019 DOI:10.1242/bio.044024). Here, the authors build on these findings by characterizing triple mutants *aip5Δ bud6Δ bnr1Δ* and *aip5Δ bud6Δ bni1Δ* (lethal). However, separation of function mutations in Bud6 that abolish direct binding to formins (*bud6-35*) and actin (*bud6-8*) are available (Graziano et al. 2011 DOI: 10.1091/mbc.E11-05-0404; Graziano et al. 2013 DOI: 10.1083/jcb.201212059). Further, the authors have characterized mutations in Aip5 that abolish Bni1 NPF activity and actin binding (DOI: 10.1038/s41467-019-13125-1), which could be used to address the importance of Aip5-actin interactions in vivo.

   Suggestion: Genetic interactions, e.g., between *bud6-35* and *aip5Δ*, *aip5Δ bnr1Δ*, or *aip5Δ bni1Δ,* would strengthen the authors' conclusions that Bud6 and Aip5 NPF activities are important in vivo. Similar experiments could be carried out in strains expressing mutant Aip5 impaired in actin binding. Each of the proteins studied here is multi-functional, so using point mutants (instead of full gene deletions) would provide a more rigorous test of the importance of the NPF funtions in vivo.

   These suggested experiments went to the details in understanding the detailed and specific interaction sites using a genetic approach, which is out of the scope of this work, considering the evidence we had to support our conclusions and multiple experimental systems we used to compare with each other. The suggested genetic experiments to identify the specific positions would be helpful for detailed analysis in the future, which would add good knowledge in addition to our multiple mutants data here using single, double, and triple mutants.

   1. In theory, the possibilities for these genetic experiments are numerous, no matter some point mutations of each member with clear structural information or not. If we really consider the interactions, we also need to consider Act1, which has more than 20 mutants in the ts-library. We currently do not have a robotic system in the lab to carry out such extensive large-scale genetic experiments. Reviewer #1 asked though several top genetic labs can do that such as Boone lab in Canada.

   2. Here is some additional information for your kind consideration. Obtaining the genetic materials from overseas and handling the MTA is rather difficult at this moment during the pandemic. In another revision work about the plant actin study, the MTA for a material request was revised multiple rounds back and forth between two Universities and only went through halfway after six months. Eventually, we got some very kind considerations from both the editorial office and Reviewer about the situation.

Reviewer 2

Review on the manuscript "A teamwork promotion of formins-mediated actin nucleations by Bud6 and Aip5 in Saccharomyces cerevisiae" by Ying Xie et al.

Xie and colleagues report on the interplay between the yeast formins, and the nucleation promoting factors Bud6 and Aip5 in the formation of actin filaments. The authors provide evidence that the simultaneous interaction of Bud6 and Aip5 with the yeast formin Bni1 has a synergistic effect on the complex's ability to

nucleate actin filaments. A synergistic effect of the two NPFs was not measurable for the Bnr1-Bud6-Aip5 complex.

A Monte Carlo simulation predicts a structure of the Bni1-Bud6-Aip5 as well as the Bnr1-Bud6-Aip5 complex. By focusing on the combined biochemical activity of two different nucleation promoting factors the authors provide a more realistic picture of how actin nucleation might be achieved in the cell. The manuscript is therefore a valuable contribution to the field that merits publication.
Below I summarize my critique and suggestions that might help to strengthen the conclusions of the paper.

1.) The authors work with fragments of Bud6, Bni1, Bnr1 and Aip5 to postulate a network of factors that influence actin nucleation. Aip5 and Bud6 interact physically with each other. With Aip5 (1110-1234) and Bud6 (550-789) two fragments of the proteins were chosen in this work that lack the binding site between the two proteins (Bud6 1-144); Aip5 (1000-1125) (Glomb et al., 2019). This is a very unfortunate choice as the physical interaction between the two actin nucleation factors might change the properties of the trimeric Bni1-Aip5-Bud6 complex. An experiment that measures actin filament formation in the presence of Aip5 (1000-1234) and the full length Bud6 would resolve some of the open questions. If this is not feasible, the authors might at least give room for a fair discussion of the limitations of their current measurements.

