Aurora A phosphorylates Ndel1 to reduce the levels of Mad1 and NuMA at spindle poles

Pawel Janczyk, Eliza Zylkiewicz, Henry De Hoyos, Thomas West, Daniel Matson, Won-Chan Choi, Heather Raimer, Zygmunt Derewenda, and P. Stukenberg

Corresponding author(s): P. Stukenberg, Univ. of Virginia Medical School

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

RE: Manuscript #E21-09-0438
TITLE: "Aurora A phosphorylates Ndel1 to control dynein stripping at spindle poles"

Dear Prof. Stukenberg:

Two expert reviewers have now seen your manuscript. Both reviewers agree that the data support the conclusion that Ndel1 phosphorylation at S285 by Aurora A has an inhibitory effect on dynein function at spindle poles preventing accumulation of SAC proteins there. The study will motivate future investigations into the molecular mechanism underlying this regulation. The reviewers also raised a number of concerns that should all be addressable without performing further experiments.
We look forward to receiving your revised manuscript, together with a letter indicating the changes you have made and a point-by-point reply to the reviewers' concerns.

Sincerely,
Thomas Surrey
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Stukenberg,

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 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.

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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):

Janczyk and colleagues describe a novel Aurora A phosphorylation site (S285) in the dynein co-factor Ndel1. A phospho-specific antibody is generated and the role of Ndel1 S285 phosphorylation is examined in the context of SAC signaling and spindle assembly. Inhibition of Aurora A activity and expression of the Ndel1 S285A mutant (after depletion of endogenous Ndel1 by RNAi) is shown to result in spindle pole accumulation of Mad1 and NuMA in HeLa cells, and Ndel1 S285A causes premature exit from mitosis in low nocodazole, suggesting partially defecting SAC signaling. In Xenopus egg extract, Ndel1 S285A is more efficient at rescuing microtubule aster formation than the wild-type protein. The authors conclude that Ndel1 phosphorylation at S285 has an inhibitory effect on dynein. Specifically, they propose that Aurora A activity at spindle poles prevents SAC proteins that are stripped from kinetochores by dynein from reaching spindle poles.

Overall the data support the idea that phosphorylation of Ndel1 at S285 negatively regulates dynein, although there is no insight into how this regulation occurs at the molecular level. The study also offers a plausible explanation for the 20-year-old observation that lowering ATP levels by azide/deoxyglucose treatment results in spindle pole accumulation of SAC proteins. The study will therefore be of interest to the mitosis and dynein communities.

Specific points:

- Page 3:"Cytoplasmic dynein" should be cytoplasmic dynein 1.

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Reviewer #2 (Remarks to the Author):

This is a nice study reporting a novel Aurora kinase target, S285, on the dynein-associated protein, Ndel1. The authors make a convincing case for phosphorylation of this residue in regulating Mad1 accumulation at spindle poles, full activity of the spindle assembly checkpoint, accumulation of NUMA at spindle poles and aster formation as assessed in Xenopus egg extracts. The overall model of phosphorylation of S285 on Ndel1 downregulating dynein activity is consistent with the data but will require further conformation in future experiments. The authors should consider the following suggestions, most or all of should be addressable without further experiments.

1. I am confused about some aspects of the immunolabeling. Mid page 17, methods section mentions blocking with mouse or rabbit serum then using directly conjugated antibodies. Just beyond that the text talks about primary and secondary antibodies. Please clarify.

2. It would be useful to other researchers to explain more precisely how MLN8054 affected labeling with the ACA antibody.

3. There are other issues with immunolabeling that should be clarified. The methods suggest that centrosomes were identified by microtubule labeling. However, in some instances, 1.5 uM MLN in Fig 1A, there appear to be almost no intact microtubules.

4. Also please explain the effects of the drug treatments on spindle microtubules since they appear reduced with increasing

MLN8054 drug concentration. Again, did that hamper identification of spindle poles? In Fig1E, in the cells treated with CC1, how were poles identified and then quantified if they had little Mad1 label?

5. Further did the authors test if the disruption of spindle microtubules itself might affect Mad1 distribution at poles without Aurora A inhibition? In FigS1C, it appears that the Mad1/tubulin ratio is used. One would expect disruption of microtubules to affect the reliability of these measurements?

6. The DeLuca lab has reported that in addition to its concentration at spindle poles, Aurora A is also bound to Incenp at centromeres where it phosphorylates Hec1. The authors should discuss how this finding might impact their models of dynein regulation on the mitotic spindle. It would be of interest to know if the phosphorylation of Ndel S285 at kinetochores of prometaphase cells was attributable to Aurora A or Aurora B or both.

