JCB

Phosphorylation and Pin1 binding to the LIC1 subunit selectively regulate mitotic dynein functions

Amrita Kumari, Chandan Kumar, Rajaiah Pergu, Megha Kumar, Sagar Mahale, Neeraj Wasnik, and Sivaram Mylavarapu

Corresponding Author(s): Sivaram Mylavarapu, Regional Centre for Biotechnology

Review Timeline:

Submission Date:	2020-05-26
Editorial Decision:	2020-07-13
Revision Received:	2021-05-13
Editorial Decision:	2021-07-07
Revision Received:	2021-08-28
Editorial Decision:	2021-09-07
Revision Received:	2021-09-16

Monitoring Editor: Daniela Cimini

Scientific Editor: Tim Spencer

Transaction Report:

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

DOI: https://doi.org/10.1083/jcb.202005184

July 13, 2020

Re: JCB manuscript #202005184

Dr. Sivaram V S Mylavarapu
Regional Centre for Biotechnology
Laboratory of Cellular Dynamics
NCR Biotech Science Cluster, 3rd Milestone
Faridabad Gurgaon Expressway
Faridabad, Haryana 121001
India

Dear Dr. Mylavarapu,

Thank you for submitting your manuscript entitled "Phosphorylation and Pin1 binding to the LIC1 subunit selectively regulate mitotic dynein functions". The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

As you will see, both reviewers have voiced enthusiasm for the premise of your work, but also have significant concerns. In particular, they feel that the conclusions are not adequately supported by the data and have raised a number of substantive concerns on experimental design, as well as data analysis and interpretation.

Based on the extent of revisions that would be necessary to address the reviewers' concerns, we cannot consider your manuscript for publication in JCB at this time. If you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

However, given interest in the topic, we would be open to an appeal of this decision and resubmission to JCB of a significantly revised and extended manuscript that completely addresses the reviewers' concerns. If you would like to resubmit this work to JCB, you may contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system once you have completed your revisions. Please note that priority and novelty would be reassessed at resubmission and the paper would, of course, be subject to re-review by the same reviewers (if possible).

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Daniela Cimini, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

In this manuscript, the authors demonstrate that the phosphorylation of dynein light intermediate chain C-terminal domain (LIC1-CTD) at three conserved Cdk1 sites is important for mitotic progression. Mitotic cells are severely delayed when LIC1 does not acquire these phosphorylation (prolonged metaphase arrest), due to apparently defective satisfaction of the spindle assembly checkpoint (SAC). These phosphorylation seems to be important to load LIC1 and the dynein motor to prometaphase kinetochores. These post-translational modifications are also important to recruit peptidyl prolyl isomerase, Pin1, to a subpopulation of high-load bearing dynein complexes containing dynein heavy chain (DHC) and its interaction partners, Lis1 and NudE. One dynein function that Pin1 recruitment is required for is the proper engagement of the centrosomes with the NE during in mitotic prophase. Over all, I feel that a role for LIC1 and its phosporylation in SAC is not novel enough. It has also been shown that mitotically phosporylated dynein IC associates weakly with the dynactin (& possibly spindly) containing dynein complexes and that this process is critical for the stripping of checkpoint complexes from kinetochores at metaphase. Their results with Pin1 binding to a particular dynein subcomplexes in response to Cdc2 phosphorylation and the zebrafish studies however are quite interesting. I suggest the following changes to improve the quality of this manuscript:

i. Suggested changes in the abstract: "One way dynein's cargo-binding selectivity is regulated.....". It might be better not to use "recruit" unless PIN1 staining can be shown at sites of dynein localization in a G2/M phase specific manner.

ii. In the experiments in Fig. 1, it would have been better to design an siRNA resistant version of hLIC1 instead of using rLIC1. Since the phosphorylated dynein IC/LIC with Lis1/Nde1 is functional in prometaphase, one would expect that this interaction and consequent Pin1 binding is required for chromosome capture and alignment. But the phenotype is not indicative of that. Have the authors explored this possibility thoroughly?

iii. In Fig. 2 and 3, HeLa cells normally have a mitotic index of 4-6%; so I am really intrigued by the observed number of 2% and I am worried that this positively skews the effect observed with the use of various mutants.

iv. Please show the LIC1 blot in Fig. 3b. The data from Fig. 3 g-h show quite a few normally stretched kinetochores. This suggests to me that the kinetochore microtubule attachments are formed normally. Also IC and LIC targeting is found to be reduced to prometaphase kinetochores. But It has been shown that dynein function is required for chromosome biorientation and also for the formation of stable kinetochore microtubule attachments. In light of these points, I feel that it is important to assay for kinetochore microtubule attachment stability. It will be generally interesting to test if LIC1 function is required in this regard considering the observed phenotype of a metaphase-like cell arrest and accumulation of SAC proteins at kinetochores in these cells.

v. Fig. 4: Is there a way to check for DHC staining in these experiments? I feel that Fig. 4 and 5 could be pooled as they are roughly making the same point. Most of the Fig. 4 outside of IC recruitment is better to be included as supplemental data anyways.

vi. Fig. 5: Is it necessary that there should be an observed difference in Mad1/2 between the wt and mutant LIC1 eluates? Remember that the SAC complexes at kinetochores, especially, Mad1/2 are not engaged with dynein in prometaphase. On the other hand, what is found with regard to reduced Zw10 levels makes some sense (also as you see IC targeting) as Zw10 is thought to target dynein to kinetochores in prometaphase, possibly independent of dynactin. However, as said before the lack of defects in chromosome capture and/or congression in prometaphase cells does not really fit with the authors ideas.

vii. In Fig. 6I, again, why have the authors used rat and not human LIC1? Please clarify whether these are full-length LIC1 constructs or if they are deletion fragments?

viii. Fig. 7: It is very intriguing that the adaptor Hook2 is coming down with a dynein subcomplex which does not contain dynactin because work from Andrew Carter's cryoEM studies and Mckenney, Vale et al publications suggest that dynein adaptors can bind only after a processive dynein-dynactin complex is formed. This seems to be especially true for BicD2. This result also does not quite fit with the idea that Hook2 is required for proper p150 localization/function and binding to dynein in G2 and M phase (even though the authors do talk about Hook2-containing dynein-dynactin complexes in the discussion). Moreover, I see enough of p150 coming down in Fig. 7A. So, I would be very careful about how this result is stated (for eg: "dynactin-free") especially in the abstract. I would also like to see another subunit of the dynactin complex that is probed for in Fig. 7A and D. Even if we imagine a scenario where there exists two fractions of dynein in prometaphase, one that is load-bearing (as described in this manuscript) and another one that is processive (containing dynactin) and both of these fractions contain Hook2, then one would still expect at least a moderate phenotype of defective chromosome capture and congression when Hook2 is perturbed. Is this the case?

ix. I think that a more elaborate analysis is needed for Pin1 recruitment and function in the context of dynein as prophase centrosome uncoupling is not yet on of the key established function of dynein (even considering the involvement of Hook2 in this process). Have the authors performed an in-depth analysis of interphase functions of dynein, such as vesicle motility? It is known that Cdc2 phosphorylation releases dynein from membranes, but is Pin1 binding in some way connected to this function so that membrane/vesicle binding could be regulated?

x. It has been shown in Whyte, Vaughan et al, JCB, 2008, that phosphorylation of Dynein IC in prometaphase enables it to bind better to ZW10 (RZZ complex) for the purpose of targeting dynein to kinetochores. However, neither LIC1 phosphorylation nor Pin1-binding to LIC1 seems to be required for RZZ binding. In this scenario, I wonder how one could delineate the consequences of DIC phosphorylation from that of LIC1 phosphorylation by Cdc2/Cdk1. I wonder if it is known whether it is the same kinase that phosphorylates DIC. In any case, the authors should absolutely cite Whyte et al, 2005, in their work and discuss the relevant details in the context of their observations.

Minor points:

i. The institutional citation number for coauthor, Megha Kumar, might be incorrect.

ii. For the ease of the readers, it might be better to mention in Fig. 5A & B or the associated legend that 'prometaphase' = 'nocodazole-treated'. Similarly for Fig. 8A, G2/prophase = 8 hrs post double thymidine release.

iii. It might be slightly incorrect to equate Histone H2B-GFP labelling to the nuclear envelope at the resolution of live imaging carried out in Fig. 8C. It is interesting that the same mitotic cells with the defective coupling of centrosomes to NE also exhibit mitotic delay and arrest. But I am not able to discern the logic behind how the defective coupling is related to defective checkpoint inactivation and the accumulation of SAC proteins at kinetochores in metaphase-arrested cells. Please explain.

iv. Line # 55, please add "LICs are one of the most important mediators....."

v. In line # 160, what do the authors mean by "early" vertebrate zebrafish?

vi. In line # 373, it might be better to say prometaphase 'U2OS' lysates.

vii. In line # 397, "reported earlier" might be better than "reported above".