Thanks for the excellent suggestion. We agree with the Reviewer that the interaction between Aip5 (1000-1234 aa) and Bud6 (1-144 aa) is likely to tune the complex assembly and thereby activities in actin polymerization. Due to the technical challenges in producing these full-length proteins, we are unable to describe the complex assembly modes between full-length formin and NPFs. We have now described such interaction and explained the technical challenges for full-length work in the revised manuscript on page 4. We have also better discussed the importance of the functional macromolecular assembly, which has higher-order connectivity than the minimum-reconstitution system used in this work on page 7. Indeed, this is a general challenge in studying the actin regulatory proteins. In many cases, full-length recombinant proteins are challenging to obtain to reflect different functional modules of the targeted proteins. But the domain-based studies would still add necessary knowledge to the field by providing minimum-component/domain-reconstitution-based biochemical results.

In that respect, the statement of the authors in the discussion: “Although Aip5C does not interact with Bud6C, whether the full-length Aip5 and Bud6 would collaborate to promote formin function has yet to know, because the intrinsically disordered regions of Aip5 and Bud6 could drive macromolecular assembly at heterogeneous states under different cellular conditions (8,36)” is at least misleading and difficult to understand. The two proteins are known to form a complex in vivo.

We apologize for the confusion and misleading description. In addition to the IDR-created multivalent interactions between complex members, we have also described the reported interactions between Aip5(1000-1234) and Bud6(1-144) from Glomb 2019.

2.) The authors perform a simulation to predict the structure of the trimeric Bni1Aip5-Bud6 and the Bnr1-Aip5-Bud6 complexes. The predictions are very specific. Testing these predictions would strengthen the proposed models of the simulations. I highly recommend to use the outcome of the simulation to construct minimal mutants of the fragments that are predicted to not interact and to test their properties with the biochemical assays.

We appreciate the suggestions and want to express the challenges in performing point mutagenesis-based biochemical assays for the simulation predicted IDR. Multiple IDR enables multivalent and weak interactions. The interaction between IDR and binding partners is often not single site-specific, which is often different from the well-defined interface from the crystal packing, such as from X-Ray crystallography. The interactions often have a bulk effect towards the lowest energy states with multiple weak associations with conformational flexibility. For example, our JBC paper (https://pubmed.ncbi.nlm.nih.gov/31653702/) used MD simulation and biochemical studies to understand how an IDR region of 37 amino acid of Arabidopsis profilin homolog 3 (AtPRF3) interacts with the other sites of AtPRF3 and cooperatively engage the polyP of plant formin. We have spent dedicated effort and a long time to mutate the profilin sites based on the MD results for two rounds. And we found that after each round of mutagenesis, the AtPRF3 can still bind to the formin polyp by evolving new binding modes, which were all validated by biochemical interaction assays. Here, we did not claim the specific binding site from the MD simulation that drives complex assembly. Instead, we described the MD result by proposing the positional relationship between two NFPs and formin IDR. We propose the multi-site electrostatic interactions and hydrogen bonds contribute to the trip-protein complex, shown in Fig. S5. We think specific amino-acid-based interactions need to brace the dynamic and conformational flexibility of IDR to have a holistic view of the underlying mechanism for complex assembly between formin IDR and two NPFs. For these reasons, it is challenging to identify the best strategy in mutating the sites for additional stimulation and biochemical assays, and the point mutagenesis-based validation might not be able to draw a clear conclusion to support the claims of this study. Now, we have discussed these points in the result and discussion part of MD results for the above reasons.

3.) Minor points:

3.1 Page 2 line 39: in the presence of profilin (Pfy1)….
Revised now

3.2 Page 2 last line: reminiscent instead of reminiscence.
Corrected now

3.3 Page 3 line 25: further activated instead of has further activated.
Corrected now

3.4 Page 3 line 48: stoichiometry
Corrected now

3.5 Figures 1 and 2 A,B: Are we looking at the averages of more than one measurement for each condition? Please indicate.
Yes, we showed the final results by using the average value from three independent biological replicates. It is specified on page 9 now.

3.6 Fig 2 F “Anosotropy”; Fig 1E “Anistropy” .

Corrected now

3.7 Page 7 Discussion: “By having long IDR, Aip5 accumulates at the bud tip for cell polarization….” This statement is misleading as it imposes that phase separation might drive Aip5 into the bud tip. However, it was shown that a direct interaction between Spa2 and Aip5 localizes Aip5 at the bud tip.