7. Please reconcile. The text on page 8 indicates that the p285 antibody was present on prometaphase kinetochores but was absent from most kinetochores by anaphase. The legend to Fig 2E indicates that pS285 signal co-localizes with Ndc80 at most kinetochores in both prometaphase and metaphase. From the single example shown, it appears that the signal may be lost on metaphase kinetochores that are most aligned to the spindle equator but remains on the kinetochores located further away from the plate. Some quantification here would be helpful.

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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 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 contanct 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

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- Figure 1C; Figure 4D and F; Figure S1C and F: Since multiple conditions are compared to each other, the student t-test should be substituted by ANOVA/post hoc test.

We have made these changes. The statistics were all still significant using the ANOVA.

- Figure 2A and page 23: "two Aurora sites are highly conserved among vertebrates". To make that claim, more vertebrates need to be included in the alignment (human, rodent, and frog sequences are not sufficient).

The statement has been changed to "two Aurora sites are conserved in frogs, rodents and humans"

- Figure 2C: Ndel1 S194A is shown in the figure but not mentioned in the text.

We have modified the text so that it is included.

- Figure 2E: images have no scale bar.

Thank you a scale bar has been added

- Figure 3D: WT eventually catches up with S285A, so I don't quite understand how this can explain the difference in confluency shown in Figure 3C.

Thank you for this comment as the Y-axis label was not clearly stating what we had quantified and has been changed.. If there is still confusion we point out that 3D is a cumulative frequency plot and only cells that escaped were quantified, so eventually 100% of both populations eventually escape the mitotic block. The point is that the rate that mutant exits is different from the rate of the WT protein.

- Figure 4: the title of this figure in the figure legends has nothing to do with what is shown in the figure.

*We apologize as we changed the figures in a late revision and forgot to change the legend. The new legend is "***Phosphorylation of Ndel1 on Ser285 also limits the localization of NuMA to spindle poles and negatively regulates dynein dependent aster formation in *Xenopus* egg extracts."**

- Figure S3D and E: it is not clear which image series is WT and which is S285A.

Sorry for the confusion but S3D and E are not showing WT vs mutant but are examples of the phenotypes that are quantified for both conditions.

Reviewer #2 (Remarks to the Author): This is a nice study reporting a novel Aurora kinase target, S285, on the dynein-associated protein, Ndel1. The authors make a convincing case for phosphorylation of this residue in regulating Mad1 accumulation at spindle poles, full activity of the spindle assembly checkpoint, accumulation of NUMA at spindle poles and aster formation as assessed in Xenopus egg extracts. The overall model of phosphorylation of S285 onNdel1 downregulating dynein activity is consistent with the data but will require further conformation in future experiments. The authors should consider the following suggestions, most or all of should be addressable without further experiments.

1. I am confused about some aspects of the immunolabeling. Mid page 17, methods section mentions blocking with mouse or rabbit serum then using directly conjugated antibodies. Just beyond that the text talks about primary and secondary antibodies. Please clarify.

Thank you for noticing this we have changed the text to clarify how we used directly conjugated antibodies to enable the three color visualization in frog cells where ACA is not useful.

2. It would be useful to other researchers to explain more precisely how MLN8054affected labeling with the ACA antibody.

There was a dose dependent reduction of ACA (~2x at highest concentrations of MLN). We don't understand this but it was reproducible. We have added an additional quantification to the supplemental data that shows that the accumulation of Mad1 at poles and reduction of Mad1 at kinetochores is still significant if we ratio them to ACA. However, because of the reproducibility of the ACA reduction we feel that it would be slightly misleading to divide the pole staining by ACA as it would over-represent the effect of Aurora inhibitors. We have added clarifying text to the figure legends.

3. There are other issues with immunolabeling that should be clarified. The methods suggest that centrosomes were identified by microtubule labeling. However, in some instances, 1.5 uM MLN in Fig 1A, there appear to be almost no intact microtubules.

Even though the tubulin staining was weaker after MLN treatment there were still obvious spindle poles in these cells.

4. Also please explain the effects of the drug treatments on spindle microtubules since they appear reduced with increasing MLN8054 drug concentration. Again, did that hamper identification of spindle poles? In Fig1E, in the cells treated with CC1, how were poles identified and then quantified if they had little Mad1 label?

The effects of Aurora inhibition on the spindle is known and not the focus of this work. Even after inhibition the microtubules had an obvious focal point that could be used to identify the spindle pole. The experiment with CC1 was trickier to quantify because we did not have a channel to also stain for microtubules. However, it was not hard to distinguish whether the Mad1 staining had two strong signals that were outside DNA staining masses compared to numerous kinetochore stains that were weaker and scattered within the DNA masses.