Reviewer #2 (Comments to the Authors (Required)):

Kumari and colleagues explore the role of phosphorylation in the C-terminal cargo-binding region of dynein light intermediate chain in regulating mitotic events. Indeed, cytoplasmic dynein regulates several key steps during cell division. These steps require the association the light intermediate chain 1 (LIC1) with a repertoire of cellular adaptors. Here the authors report that phosphorylation of the putative Cdk1/Cyclin B1 target sites S839, S405 & T408 is required for the timely mitotic progression in human cells and that phosphorylation-defective mutants leads to severe developmental defects in zebrafish embryos. Based on their data the authors propose that the mitotic delay observed in the presence of phosphorylation defective LIC1 mutants is due reduced dynein loading on kinetochores, preventing the silencing of spindle assembly checkpoint. Based on pull-down experiments the authors find that LIC1 phosphorylation is crucial for the association of dynein with the RZZ-Spindly-dynactin complex on kinetochores and the propyl isomerase enzyme Pin1, another key mitotic regulator. Finally, the authors that LIC1 phosphorylation also favours the the binding to Pin1, Nde1-LIS1 and CENP-F during Late G2 phase to ensure the attachment of centrosomes to the nuclear envelope at mitotic entry.

The study addresses an interesting and novel topic, providing a detailed analysis of the potential roles of LIC1 phosphorylation during cell division. If all the findings were to be confirmed, this would be an exciting and compelling study for Journal of Cell Biology. The problem, however, is that the main conclusions of the paper are not always well supported by the presented data, as key controls are missing. At this present stage this raises major issues about the robustness of the presented results, and addressing this issues will require a large amount of work. Specifically:

1.) All the microscopy experiments (live and fixed cells) presented in Figures 1-4 and 8 rely on the transient over-expression of LIC1 mutants. The authors present the data as if all the cells are transfected the different constructs, but this is a misleading assumption. Even in the best case scenario, only 50-60% of HeLa will be expressing an endogenous protein after transient transfection. Since the transfected constructs contain to my knowledge no fluorescent tag, the

authors cannot ascertain the expression level of the constructs in any given cells or know if a particular cells is transfected or not. Moreover, even tough the authors cannot know if the different mutants are expressed at similar levels, as some mutants might be poorly transfected but highly expressed, while other mutants might be expressed at lower levels but in a higher percentage of cells. The authors must repeat those experiments with a fluorescent version of their protein, and analyze the behavior of only the transfected cells and also report the behavior of non-transfected cells. Only such a comparison will reveal the true phenotype of the different mutants.

2) In figure 2A, authors present the expression levels of rLIC1 mutants in an LIC1 siRNA background. However, the level of expression endogenous LIC1 is missing. Since overexpression of dynein complex subunits can disrupt the dynein complex and its motor activity, it is essential that the authors confirm that the level of expression of the rescue constructs is similar to the endogenous protein levels. A similar concern arises in Figure 1C, where the authors express the different mutants over the endogenous protein, the authors need to test for the expression level compared to the endogenous protein.

3) In Figure 3 and 4 the authors compare the effect of the SST mutant compared to the AAA mutant, but in all experiments we don't know what is the ground state, and what are the levels of the different proteins or inter-kinetochore distances in untransfected cells. this information is important to assess the effect of the two mutants. In this context, it is also interesting that expression of the AAA mutant leads to an increase in inter-kinetochore distances. How do the authors interpret this, since both the cells expressing the SST or the AAA mutant are in the same stage of the cell cycle. Does this suggest that expression of the AAA mutant leads to elevated pullling forces on kinetochores, independently of its effect on SAC proteins?

4) The quantifications of pulldown experiments presented in Figure 5 and Figure 7F were performed on saturated exposures which can lead to over/under interpretation of the real results and must be performed on unsaturated exposures to allow robust conclusions.

5) In Figure 8C, authors report the importance of LIC1 phosphorylation in regulating the attachment of centrosomes to the the nuclear envelope during late G2 phase. Throughout this study the authors assume that these residues are phosphorylated by CDK1-CyclinB1, which they also use in their in vitro phosphorylation experiments. However, fully active CDK1-CyclinB complex formation occurs only after nuclear envelope breakdown. This raises the question as to how these phosphorylation sites might impact centrosome attachment to the nuclear envelope if CDK1/CyclinB1 is not yet active. One interesting possibility would be CDK1-CyclinA? The authors should test for centrosome attachment to the nuclear envelope in G2 after Cyclin A or Cyclin B1 depletion.

6) In Figure 2F, the authors report a minor rescue with zLIC1A (phosphorylation defective) and zLIC1E (phospho-mimetic) which look very similar to LIC1 MO indicating that there is very less or no rescue. This point should be discussed in detail. Additionally there is no panel for Control MO for better comparison. Moreover, more generally, the authors may want to indicate if they tested any phosphor-mimetic mutants in the assay showed in Figure 3 and 4. Absence of positive result of course does not mean anything, but would help the reader if he/she knew if these experiments were attempted.

Minor points:
- In Figure 4I and S2B, the authors use the hLIC1-MTAP (WT/AAA) construct in a LIC1 siRNA background. However, they do not mention how and if this construct was made siRNA resistant.

This should be indicated in the Material and Methods.

- In figure 3B, It would be worth to show LIC1 levels in both the siRNA treatments.

-In figure 4I and K, the images do not represent the quantification and should be replaced. Among the three residues in LIC1-CTD (S398, S405 and T408), the findings report S405 to be majorly important in the process. Authors should also discuss the role of the other phosphorylation sites.

- page 2: in line 32, reference to minus end directed kinesins should also be made since dynein is not the only major retrograde transporter. In line 54, the studies on dynein structure and stoichiometry from Andrew Carter's, Ron Vale's and Reck-Peterson's groups should also be cited.

Patrick Meraldi

Kumari et al, pointwise response to reviewers

Manuscript title: Phosphorylation and Pin1 binding to the LIC1 subunit selectively regulate mitotic dynein functions (revision).

Manuscript number: 202005184

Dear Dr. Spencer and Dr. Cimini,

Please find below our pointwise responses to the reviewers' comments towards the revision of our manuscript for submission to JCB. I am happy to report that we have addressed all of the reviewers' concerns, thereby significantly improving the manuscript. We hope it will now be found suitable for publication in JCB.

The responses are highlighted in blue font after each query below.

Sincerely,

Sivaram Mylavarapu

(corresponding author).

Reviewer 1:

In this manuscript, the authors demonstrate that the phosphorylation of dynein light intermediate chain C-terminal domain (LIC1-CTD) at three conserved Cdk1 sites is important for mitotic progression. Mitotic cells are severely delayed when LIC1 does not acquire these phosphorylation (prolonged metaphase arrest), due to apparently defective satisfaction of the spindle assembly checkpoint (SAC). These phosphorylation seems to be important to load LIC1 and the dynein motor to prometaphase kinetochores. These post-translational modifications are also important to recruit peptidyl prolyl isomerase, Pin1, to a subpopulation of high-load bearing dynein complexes containing dynein heavy chain (DHC) and its interaction partners, Lis1 and NudE. One dynein function that Pin1 recruitment is required for is the proper engagement of the centrosomes with the NE during in mitotic prophase. Over all, I feel that a role for LIC1 and its phosporylation in SAC is not novel enough. It has also been shown that mitotically phosporylated dynein IC associates weakly with the dynactin (& possibly spindly) containing dynein complexes and that this process is critical for the stripping of checkpoint complexes from kinetochores at metaphase. Their results with Pin1 binding to a particular dynein subcomplexes in response to Cdc2 phosphorylation and the zebrafish studies however are quite interesting. I suggest the following changes to improve the quality of this manuscript:

Comment i. Suggested changes in the abstract: "One way dynein's cargo-binding selectivity is regulated.....". It might be better not to use "recruit" unless PIN1 staining can be shown at sites of dynein localization in a G2/M phase specific manner.

Response: We thank the reviewer for this comment. As suggested, we have now performed immunostaining of Pin1 and observed its colocalization with LIC1-MTAP (both SST and AAA) at prophase centrosomes and on the metaphase spindle, however Pin1 does not appear to be localized at mitotic kinetochores (fig. 5E). Since we observe Pin1 localization at these mitotic sites despite the AAA mutation, we have now modified the text to indicate that LIC1 phosphorylation engages Pin1 to the dynein complex (abstract).