Sorry for not making this clear enough. Aip5 IDR contributes complex assembly that contributes to the “accumulation” of Aip5 at the tip, whereas Spa2 and Spa2-assemblies recruit the Aip5 to the bud tip. We have now revised the description to avoid misleading on page 7.

3.8 Page 7 Discussion: “During cell signal transductions under physiological and pathological conditions, IDR creates diverse states of macromolecular assembly in spatiotemporally-regulated manners, which could provide tunable activities of the functional complex, such as NF-NFPs.” Here and in other parts of the discussion (see point 1) the accumulation of ill-defined terms makes it very difficult to follow the argument of the authors. Please be more precise.

Sorry for not elaborating the research findings in detail, such as the LLPS-tuned actin nucleation in T-cell immunity and during pathogen-host interactions reported by two of our recent publications. We have added the corresponding references to provide more specific examples as clarification. Please see the revised sentences on page 8.

3.9 Page 8: “where 3 μM yeast profilin was added as an indication”. ??
Corrected now to “where 3 μM yeast profilin was added.”

RE: Manuscript #E21-06-0285R
TITLE: "A teamwork promotion of formins-mediated actin nucleations by Bud6 and Aip5 in Saccharomyces cerevisiae"

Dear authors:

You will be pleased that both reviewers feel that you responded adequately to their requests for revisions, so your paper is acceptable scientifically for publication in MBoC.

On the other hand, reviewer 1 notes that "The manuscript is full of grammatical errors and awkward sentences, as well as inconsistent use of acronyms, etc." My own reading confirms that the construction of many sentences is awkward, which may be difficult for someone who did not grow up speaking English to detect. Many sentences have minor grammatical errors that may also be difficult to detect.

Therefore, I recommend that you get help from someone with expertise in English writing to repair these problems and polish up your manuscript. This person would not necessarily need to be an expert on the content to give you the help you need.

After revision of the text, I should be able to accept the paper without further review.

Tom Pollard

Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Miao,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL):
Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

See attached.

Reviewer #2 (Remarks to the Author):

Review on the manuscript "A teamwork promotion of formins-mediated actin nucleations by Bud6 and Aip5 in Saccharomyces cerevisiae" by Ying Xie et al.
The authors addressed my comments.
They choose to not respond by performing additional experiments but by changes in the text of the revised manuscript. As this leaves some of the questions unanswered, I hope that the authors stick to their promise and keep working on this interesting project.

The authors have made significant improvements to the text, and added a model figure for clarity. These improvements increase the accessibility and impact of the work. While not all of the comments were adequately addressed, some are minor and can be dismissed. There are three places (below) where important issues still remain, but can be fixed with small revisions to the text.

1) **Comment:** In the Results the authors state, "*We previously characterized NFs and NPFs in vivo to understand their physiological- relevant range of concentrations and stoichiometry at their concentrated sites. In addition to our previously measured in vivo Aip5 and Bni1 concentration at the bud tip, Aip5 (~120 nM) and Bni1 (~80 nM) (24).*" However, reference 24 in the reference list is: Li, J., Henty-Ridilla, J. L., Staiger, B. H., Day, B., and Staiger, C. J. (2015) Capping protein integrates multiple MAMP signaling pathways to modulate actin dynamics during plant innate immunity. Nat Commun 6, 7206. This does not appear to be the correct citation. Additionally, how information about the cellular concentrations of these proteins influenced the design of their in vitro biochemical assays or model for how the tripartite protein complexes Bud6-Aip5-(Bni1/Bnr1) function is not made clear.
Suggestion 1: Check that the correct paper is referenced, Xie et. al 2019, reference #8.
We apologize for the wrong citation, which has been corrected now.

Suggestion 2: In the results, clarify the motivation for quantifying cellular concentrations of Aip5 and Bnr1, and expand on how these concentrations are related to the in vitro assays.

Quantifying cellular protein concentration could provide a relative concentration between two NPFs, Aip5 and Bud6, and yeast formin protein with physiology relevance. Such protein concentration would inform the design of in vitro assays for two types of formin-NPFs complexes, Bni1-Aip5-Bud6 and Bnr1-Aip5-Bud6, which facilitate the interpretation of the activity comparison between two types of NF-NPFs complex. We added these clarifications now on page 4.