5. Further did the authors test if the disruption of spindle microtubules itself might affectMad1 distribution at poles without Aurora A inhibition? In FigS1C, it appears that theMad1/tubulin ratio is used. One would expect disruption of microtubules to affect there liability of these measurements?

The reviewer's point is valid and demonstrates why identification of a point mutant on Ndel1 that recapitulates the phenotype is such a critical experiment to address this and many other similar concerns about indirect effects of Aurora inhibitors.

6. The DeLuca lab has reported that in addition to its concentration at spindle poles, Aurora A is also bound to Incenp at centromeres where it phosphorylates Hec1. The authors should discuss how this finding might impact their models of dynein regulation on the mitotic spindle. It would be of interest to know if the phosphorylation of NdelS285 at kinetochores of prometaphase cells was attributable to Aurora A or Aurora B or both.

We agree that the regulation of this site at kinetochores is an important future direction and have a paragraph dedicated to this point in the discussion. We have modified the paragraph to address the interesting idea that Aurora A could also be regulating Ndel1 at kinetochores.

7. Please reconcile. The text on page 8 indicates that the p285 antibody was present on prometaphase kinetochores but was absent from most kinetochores by anaphase. The legend to Fig 2E indicates that pS285 signal co-localizes with Ndc80 at most kinetochores in both prometaphase and metaphase. From the single example shown, it appears that the signal may be lost on metaphase kinetochores that are most aligned to the spindle equator but remains on the kinetochores located further away from the plate. Some quantification here would be helpful.

Although it may be that there is reduction of the pS285 at kinetochores in metaphase we still see some signal there so would rather not draw a strong conclusion. We now state that we see signal at centrosomes and kinetochores in mitotic cells.

8. The authors posit a model "suggesting that Aurora phosphorylation of Ndel1 S285 is to limit dynein dependent transport of SAC proteins." Aurora A inhibition and mutant expression induces accumulation of Mad1 at kinetochores but doesn't really address transport.

We have added a figure that shows that Mad1 also accumulates on the spindle which provides additional evidence that the accumulation at poles is caused by dynein stripping and lack of cargo release.

9. The authors suggest that expression of S285A Ndel1 produces a defect in the SAC allowing mutant-expressing cells, but not cells expressing the wild type Ndel1 to proliferate over several days in the presence of 20 nM nocodazole (Fig 3C). They ascribe this difference to the data in Fig 3D showing that mutant cells escape mitotic arrest in this concentration of nocodazole more quickly than cells expressing the wild type protein. However, cells expressing the wild type protein are only comparatively delayed in escape but 100% do escape. This seems inconsistent with the data in Fig 3C showing almost no increase in confluency for cells expressing the wild type protein. It is also a leap to ascribe the difference in proliferation solely to the effect on the SAC. Do cells expressing the mutant protein show any differences in proliferation in the absence of nocodazole? This is particularly important since, according to Fig S3A, the mutant protein seems to be expressed at higher levels than the wild type, an item which the authors should point out. It would also be informative for the authors to contrast, if they have the data, effects on proliferation, SAC potency and NUMA accumulation on siRNA depletion of Ndel1 in the absence of rescue.

We had quantified the proliferation of cells in the absence of nocodazole during the original experiment but left the data out for simplification. They clearly show that there was no difference in proliferation rate in the absence of nocodazole and these data have been added to supplemental figure 3B. We have also changed the text to not directly connect the proliferation and slippage from nocodazole arrest.

We have also added a line of text about the levels of rescued Ndel1 proteins: "The siRNA treatment efficiently depleted the endogenous proteins and rescued recombinant proteins were

higher than endogenous proteins (Ndel1(S285A)-GFP is also higher than Nde1-GFP (Figure S3A))".

10. Finally, the title should probably be reworded since it may be confusing. First dynein stripping is usually a feature of SAC protein removal from kinetochores. Second there is no real assessment of dynein in the manuscript. Perhaps focus on the novel phosphor site on Ndel1 as a potential regulator of SAC protein accumulation and integrity of spindle poles.

The title has been changed to:

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Dear Prof. Stukenberg:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

The revision has addressed all comments of the reviewers. The editorial changes and the new statistical analysis improve clarity and further support the conclusions of the authors. This study provides new insight into the regulation of pole formation by Aurora A kinase via the dynein regulator Ndel1. Congratulations to all authors for this interesting study.

Sincerely,
Thomas Surrey
Monitoring Editor
Molecular Biology of the Cell

Dear Prof. Stukenberg:

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.

Within approximately four weeks you will receive a PDF page proof of your article.

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We are pleased that you chose to publish your work in MBoC.

Sincerely,

Eric Baker
Journal Production Manager
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