Comment ii. In the experiments in Fig. 1, it would have been better to design an siRNA resistant version of hLIC1 instead of using rLIC1. Since the phosphorylated dynein IC/LIC with Lis1/Nde1 is functional in prometaphase, one would expect that this interaction and consequent Pin1 binding is required for chromosome capture and alignment. But the phenotype is not indicative of that. Have the authors explored this possibility thoroughly?

Response: We thank the reviewer for this comment. We have performed the chromosome congression experiments, where we do observe chromosome alignment defects in metaphase in both live cell and fixed cell imaging experiments (fig. 7A, B). We did not observe any significant reduction in the tubulin intensity at the plate following cold treatment, indicating that kinetochore-microtubule attachment stability is not impacted (fig. 7C, D). Our live cell imaging experiments in stable hLIC1-MTAP (SST and AAA) cell lines after transfecting H2B-mCherry (to visualize chromosomes), confirmed clear delays in chromosome capture and alignment to the metaphase plate, but no premature dispersal of chromosomes after alignment (fig. 7E, F; video S9). This new data has been incorporated into the manuscript (lines 240-260).

Regarding the query about the use of rat LIC1: Rat LIC1 has been demonstrated to be competent for the functional rescue of human LIC1 depletion, for efficient integration into the dynein complex as well as for its cellular localization (Sivaram et al, *EMBO J* 2009; Mahale et al, *PLoS One* 2016, Mahale et al, *Sci Rep* 2016). We had therefore used these verified LIC1-specific siRNA sequences and the rat LIC1 complementation constructs in our study.

Comment iii. In Fig. 2 and 3, HeLa cells normally have a mitotic index of 4-6%; so I am really intrigued by the observed number of 2% and I am worried that this positively skews the effect observed with the use of various mutants.

Response: We wish to draw attention to the fact that we have shown the metaphase index here, which is a fraction of the total mitotic index. We have also reported consistent results in the past (Mahale et al, *PLoS One* 2016; Mahale et al, *Sci Rep* 2016). In the revised manuscript, we have reported the mitotic index in U2OS cells (fig. 2C), and the HeLa cell data has now been moved to fig. S1K and S2I respectively).

Comment iv. Please show the LIC1 blot in Fig. 3b. The data from Fig. 3 g-h show quite a few normally stretched kinetochores. This suggests to me that the kinetochore microtubule attachments are formed normally. Also IC and LIC targeting is found to be reduced to prometaphase kinetochores. But It has been shown that dynein function is required for

chromosome biorientation and also for the formation of stable kinetochore microtubule attachments. In light of these points, I feel that it is important to assay for kinetochore microtubule attachment stability. It will be generally interesting to test if LIC1 function is required in this regard considering the observed phenotype of a metaphase-like cell arrest and accumulation of SAC proteins at kinetochores in these cells.

Response: As suggested, we have now included the LIC1 blot for the earlier figure 3b (now fig. S4B). We have also performed the kinetochore-microtubule attachment stability assays using fixed and live cells, as stated above for comment ii. Our data suggest that LIC1 depletion does not result in any significant loss of kinetochore-microtubule attachment stability. This data has been incorporated into the manuscript in fig. 7 and lines 249-260 of the text.

Comment v. Fig. 4: Is there a way to check for DHC staining in these experiments? I feel that Fig. 4 and 5 could be pooled as they are roughly making the same point. Most of the Fig. 4 outside of IC recruitment is better to be included as supplemental data anyways.

Response: In the new figure for this experiment, we have performed DHC staining in U2OS cell lines (now fig. 3A). As suggested, we have included only the new dynein loading data for the U2OS cell lines in the main figure (fig. 3), and have moved the rest of the data into the supplementary information (fig. S3A-H). As also suggested, we have moved the original fig. 5 blots to the new fig. 3E and 3G.

Comment vi. Fig. 5: Is it necessary that there should be an observed difference in Mad1/2 between the wt and mutant LIC1 eluates? Remember that the SAC complexes at kinetochores, especially, Mad1/2 are not engaged with dynein in prometaphase. On the other hand, what is found with regard to reduced Zw10 levels makes some sense (also as you see IC targeting) as Zw10 is thought to target dynein to kinetochores in prometaphase, possibly independent of dynactin. However, as said before the lack of defects in chromosome capture and/or congression in prometaphase cells does not really fit with the authors ideas.

Response: We appreciate this comment. Multiple reports in the literature have shown that the RZZ complex at unattached prometaphase kinetochores is required as a recruitment factor for dynein and the Mad1/2 proteins. RZZ is also required for the dynein-mediated removal of Mad1/2 from attached metaphase kinetochores, with a fraction of the Mad proteins also found physically interacting with the RZZ complex. Thus, RZZ is involved in both the activation and inactivation of the SAC (Defachelles et al, *Chromosome Res* 2015; Musacchio and Salmon, *Nat Rev Mol Cell Biol* 2007; Karess, *Trends Cell Biol* 2005; Kops et al. *J Cell Biol* 2005; Buffin et al. *Curr Biol* 2005). Our results are consistent with these hypotheses and the reviewer's observations, since we observe the strongest effect of LIC1 phosphomutation on Zw10, and a slightly smaller effect on Mad1 (earlier fig. 5, now fig. 3E-H).

As explained above in response to comment ii, we now also report the requirement of LIC1-CTD phosphorylation for chromosome congression, but not for maintaining the stability of kinetochore-microtubule attachments (fig. 7).

Comment vii. In Fig. 6I, again, why have the authors used rat and not human LIC1? Please clarify whether these are full-length LIC1 constructs or if they are deletion fragments?

Response: As explained above, rat LIC1 has been demonstrated to be competent for the functional rescue of human LIC1 depletion, for efficient integration into the dynein complex as well as for its cellular localization (Sivaram et al, *EMBO J* 2009; Mahale et al, *PLoS One* 2016, Mahale et al, *Sci Rep* 2016). We had therefore used these verified LIC1-specific siRNA sequences and the rat LIC1 complementation constructs in our study. Therefore, we had used rat LIC1 for our HeLa cellular rescue experiments and rat LIC1 mutants to show Pin1 binding. All of the mutants used here are full length LIC1 constructs, which has now also been indicated in the figure 4 panels for clarity.

Comment viii. Fig. 7: It is very intriguing that the adaptor Hook2 is coming down with a dynein subcomplex which does not contain dynactin because work from Andrew Carter's cryoEM studies and Mckenney, Vale et al publications suggest that dynein adaptors can bind only after a processive dynein-dynactin complex is formed. This seems to be especially true for BicD2. This result also does not quite fit with the idea that Hook2 is required for proper p150 localization/function and binding to dynein in G2 and M phase (even though the authors do talk about Hook2-containing dynein-dynactin complexes in the discussion). Moreover, I see enough of p150 coming down in Fig. 7A. So, I would be very careful about how this result is stated (for eg: "dynactin-free") especially in the abstract. I would also like to see another subunit of the dynactin complex that is probed for in Fig. 7A and D. Even if we imagine a scenario where there exists two fractions of dynein in prometaphase, one that is load-bearing (as described in this manuscript) and another one that is processive (containing dynactin) and both of these fractions contain Hook2, then one would still expect at least a moderate phenotype of defective chromosome capture and congression when Hook2 is perturbed. Is this the case?

Response: We thank the reviewer for this valuable comment. We had named the complex as “dynactin-free” due to the comparatively negligible levels of p150 that interacted with this complex. However, in cognizance of the reviewer’s concern, we have now reworded this phrase in the abstract to a more appropriate version to reflect the low levels of dynactin binding. As suggested, we have also now probed for another dynactin subunit, p50 (dynamitin), in the pull down assays [old figs. 7A and D (now fig. 5A, B), and new fig. 6G]. The p50 binding data is consistent with our p150 data, showing virtually no interaction with the Pin1-associated dynein complex. As stated above in response to multiple comments, we have also now reported effects on chromosome congression but not on kinetochore-microtubule stability (Fig. 7).

The consensus from the dynein literature shows that dynein-adaptor binding precedes, and is required for the formation of an ultra-processive dynein-adaptor-dynactin tripartite complex, in addition to also imparting cargo-binding specificity to dynein. These adaptors are therefore termed “activating adaptors”, as they facilitate more stable dynein-dynactin engagement through multi-site interactions (Lee *et al*, *Nat Comm* 2020)(Schroeder and Vale, *J Cell Biol* 2016; Lee et al, *Nat Comm* 2018; Celestino et al, *PLoS Biol* 2019; Olenick and Holzbaur, *J Cell Sci* 2019; Reck-Peterson et al, Nat Rev Mol Cell Biol 2018; Splinter et al, *Mol Biol Cell* 2012; Carter et al, *Curr Opin Struct Biol* 2016; Schlager et al, *EMBO J* 2014; McKenney et al, *Science* 2014). Adaptors

have been shown to have independent binding surfaces for binding with dynein (LIC1) and dynactin, which has also been shown for Hook2 (Dwivedi et al, *J Cell Biol* 2019). These studies clearly show that purified adaptors can directly bind to dynein LIC1 by themselves independent of dynactin binding through independent binding sites.