I have one remaining concern here, which is easy to address in the text. The authors measured local concentrations of Bnr1 at the neck, and Bni1, Bud6 and Aip5 at the bud tip, but NOT Bud6 or Aip5 at the neck with Bnr1. They should point this out to readers in the Results, to clarify the relationship between these measurements and the design of the in vitro experiments. This in no way takes away from the excellent results they have obtained, but it provides an important cautionary note about this one limitation for readers and for future researchers in this area.

We appreciate the comments. We have now clarified this point on Page 4 by explaining that two formins have differential localizations during the early polar growth. Aip5 and Bud6 could switch their localization from the bud tip to the neck along with the cell cycle progression. We measured the concentrations of two formins at both tip and neck, respectively. In contrast, we only measured Aip5 and Bud6 concentrations at one location to simplify the stoichiometry estimation of NF-NPFs at a snapshot moment along the cell cycle, without considering the migration of Aip5 and Bud6.

3. **Comment:** As noted above, the authors largely ignore that Bud6 and Aip5 interact and do not discuss how this interaction may influence cooperation between the two NPFs. Aip5 residues 1000-1131 are necessary and sufficient to interact with Bud6, and a C-terminal fragment of Aip5 (1000-1234) interacts with an N-terminal fragment of Bud6 (1-141). Furthermore, Bud6-Aip5 interaction promotes the production of actin cables in vivo (Glomb et. al 2019 DOI:10.1242/bio.044024). However, in this publication, the authors use C-terminal fragments of Bud6 and Aip5 in their in vitro experiments that lack these binding domains.

Suggestion 1: Previously (DOI: 10.1038/s41467-019-13125-1), the authors purified and characterized full-length Aip5. Why not test if full-length Aip5 and Bud6 (or fragments of these proteins that interact) synergize? If the authors are unable to purify sufficient quantities for these experiments, explain this in the Results.

Suggestion 2: The authors need to acknowledge that while the fragments they use are sufficient to bind and activate formins in vitro, Bud6-Aip5 interactions likely contribute to the mechanism in vivo.

We apologize for not discussing well the interactions between Aip5 and Bud6, although our experimental setup did not involve these interactions. Please also see our above reply to Minor Comment 3, which is similar to this Major one.

1. We have explained the reason for carrying these minimum-reconstitution experiments on formin by using the functional domains of NPFs without their intermolecular interactions. Please also see the reply in Minor Comment 3.
2. The expression of all full-length proteins of Bni1, Aip5, and Bud6 is technically challenging. We are still working on it using different expression systems, including insect cells, yeast cells, or even mammalian cells. It is the final goal to study macromolecular assembly. However, the technical challenges in purifying enough soluble proteins for biochemistry work is still a significant hurdle, which would not be solved easily. As the Reviewer suggested, we have described such challenges on page 4 now.
3. We have now also added a discussion to acknowledge the additional interaction between two NPFs (Glomb et. al 2019 DOI:10.1242/bio.044024) might further contribute to the regulation of formin activities with a higher-order assembly on pages 2,4,5,8 now.

The authors have improved the text, explaining that there are technical reasons why they used fragments of Aip5 and Bud6 rather than full-length proteins. However, I suggest a clarification about a very important detail for the field - suggested changes to text (in **BOLD**) here: "*Hence, we used a minimum-component reconstitution approach to dissect the molecular mechanism by which two NPFs work on one formin simultaneously. We reconstituted the* **tripartite-***complex interaction using* **fragments of Aip5C and Bud6C. Importantly, these fragments contain the formin-binding NPF domains of each protein, but lack other domains known to mediate direct interactions between Aip5 and Bud6 (references). It is possible that these domains could even further elevate the collaborative NPF effects of Aip5 and Bud6 on formins.**"

We made suggested changes now on page 4.