Comment ix. I think that a more elaborate analysis is needed for Pin1 recruitment and function in the context of dynein as prophase centrosome uncoupling is not yet on of the key established function of dynein (even considering the involvement of Hook2 in this process). Have the authors performed an in-depth analysis of interphase functions of dynein, such as vesicle motility? It is known that Cdc2 phosphorylation releases dynein from membranes, but is Pin1 binding in some way connected to this function so that membrane/vesicle binding could be regulated?

Response: We appreciate this comment. Several papers have documented the essential role of dynein in maintaining centrosome attachment to the prophase nuclear envelope (Dwivedi, et al, *J Cell Biol* 2019; Raaijmakers et al, *J Cell Biol* 2013; Raaijmakers et al, *EMBO J* 2012; Bolhy et al, *J Cell Biol* 2011; Splinter et al, *PLoS Biol* 2010; Gönczy et al, *J Cell Biol* 1999). However, as aptly suggested by the reviewer, we also now report a role for these LIC1 phosphorylation events on another key Hook2-Nde1-dynein complex function, namely chromosome capture and alignment (congression) in prometaphase-metaphase (as discussed above, Fig. 7).

As suggested, we have now probed the effect of LIC1-CTD phosphorylation on the dynein-mediated motility of two different interphase membrane cargoes, namely for lysosomes (using LAMP1 staining) and ER-Golgi transport [using beta-1,4-galatosyltransferase I (GalT) and COPI], using the wild type (SST) and triple mutant (AAA) LIC1 expressing stable cell lines. We have also used a recently described Pin1-specific inhibitor (BJP-06-005-3, Pinch *et al*, *Nature Chemical Biology 2020*) to show that interphase dynein functions do not appear to be affected upon either AAA phosphodeficient mutation or Pin1 inhibition (Fig. 9, lines 261-295).

In order to test the function of LIC-CTD phosphorylation and Pin1 in dynein's membrane disengagement during mitosis, we have used the Pin1 inhibitor on cell lysates and quantified the extent of Golgi fragmentation in metaphase, which is an indicator of dynein detachment from the Golgi membrane (Yadav and Linstedt *Cold Spring Harb Perspect Biol* 2011; Yadav et al 2012, *Dev Cell*). Our results reveal a requirement for LIC1-CTD phosphorylation and Pin1 activity in the complete release of Golgi membranes from mitotic dynein (fig. 9).

Comment x. It has been shown in Whyte, Vaughan et al, JCB, 2008, that phosphorylation of Dynein IC in prometaphase enables it to bind better to ZW10 (RZZ complex) for the purpose of targeting dynein to kinetochores. However, neither LIC1 phosphorylation nor Pin1-binding to LIC1 seems to be required for RZZ binding. In this scenario, I wonder how one could delineate the consequences of DIC phosphorylation from that of LIC1 phosphorylation by Cdc2/Cdk1. I wonder if it is known whether it is the same kinase that phosphorylates DIC. In any case, the authors should absolutely cite Whyte et al, 2005, in their work and discuss the relevant details in the context of their observations.

Response: We thank the reviewer for this comment. We would like to draw attention to our data (earlier fig. 5, now fig. 3E-H), which clearly shows that phosphorylation of LIC1 at the three CTD Cdk1 sites is required for efficient binding with Zw10 in mitosis. IC gets phosphorylated by Plk-1 at T89, an event similarly required for Zw10 binding (Bader et al, *J Biol Chem* 2011; Whyte, Vaughan et al, *J Cell Biol* 2008). In many of our blots, we see a retarted IC band (see figs. 5A, 5B, 6G), suggesting the possibility of mitotic IC phosphorylation. Thus, it is possible that both phosphorylated LIC1 and phosphorylated IC, if present in the same dynein complex, could positively cooperate to enhance dynein binding to the RZZ complex, although this possibility needs to be experimentally tested. Phosphorylation of IC at S84 leads to stronger Nde1 binding at the expense of dynactin (Jie et al 2017, *Structure*). Phosphorylation at T89, the likely mitotic equivalent of S84, also leads to reduced dynactin binding, but increased Zw10 binding (Whyte et al 2008, *J Cell Biol*). These correlated observations suggest the possibility of a coordinated regulation of selective dynein complex formation through the phosphorylation of different dynein subunits, perhaps to generate a variety of dynein complexes. The resultant binding of Zw10 to the dynein complex is likely to be dictated by the intricate spatiotemporal interplay between these phosphorylation events and merits an independent investigation. Our data show however, that Pin1 does not appear to be required for dynein-Zw10 binding in mitosis (figs. 5, 6), since its interaction is confined predominantly to the Nde1-Lis1-CENPF-dynein complex, and not to the RZZ-spindly-dynactin-dynein complex.

Minor points:

Comment i. The institutional citation number for coauthor, Megha Kumar, might be incorrect.

Response: The citation number has been corrected.

Comment ii. For the ease of the readers, it might be better to mention in Fig. 5A & B or the associated legend that 'prometaphase' = 'nocodazole-treated'. Similarly for Fig. 8A, G2/prophase = 8 hrs post double thymidine release.

Response: These additions have been made in the respective figure legends (figs. 3, 5-8, S3, S4).

Comment iii. It might be slightly incorrect to equate Histone H2B-GFP labelling to the nuclear envelope at the resolution of live imaging carried out in Fig. 8C. It is interesting that the same mitotic cells with the defective coupling of centrosomes to NE also exhibit mitotic delay and arrest. But I am not able to discern the logic behind how the defective coupling is related to defective checkpoint inactivation and the accumulation of SAC proteins at kinetochores in metaphase-arrested cells. Please explain.

Response: We thank the reviewer for this comment. We appreciate that the boundary of the H2B-mCherry signal in G2/prophase may not be a precise measure of the NE at the x-y resolution of confocal imaging. However, we have followed the published literature that has used the H2B-mCherry signal for the purpose of making centrosome-NE distance measurements (Bolhy *et al,* 2011, *J Cell Biol*), since it makes a reasonable approximation of the NE to be able to measure

the displacement of the centrosome from it. As regards the same cells exhibiting centrosome-NE attachment defects and SAC-related prolonged metaphase arrest, we had only tried to use the presence of two distinct AAA-induced phenotypes in the same cell as validation of expression of the SST/AAA rLIC1 constructs (since we did not, at the time, have any fluorescent reporter attached to validate expression). In our significantly revised manuscript, this data has been replaced with SST/AAA MTAP stable cell lines that show fluorescence signals to confirm expression in the cells analyzed, which confirm our earlier observations (fig. 6).

Comment iv. Line # 55, please add "LICs are one of the most important mediators....."

Response: We have now modified this line keeping in mind the reviewer's suggestion (lines 47-50).

Comment v. In line # 160, what do the authors mean by "early" vertebrate zebrafish?

Response: We have now modified the text and not used this phrase in the significantly modified manuscript.

Comment vi. In line # 373, it might be better to say prometaphase 'U2OS' lysates.

Response: We have now modified the text as per the reviewer's suggestion and included tge names of the cell lines (lines 189-190).

Comment vii. In line # 397, "reported earlier" might be better than "reported above".

Response: We have now modified the text and not used this phrase in the significantly modified manuscript.

Reviewer #2 (Comments to the Authors (Required):

Kumari and colleagues explore the role of phosphorylation in the C-terminal cargo-binding region of dynein light intermediate chain in regulating mitotic events. Indeed, cytoplasmic dynein regulates several key steps during cell division. These steps require the association the light intermediate chain 1 (LIC1) with a repertoire of cellular adaptors. Here the authors report that phosphorylation of the putative Cdk1/Cyclin B1 target sites S839, S405 & T408 is required for the timely mitotic progression in human cells and that phosphorylation-defective mutants leads to severe developmental defects in zebrafish embryos. Based on their data the authors propose that the mitotic delay observed in the presence of phosphorylation defective LIC1 mutants is due reduced dynein loading on kinetochores, preventing the silencing of spindle assembly checkpoint. Based on pull-down experiments the authors find that LIC1 phosphorylation is crucial for the association of dynein with the RZZ-Spindly-dynactin complex on kinetochores and the propyl isomerase enzyme Pin1, another key mitotic regulator. Finally, the authors that LIC1 phosphorylation also favours the the binding to Pin1, Nde1-LIS1 and CENP-F during Late G2 phase to ensure the attachment of centrosomes to the nuclear envelope at mitotic entry.

The study addresses an interesting and novel topic, providing a detailed analysis of the potential roles of LIC1 phosphorylation during cell division. If all the findings were to be confirmed, this would be an exciting and compelling study for Journal of Cell Biology. The problem, however, is that the main conclusions of the paper are not always well supported by the presented data, as key controls are missing. At this present stage this raises major issues about the robustness of the presented results, and addressing this issues will require a large amount of work. Specifically:

Comment 1.) All the microscopy experiments (live and fixed cells) presented in Figures 1-4 and 8 rely on the transient over-expression of LIC1 mutants. The authors present the data as if all the cells are transfected the different constructs, but this is a misleading assumption. Even in the best case scenario, only 50-60% of HeLa will be expressing an endogenous protein after transient transfection. Since the transfected constructs contain to my knowledge no fluorescent tag, the authors cannot ascertain the expression level of the constructs in any given cells or know if a particular cells is transfected or not. Moreover, even tough the authors cannot know if the different mutants are expressed at similar levels, as some mutants might be poorly transfected but highly expressed, while other mutants might be expressed at lower levels but in a higher percentage of cells. The authors must repeat those experiments with a fluorescent version of their protein, and analyze the behavior of only the transfected cells and also report the behavior of non-transfected cells. Only such a comparison will reveal the true phenotype of the different mutants.

Response: We thank the reviewer for this comment. As suggested, we have now generated fluorescently tagged (MTAP, which also encodes a YFP tag) wild type and 7 different phospho-deficient hLIC1 variants and their respective stably expressing U2OS cell lines, and compared to the untransfected U2OS cells to address these concerns. We have performed all of these experiments involving live cell imaging, mitotic index counts and prometaphase loading of dynein subunits in these stable cell lines and show the fluorescence expression of the LIC1 constructs in the cells analyzed (wild type SST and mutants) for each experiment. Our results from these experiments (new figs. 1-3, fig. 6) confirm the trends seen with the HeLa cell experiments. We have used this approach for our other experiments suggested during the revision as well (figs. 7-9). These results and the accompanying text have been incorporated throughout the manuscript.

Comment 2) In figure 2A, authors present the expression levels of rLIC1 mutants in an LIC1 siRNA background. However, the level of expression endogenous LIC1 is missing. Since overexpression of dynein complex subunits can disrupt the dynein complex and its motor activity, it is essential that the authors confirm that the level of expression of the rescue constructs is similar to the endogenous protein levels. A similar concern arises in Figure 1C, where the authors express the different mutants over the endogenous protein, the authors need to test for the expression level compared to the endogenous protein.

Response: We thank the reviewer for this comment. We have now addressed this concern by using MTAP-tagged hLIC1 stable lines (see comment above), where we also report the relative levels of endogenous and transgenically expressed LIC1 proteins (which differ in molecular weight by about 33 kD, the size of the MTAP tag) by probing with an anti-LIC1 antibody. As explained above, this new data has been incorporated into the manuscript (new figs. 1, 2) and the associated text (lines 85-108).

We were unable to earlier report the relative expression of endogenous and myc-tagged rLIC1 proteins because they are too close in molecular weight (different only by the molecular weight of the small myc tag) to be differentiated clearly on SDS-PAGE/ Western blots in the same gel. However, we have now probed replicate blots with an anti-LIC1 antibody, which shows a higher intensity of the LIC1 band (combination of band intensities of the virtually overlapping endogenous and myc-rLIC1 bands) only in the transfected, but not in the untransfected samples (fig. S1D).

Comment 3) In Figure 3 and 4 the authors compare the effect of the SST mutant compared to the AAA mutant, but in all experiments we don't know what is the ground state, and what are the levels of the different proteins or inter-kinetochore distances in untransfected cells. this information is important to assess the effect of the two mutants. In this context, it is also interesting that expression of the AAA mutant leads to an increase in inter-kinetochore distances. How do the authors interpret this, since both the cells expressing the SST or the AAA mutant are in the same stage of the cell cycle. Does this suggest that expression of the AAA mutant leads to elevated pullling forces on kinetochores, independently of its effect on SAC proteins?

Response: As suggested by the reviewer, and also stated in our responses above, we have now repeated the experiments of the earlier figure 4 in hLIC1 SST (WT) and AAA stable U2OS cell lines. We have included the untransfected cells' data, as well as the LIC1 depletion data as the relevant controls for prometaphase loading (new fig. 3). We similarly tried repeating the experiments of the earlier figure 3 (metaphase SAC protein removal) in the stable U2OS lines, but were unable to obtain clear Zw10 kinetochore staining in either these stable lines or in untransfected U2OS cells with our antibody despite multiple efforts, precluding us from reliably quantifying kinetochore Zw10 levels. For this reason, we have not quantified the inter-kinetochore distances for these new experiments. The rescue data for the SST and AAA in HeLa cells to assess the specific contribution of phosphorylation at the three cdk1 sites has now been moved to fig. S4.

The increased inter-kinetochore distances observed upon treatment with the proteasome inhibitor MG132 (to prevent anaphase onset) are to be normally expected at late metaphase, since the poleward pulling forces due to microtubule depolymerization would try to segregate the chromosomes, but are unable to as the proteasome is kept inactive. Therefore, we have used the increased inter-kinetochore distance (due to inter-kinetochore tension) as a marker for late metaphase cells. Our new data shows that LIC1-CTD mitotic phosphorylation has no significant impact on the stability of Kt-MT attachments at metaphase (new fig. 7). Under normal conditions, the MT attachment-sensing SAC components are known to be removed from kinetochores soon after initial attachment (Howell *et al* 2001, *J Cell Biol*). Therefore, the persistence of significant amounts of these attachment sensing SAC proteins (e.g. Mad1, Zw10 – fig. S4) at kinetochores even at late metaphase with the AAA mutant is likely to be a consequence of the inability of AAA-LIC1 dynein to efficiently bind and remove these SAC components from mitotic kinetochores. Consistent with this idea, we show reduced binding of AAA-hLIC1 to Zw10 and Mad1 in mitosis compared to SST, with this weakening being even more pronounced in metaphase (new fig. 3). Since this failure of SAC inactivation has been reported earlier upon hLIC1 depletion in HeLa cells (Mahale *et al, PLoS One* 2016; Sivaram et al, *EMBO J* 2009), our present data from HeLa cells

suggest that cdk1-mediated phosphorylation is the major contributor to this function of mitotic LIC1.

Comment 4) The quantifications of pulldown experiments presented in Figure 5 and Figure 7F were performed on saturated exposures which can lead to over/under interpretation of the real results and must be performed on unsaturated exposures to allow robust conclusions.

Response: We thank the reviewer for this comment. We have checked all of our blots for these figures again carefully, especially those used for the quantification analyses. The standard software we have used [ImageJ and the ImageQuant (GE)] to acquire and quantify the band intensities do not show any evidence of signal saturation - we report quantifications only with unsaturated exposures. The only blots that are saturated are the Pin1 blots; however, we have not used any of these for quantitative analysis, as they represent a qualitative pulldown of the bait protein. These data are now in fig. 3 and fig. 5.

Comment 5) In Figure 8C, authors report the importance of LIC1 phosphorylation in regulating the attachment of centrosomes to the nuclear envelope during late G2 phase. Throughout this study the authors assume that these residues are phosphorylated by CDK1-CyclinB1, which they also use in their in vitro phosphorylation experiments. However, fully active CDK1-CyclinB complex formation occurs only after nuclear envelope breakdown. This raises the question as to how these phosphorylation sites might impact centrosome attachment to the nuclear envelope if CDK1/CyclinB1 is not yet active. One interesting possibility would be CDK1-CyclinA? The authors should test for centrosome attachment to the nuclear envelope in G2 after Cyclin A or Cyclin B1 depletion.

Response: We thank the reviewer for suggesting this interesting possibility. We have now used sequence-specific anti-cyclin A2 and anti-cyclin B1 siRNAs and antibodies (Hégarat et al, *EMBO J* 2020) to test the possibility of cyclin A2 also contributing to centrosome-NE engagement in prophase. Our experiments reveal that cyclin A2 does not have any significant role in maintaining proper centrosome-NE attachment, while cyclin B2 does (fig. 6). These results have been incorporated into the relevant section of the Results (lines 213-225).

Comment 6) In Figure 2F, the authors report a minor rescue with zLIC1A (phosphorylation defective) and zLIC1E (phospho-mimetic) which look very similar to LIC1 MO indicating that there is very less or no rescue. This point should be discussed in detail. Additionally, there is no panel for Control MO for better comparison. Moreover, more generally, the authors may want to indicate if they tested any phosphor-mimetic mutants in the assay showed in Figure 3 and 4. Absence of positive result of course does not mean anything, but would help the reader if he/she knew if these experiments were attempted.