4. **Comment:** The authors only use one approach to characterize interactions in the Bud6-Aip5-Formin triprotein complex, and the limitations of this approach leave it unclear how each protein influences complex formation. In Figure 2F, the authors use anisotropy to quantify how increasing concentrations of Bud6C alters the rotational diffusion of Aip5C-A488 in the presence of a constant concentration of either formin (Bni1 or Bnr1). They state, "*Since Aip5C and Bud6C do not have evident interaction, we next added increasing concentrations of Bud6C to the pre-mixed sample of Alex488 labeled Aip5C and Bni1FH1C, in which the changes in anisotropic value would reflect the interaction changes between Aip5 and Bni1FH1C*." How can the authors conclude that a change in anisotropic value reflects altered interactions between Aip5C and the formin, rather than changes in anisotropy due to Bud6C joining the complex of Aip5C + formin, thus changing the rotational diffusion of the entire complex?
Suggestion: In their 2019 publication (DOI: 10.1038/s41467-019-13125-1), the authors used anisotropy to quantify the affinity of Bni1FH1C for Aip5C by using a fixed concentration of labeled Aip5C and adding increasing concentrations of Bni1FH1C. By this approach, they measured a Kd of 16 nM. To determine if Bud6 enhances the affinity between Aip5C and Bni1FH1C, the authors should repeat this experiment with a fixed high concentration of Bud6 in molar excess of the other two proteins (Aip5 and the formin). This should also be done for Bnr1FH1-C. If the presence of Bud6 in the assay alters the Kd, then they can conclude that Bud6 influences the affinity of Aip5C and either formin. This same approach can be used to determine if Aip5C alters the interaction between Bud6 and either formin by using Alex488 labeled Bud6C with a molar excess of Aip5 and increasing concentrations of either formin.

   First, "…., thus changing the rotational diffusion of the entire complex…." The question Reviewer 1 raised is rather challenging to address, which we felt is out of the scope of this report. Such precision description requires titration experiments with NMR-based structural determination that would best characterize if it has to be fully addressed. We currently do not have such capacity to perform such a level of structural studies and draw such specific conclusions.

   Second, the purpose of the experiment here is to understand the cooperation between two NFPs on one formin. This is slightly different from our 2019 published NC paper, where we only have one NF and one NFP. We tried to understand the interaction between two proteins, which can be done by fixing either one and adding an increasing concentration of the other. Here, to know how one NPF will affect another NPF function of an existing NFP-NF pair, we have to add an increasing concentration of NPF instead of a formin on its own. Sorry for any potential confusion; we hope our explanation here elaborates better on the difference between the two types of purpose and experimental designs.

   Nevertheless, in vivo, all three proteins work together at the same time. And our simulation works here were performed with three proteins together simultaneously without having an order of timing.

There is still an issue here to fix. In the revised manuscript, they now state, "*We have performed an anisotropic assay to characterize the change of interactions between Aip5 and formin by supplement of Bud6…We found that Bud6C started to obviously enhance the Aip5 (60 nM) and Bni1 (30 nM) interaction starting from a lower concentration and lower stoichiometry than for the pair of Aip5 (60 nM) and Bnr1 (30 nM)*." The data are good, but the interpretation is rather narrow. The probe is on Aip5C, and its signal changes in the presence of either formin; this makes sense. Increasing concentrations of Bud6C further increase the signal; great data. The authors interpret this to mean that Bud6C enhances Aip5C-formin interactions; however, it is possible (and seems more likely to me) that Bud6C joins the Aip5C-formin complex, increasing its tumbling radius and increasing the signal. Therefore, it is impossible to conclude that Bud6C enhances Aip5C binding to the formin. The authors should explain this in the text, and remove the claims about changing Kds between Aip5-formin

interactions. In anisotropy assays, Kds can only be derived when there are two proteins in the reaction. Once there are three present, interpretations multiply.

Appreciate the point, We agree with the reviewer and now modified the explanation now on page 4 to avoid overstatement.

RE: Manuscript #E21-06-0285RR
TITLE: "A teamwork promotion of formins-mediated actin nucleation by Bud6 and Aip5 in Saccharomyces cerevisiae"

Dear Prof. Miao:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell. Thanks for the detailed account of the revisions made in response to our reviewer. I am satisfied with these revisions and pleased to recommend acceptance of your paper. Thank you for submitting you work to MBoC.

Tom Pollard

Sincerely,
Thomas Pollard
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Miao:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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