Response: As suggested, we have now included the data from control MO injections (MO sequence included in the Materials and Methods), which showed no abnormalities (new fig. 2E-G). We have also discussed the phenotypes observed with either zLIC1A or zLIC1E rescue experiments, which we interpret as a requirement for the normal cycle of phosphorylation and dephosphorylation that could be essential for proper dynein function at different stages of the cell

cycle during early development (lines 120-122 and 311-316). While neither "locked" mutant is able to efficiently rescue the LIC1-MO phenotype, the zLIC1E (constitutively phosphorylated mimic) appears to be slightly better than zLIC1A (fig. 2F, G).

Also, as suggested, we have now reported data with the hLIC1-EEE stably expressing in U2OS cells and performed the experiments in the earlier figure 4 (now fig. 3A-D). Our results reveal that SST (wild type) and EEE (constitutively phosphorylated mimic) showed a similar ability to rescue hLIC1 depletion phenotypes with respect to the prometaphase loading of dynein subunits. As explained above, we were unable to obtain good Zw10 kinetochore staining despite multiple attempts, which precluded our efforts to perform the metaphase SAC protein (Zw10) quantification in the stable EEE U2OS cells as well.

Minor points:

Comment i): In Figure 4I and S2B, the authors use the hLIC1-MTAP (WT/AAA) construct in a LIC1 siRNA background. However, they do not mention how and if this construct was made siRNA resistant. This should be indicated in the Material and Methods.

Response: We thank the reviewer for this comment. We used sub-optimal LIC1 siRNA treatment in high expression stable lines to simultaneously achieve efficient endogenous hLIC1 depletion and exogenous LIC1-MTAP expression for rescue experiments, as confirmed by the blots in fig. 1E. We also confirmed expression of the MTAP-tagged hLIC1 constructs in all rescue experiments using the YFP fluorescence in the cells analyzed (figs. 1, 3, 5-9). We have described these details both in the Results (lines 92-94) and in the Materials and Methods (lines 429-432).

Comment ii): In figure 3B, It would be worth to show LIC1 levels in both the siRNA treatments.

Response: We have now included the LIC1 blots in the figure (fig. S4B).

Comment iii): In figure 4I and K, the images do not represent the quantification and should be replaced.

Response: We thank the reviewer for this observation. We have now replaced the images with more representative ones (fig. 3B).

Comment iv): Among the three residues in LIC1-CTD (S398, S405 and T408), the findings report S405 to be majorly important in the process. Authors should also discuss the role of the other phosphorylation sites.

Response: We thank the reviewer for this comment. Our data with transient expression of the rLIC1 WT and mutants in HeLa confirms the importance of the three CTD phosphosites, while hinting at the possibility of a hierarchy of importance in mitosis, with S405 possibly playing a major role (figs. S1, S2). These observations are consistent with the highest sequence conservation of (the equivalent of) S405 in vertebrate LIC1 orthologs as well as with the zebrafish data (fig. 2). Our new data with stably expressing U2OS cell lines do not clearly demonstrate a hierarchy between the sites (figs. 1, 2) - rather they confirm the importance of phosphorylation in this cluster

(the 398-408 region) towards mitotic dynein function. We have therefore discussed the idea of the importance of this region of LIC getting phosphorylated, also seen for other proteins that contain clustered phosphosites (Schweiger and Linial, 2010, *Biol Direct*) and Yachie et al., 2009, *Mol Cell Proteom*) in the Discussion (lines 344-351).

Comment v): page 2: in line 32, reference to minus end directed kinesins should also be made since dynein is not the only major retrograde transporter. In line 54, the studies on dynein structure and stoichiometry from Andrew Carter's, Ron Vale's and Reck-Peterson's groups should also be cited.

Response: We thank the reviewer for this comment. We have now referred to the minus end-directed kinesin family (lines 34-37). Due to constraints of character limits, we could not cite the individual papers from the Vale, Carter and Reck-Peterson groups. However, we have cited a recent comprehensive review written by the same authors (Reck-Peterson et al 2018, *Nat Rev Mol Cell Biol*) which thoroughly discusses several aspects of dynein's composition, stoichiometry, structure, dynactin recruitment through adaptors and function (lines 44-45 of this paragraph).

July 7, 2021

Re: JCB manuscript #202005184R-A

Dr. Sivaram V S Mylavarapu
Regional Centre for Biotechnology
Laboratory of Cellular Dynamics
NCR Biotech Science Cluster, 3rd Milestone
Faridabad Gurgaon Expressway
Faridabad, Haryana 121001
India

Dear Dr. Mylavarapu,

Thank you for submitting your revised manuscript entitled "Phosphorylation and Pin1 binding to the LIC1 subunit selectively regulate mitotic dynein function". The manuscript has been seen by the original reviewers whose full comments are appended below. While the reviewers continue to be overall positive about the work in terms of its suitability for JCB, some important issues remain.

You will see that the reviewers have raised concerns about a number of issues, including the small effect size of some of the experiments, the need for another Golgi marker to corroborate your data, and the accuracy and organization of some of the figures. They have also asked that you provide further discussion for several of these remaining issues.
Reviewer #2 has also raised some concerns about the methods used in the statistical analyses of your data. In some cases, it is certainly appropriate to directly and individually compare each experimental condition with the control (and, thus, a t-test may be suitable, provided that the data shows a gaussian distribution) but we recommend that you consult with a statistician to determine the best methods for the statistical analysis of your data. If they advise that you use a different statistical approach then please reanalyze the data accordingly and provide the new statistical analyses in the revised manuscript.

Our general policy is that papers are considered through only one revision cycle; however, given that the suggested changes are relatively minor we are open to one additional short round of revision. Please note that I will expect to make a final decision without additional reviewer input upon resubmission.

Please submit the final revision as quickly as time allows (within one month, preferably, but if lab closures due to COVID-19 prevent you from completing the revisions in this time frame just let us know and we can work out a suitable revision schedule) along with a cover letter that includes a point by point response to the remaining reviewer comments.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact the journal office with any questions at cellbio@rockefeller.edu.

Sincerely,

Daniela Cimini, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This is indeed a much improved manuscript with a lot more supportive data and the authors have satisfactorily addressed most of my concerns to a substantial extent. But a few points that are listed below still remain to be addressed, most of which pertains to the new data presented in the revised manuscript.

(i) Now that the experiment related to my comment i in the original review is done, it might be better to switch back to the previous wording in the abstract text, ie "one way that dynein cargo-binding is regulated....." to leave way for other, and possibly LIC1-independent mechanisms.

(ii) Related to my original comment ii, in Fig. 7C, the merge image looks different from the individual channels for siLIC1+SST. Additionally, I am keen to know the fate of the mitotic cells in the control and mutant-expressing cells in Fig. 7E.

(iii) Even though the authors have now found evidence for defective chromosome alignment, I am still very intrigued by the fact that the effect is so minimal as compared to what could be caused if LIC1-CTP phosphorylation along with Pin1-binding was required for dynein kinetochore recruitment. It would be good if they can provide more explanation to this effect.

(iv) The new data in Fig. 8 is quite interesting, but it might be better to confirm with another Golgi marker to strengthen it further. In Fig. 8B, please clarify '+AAA+Pin1i' is not significant to which other condition in the histogram.

(v) I feel that the new Fig. 9 or at least parts of it showing that LIC1-CTP phosphorylation and Pin1 binding is not required for interphase membrane transport could be supplemental data.

Reviewer #2 (Comments to the Authors (Required)):

In this revised version Kumari and colleagues address the role of phosphorylated LIC during human cell division. Using both fixed and live cell imaging-based assay and well as biochemical purification they show that the dynein light intermediate chain LIC1 must be phosphorylated to interact with different dynein subcomplexes in mitosis. In particular they point to an interaction with the Lis1-Nde1-Hook2 complex at spindle poles and centrosomes in conjugation with the prolyl-isomerase Pin1 and with the spindly-dnyactin complex at kinetochores to remove spindle assembly checkpoint proteins.

Compared to the first submission the authors have provided many novel experiments and have addressed most concerns of the reviewers, they have done a thorough and excellent job. The manuscript is therefore of much higher quality. I have nevertheless one major concern with regard to the statistical analysis, which the authors should address to validate the significance of their result. Should this revised analysis confirm their results, I believe that this study could be of sufficient quality and interest for the general readership of Journal of Cell biology.

Major points:

- One major concern is that the authors use for all their statistical analysis a simple student t-test, comparing every single condition with the respective control. Such a comparison is only possible if two criteria are met: the distribution should be gaussian, and the set of experiment should only contain two conditions. This is, however, not the case in most presented data. Some of the measures are by experience not gaussian, such as the mitotic timing. In such cases one should use non-parametric tests. Second, in most experiments the users are comparing 4-9 different conditions. In this case, one has to correct for the fact that several conditions were tested in parallel. In case of parametric tests, this would be an ANOVA test, or in the case of non-parametric set of data a Kruskal-Wallis test (both available in PRISM). This is not a non-trivial issue, as many "significant" results can turn out to be non-significant any morewhen applying such a correction. The authors should therefore provide the results of such a revised analysis.

Minor points:

- The zebrafish results are interrupting the flow of the presented biochemical/cell biological analysis in human cells, and I would suggest to present these results at the end of the manuscript, for an in vivo validation, as this might help to improve the flow of this very dense manuscript.

- in several assays the AAA-mutant has a dominant-negative impact, as its expression not only fails to rescue LIC depletion, but instead seems to worsen the phenotype. The authors might want to consider speculate about the significance of these results in 1-2 sentences in the discussion.

Phosphorylation and Pin1 binding to the LIC1 subunit selectively regulate mitotic dynein functions (manuscript number 202005184R-A).

Reviewer #1 (Comments to the Authors (Required)):

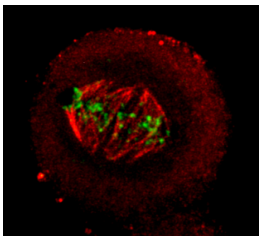
This is indeed a much improved manuscript with a lot more supportive data and the authors have satisfactorily addressed most of my concerns to a substantial extent. But a few points that are listed below still remain to be addressed, most of which pertains to the new data presented in the revised manuscript.

(i) Now that the experiment related to my comment i in the original review is done, it might be better to switch back to the previous wording in the abstract text, ie "one way that dynein cargo-binding is regulated....." to leave way for other, and possibly LIC1-independent mechanisms.

Response: This line has been reworded as suggested.

(ii) Related to my original comment ii, in Fig. 7C, the merge image looks different from the individual channels for siLIC1+SST. Additionally, I am keen to know the fate of the mitotic cells in the control and mutant-expressing cells in Fig. 7E.

Response: The merged image in fig. 7C (new fig. 6C) depicts a single z-plane shown from the z-stacks to be able to better visualize the kinetochore-microtubule attachment, and therefore appears different from the individual siLIC1+SST channels (which is a maximum projection of all planes). We had already mentioned this in the figure legend, which we have now also highlighted with an asterisk in the merged panel. *For your perusal, we have now included a merged panel of the maximum projection below.* Regarding the fate of the cells in fig. 7E (new fig. 6E), these cells remained arrested in metaphase till the end of our movies (approx. 350 min), since they are MG132-treated. We had specifically curtailed live cell imaging beyond this point to avoid measuring metaphase plate dispersal due to cohesion fatigue, which typically sets in after prolonged metaphase arrest for over about 350 min (Raaijmakers et al, J Cell Biol 2013; Daum et al., Curr. Biol., 2011; Stevens et al., PLoS ONE, 2011).



(iii) Even though the authors have now found evidence for defective chromosome alignment, I am still very intrigued by the fact that the effect is so minimal as compared to what could be caused if LIC1-CTP phosphorylation along with Pin1-binding was required for dynein kinetochore recruitment. It would be good if they can provide more explanation to this effect.

Response: In our study, we observed that LIC1-CTD phosphorylation is required for kinetochore recruitment/ localization of dynein (new fig. 2). Growing evidence is revealing that the kinetochore-dynein population does not make significant contributions to chromosome congression (Raaijmakers et al, J Cell Biol 2013; Gassmann et al., Genes Dev, 2010; Maiato et al., Biol., 2017). Rather, the prominent defects in chromosome alignment observed after dynein depletion have been attributed to spindle microtubule localized dynein for the initial poleward movement of a subset of prometaphase chromosomes (peripherally located with respect to the spindle), through the side-on, dynein-mediated association of these chromosomes with microtubules (Maiato et al., Biol., 2017; Auckland and McAinsh, J cell Sci., 2015).

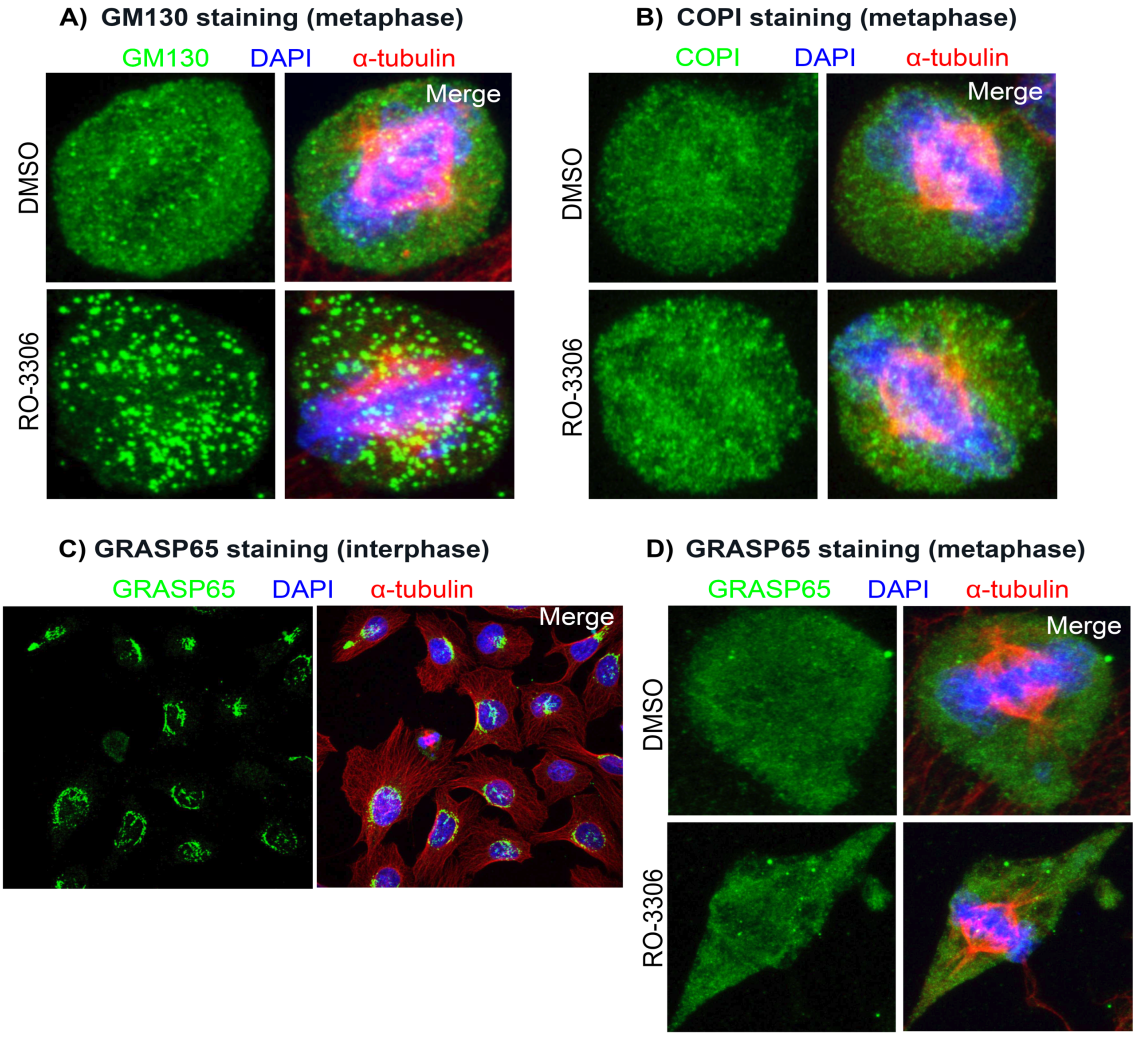
Misalignment could also be indirectly due to defects in spindle microtubule assembly/organization (Raaijmakers et al, J Cell Biol. 2013; Maiato et al., Biol. 2017, Raaijmakers and Medema, Chromosoma, 2014). Moreover, we have shown that Pin1 does not bind to the dynein-dynactin complex; rather, it interacts exclusively with the Hook2-Nde1-Lis1 containing mitotic dynein complex (new fig. 4). Neither Hook2 (Dwivedi et al J Cell Biol., 2019) nor Pin1 (this study, St-Denis et al., J cell Sci., 2011) have been shown to localize at mitotic kinetochores, suggesting that the Hook2-Nde1-Lis1-dynein complex, possibly bound to Pin1, is majorly localized on the spindles and/or the poles and may be primarily responsible for chromosome alignment. This idea is consistent with models proposed for dynein function in chromosome congression (Maiato et al., Biol., 2017; Auckland and McAinsh, J cell Sci., 2015). In addition, our study only probes the role of the phosphorylation of LIC1-dynein in mitotic functions. LIC2-dynein, a separate dynein complex, plays an independent role in chromosome alignment in mammalian cells (Mahale et al, Sci Rep 2016), and co-depletion of LIC1 and LIC2 leads to drastic congression defects Mahale et al, Sci Rep 2016; Raaijmakers et al, J Cell Biol 2013). Therefore, LIC1 and its mitotic phosphorylation are likely to contribute only partially to chromosome congression. We have now discussed these points in the manuscript (lines 353-361).

(iv) The new data in Fig. 8 is quite interesting, but it might be better to confirm with another Golgi marker to strengthen it further. In Fig. 8B, please clarify '+AAA+Pin1i' is not significant to which other condition in the histogram.

Response: As suggested, we have now attempted to repeat the Golgi fragmentation experiments using two other Golgi markers, beta COP and GRASP65. Our experiments revealed that these markers, though widely used in Golgi studies, do not serve as good reporters for quantifying mitotic Golgi fragmentation by confocal microscopy. GM130 (already used in our manuscript, new fig. 7) is the most widely used marker for measuring mitotic Golgi fragmentation (Wortzel et al, iScience, 2021; Guizzuntia and Seemann, Proc. Natl. Acad. Sci. 2016; Kienzle et al., Mol. Biol Cell, 2012; Tang et al., Biol Open, 2012; Tang et al., Traffic, 2010, Shima et al., J Cell Biol. 1998; Lowe et al., Cell, 1998). GRASP65 staining is expected to be similar to GM130 staining, since both stay in a stable complex through mitosis (Barr et al., cell, 1997). However, the antibody we used for GRASP65, which stained interphase Golgi stacks robustly, unfortunately did not stain punctate Golgi spots upon inhibiting Cdk1 activity, precluding us from quantifying this phenotype with this marker. Fluorescently tagged constructs of GRASP65 in stable cell lines have been shown to stain partially fragmented Golgi punctae during mitosis (Tang et al., Biol Open, 2012; Tang et al., Traffic, 2010), however we could not perform these experiments due to the considerable amount of time that would

have been required. Our experiments with the beta COP antibody worked well to assess interphase Golgi organization (new fig. 8). However, this antibody was also unable to detect large Golgi punctae in mitosis upon inhibiting Cdk1. We surmise that the COPI positive Golgi punctae in mitosis are too small to be detected through diffraction-limited confocal imaging, which is consistent with the literature (Misteli and Warren, J Cell Biol. 1994; Sönnichsen et al., J Cell Biol. 1996; Shorter and Warren, Annu. Rev. Cell Dev .Biol. 2002).

Following our revised statistical re-analysis (as suggested by reviewer 2), we have now clearly indicated the various comparisons in the figure.



(v) I feel that the new Fig. 9 or at least parts of it showing that LIC1-CTP phosphorylation and Pin1 binding is not required for interphase membrane transport could be supplemental data.

Response: We appreciate this suggestion. However, we wish to retain this data in the new main Fig. 8 to emphasize the exclusive role of these phosphorylation events during mitosis.

Reviewer #2 (Comments to the Authors (Required)):

In this revised version Kumari and colleagues address the role of phosphorylated LIC during human cell division. Using both fixed and live cell imaging-based assay and well as biochemical purification they show that the dynein light intermediate chain LIC1 must be phosphorylated to interact with different dynein subcomplexes in mitosis. In particular they point to an interaction with the Lis1-Nde1-Hook2 complex at spindle poles and centrosomes in conjugation with the prolyl-isomerase Pin1 and with the spindly-dnyactin complex at kinetochores to remove spindle assembly checkpoint proteins.

Compared to the first submission the authors have provided many novel experiments and have addressed most concerns of the reviewers, they have done a thorough and excellent job. The manuscript is therefore of much higher quality. I have nevertheless one major concern with regard to the statistical analysis, which the authors should address to validate the significance of their result. Should this revised analysis confirm their results, I believe that this study could be of sufficient quality and interest for the general readership of Journal of Cell biology.

Major points:

- One major concern is that the authors use for all their statistical analysis a simple student t-test, comparing every single condition with the respective control. Such a comparison is only possible if two criteria are met: the distribution should be gaussian, and the set of experiment should only contain two conditions. This is, however, not the case in most presented data. Some of the measures are by experience not gaussian, such as the mitotic timing. In such cases one should use non-parametric tests. Second, in most experiments the users are comparing 4-9 different conditions. In this case, one has to correct for the fact that several conditions were tested in parallel. In case of parametric tests, this would be an ANOVA test, or in the case of non-parametric set of data a Kruskal-Wallis test (both available in PRISM). This is not a non-trivial issue, as many "significant" results can turn out to be non-significant any morewhen applying such a correction. The authors should therefore provide the results of such a revised analysis.

Response: We thank the reviewer for this suggestion. As advised, we have now revisited all of the data and analysed most of the data using either the one-way ANOVA (for normal distributions of multi-condition data), or the Kruskal-Wallis test (for non-normal distributions of multi-condition data). Distribution of normality was assessed using the D'Agostino and Pearson test. We used post-hoc analysis of multiple comparisons using the Tukey's test. All analysis was performed through the GraphPad Prism software. We have noted these details in the respective figure legends and in the Materials and Methods. We are happy to note that the trends shown in all of the data were confirmed by these analyses, and in some cases the significance values have improved. We have now included the raw data points (shown as scatter plots) for several figures, which depicts the data distribution and has also helped to strengthen the statistical analysis (e.g. new figs. 1G, 6F, 7B, S1G etc).

Minor points:

- The zebrafish results are interrupting the flow of the presented biochemical/cell biological analysis in human cells, and I would suggest to present these results at the end of the manuscript, for an in vivo validation, as this might help to improve the flow of this very dense manuscript.

Response: As suggested, we have now moved the zebrafish results as a figure on its own at the end of the manuscript (new Fig. 9) and moved the corresponding text to the end of the Results section. Panels A-C of the erstwhile figure 2, which contain mitotic index data from U2OS cells, have now been moved to supplementary figure S2.

- in several assays the AAA-mutant has a dominant-negative impact, as its expression not only fails to rescue LIC depletion, but instead seems to worsen the phenotype. The authors might want to consider speculate about the significance of these results in 1-2 sentences in the discussion.

Response: The mutually exclusive LIC2-dynein complex is independently required for most of dynein's mitotic functions, often redundantly (Mahale et al, Sci Rep 2016; Raaijmakers et al, J Cell Biol 2013), which could explain the apparently small effects seen upon LIC1 depletion alone. Interestingly, depletion of either of these subunits is known to cause a compensatory increase in levels of the other (unpublished observations, Scherer et al., J Cell Biol. 2014). Rescue of LIC1 depletion with AAA may possibly suppress this feedback loop, leading to a lack of compensatory rescue by LIC2-dynein. In this background, the sequestering of the natural interacting partners by the non-functional AAA-LIC1-dynein could result in the dominant negative effects that further exacerbate the LIC1 depletion phenotype. We have now discussed this in the manuscript (lines 353-361).

September 7, 2021

RE: JCB Manuscript #202005184RR

Dr. Sivaram V S Mylavarapu
Regional Centre for Biotechnology
Laboratory of Cellular Dynamics
NCR Biotech Science Cluster, 3rd Milestone
Faridabad Gurgaon Expressway
Faridabad, Haryana 121001
India

Dear Dr. Mylavarapu:

Thank you for submitting your revised manuscript entitled "Phosphorylation and Pin1 binding to the LIC1 subunit selectively regulate mitotic dynein functions". We have now assessed your revised manuscript and we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends. You are below this limit at the moment but please bear it in mind when revising.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described." Please also note that we are unable to accommodate tables in the methods section so please add your primer table to the supplementary materials, rename it "Supplementary Table 1", and then refer to it as such in the methods section.

5) In addition to your primers/oligos, please be sure to provide the sequences for all/any RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

6) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses
c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. At the moment, you are below this limit but please bear it in mind when revising.
Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

9) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

10) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

11) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

12) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

-- An editable version of the final text (.DOC or .DOCX) is needed for copyediting (no PDFs).

-- High-resolution figure and MP4 video files: See our detailed guidelines for preparing your production-ready images, https://jcb.rupress.org/fig-vid-guidelines.

-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

The license to publish form must be signed before your manuscript can be sent to production. A link to the electronic license to publish form will be sent to the corresponding author only. Please take a moment to check your funder requirements before choosing the appropriate license.

Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Daniela Cimini, PhD
Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology
