THE
EMBO
JOURNAL

POLO REGULATES SPINDLY TO PREVENT PREMATURE STABILIZATION OF KINETOCHORE-MICROTUBULE ATTACHMENTS

João Barbosa, Torcato Martins, Tanja Bange, Li Tao, Carlos Conde and Claudio Sunkel.

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Transaction Report:

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

1st Editorial Decision 8th October 2018

Thank you for submitting your manuscript to our editorial office, and please excuse the delay in getting back to you with a response, owed to both Bernd and myself being away at meetings last week. I have now had the chance to carefully read your study and to further discuss it with Bernd as well as with the other editors. Our conclusion from these considerations, I am afraid to say, was that the study in its present form does not appear to be a sufficiently compelling candidate for an EMBO Journal article. We appreciate that you have further extended your earlier finding of mitotic Polo kinase activity fluctuations at kinetochores and of Polo roles in maintaining the spindle assembly checkpoint, by genetically identifying decreased RZZ removal from kinetochores as a cause of Polo-T182D-dependent phenotypes. While these results, together with the observation of reduced Spindly association with unaligned prometaphase kinetochores and phenocopy by Spindly knockdown, should certainly be of interest to others in the field, we are however not convinced that these mainly genetic and cell biological data offer sufficiently striking new insights, in light of various previous findings on Polo kinase/Plk1 roles in regulating microtubule-kinetochore attachments, to likely also be of immediate wider significance to a broader non-expert readership. We realize that your further data employing in vitro phosphorylation assays and expression of a phosphomimetic Spindly version are consistent with a molecular scenario in which Polo phosphorylation may regulate Spindly recruitment by affecting its interaction with RZZ, but I am afraid we feel that at the level of a broad general journal such as The EMBO Journal, it would be essential to support this model with considerably stronger evidence for the physiological significance of such a mechanism, e.g. by demonstrating Polo-dependent/regulated S499 phosphorylation on endogenous Spindly during mitosis in cells, and by supporting the in vivo role of S499 phosphorylation not just by gain-of-function with the supposedly phosphomimetic version, but by also testing the functional significance and relative regulatory contribution of this site in non-phosphorylatable mutants. Should you be able to obtain such additional, more definitive evidence through further experimentation, we would in fact be happy to consider a new version of this study for in-depth review at The EMBO Journal; while at the present level of conclusiveness, we feel that the study would primarily be of field-

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specific value, and hence already in its current form well-suited for our new open-access journal Life Science Alliance (http://www.life-science-alliance.org). We briefly confirmed this by consulting with its Executive Editor, Dr. Andrea Leibfried, who be interested in considering this work at Life Science Alliance and to hence send it our for formal peer-review in case you should be interested in this opportunity.

I am sorry that I cannot be more positive for The EMBO Journal at the current stage, but hope you understand that in the case of overall already well-studied pathways, we do generally require strong support for endogenous occurrence and context as well as regulatory significance for a study to achieve the level of advance we expect from an EMBO Journal article. In any case, as mentioned we would remain open to looking again at an extended version of this work, or - upon transfer via the link below - offer immediate in-depth review at Life Science Alliance; please do not hesitate to get back to us should you have any questions in this regard.

Resubmission - authors' appeal email 2nd May 2019

We would like to submit a new version of the manuscript entitled "**Polo-mediated phosphorylation of Spindly prevents premature stabilization of kinetochore-microtubule attachments**" to EMBO journal.

Following your comments to our previous submission EMBOJ-2018-100789, we have now included additional and stronger evidence supporting the physiological significance of Polo-mediated regulation of the RZZ/Spindly/Dynein module. We examined the impact of a non-phosphorylatable Spindly mutant (S499A) on kinetochore dynamics of the RZZ complex and on kinetochore-microtubule attachments. In perfect agreement with the model we had proposed in the first submission, preventing the phosphorylation of Spindly on S499 promotes Dynein-mediated streaming of RZZ along spindle microtubules and concomitantly increases the frequency of merotelic attachments. Furthermore, you will notice that we have also expanded the *in vitro* pull-down assays to support a model in which S499 phosphorylation elicits a negative regulatory action of Spindly C-terminal region over the N-terminus to prevent its interaction with Zwilch.
These new set of results in conjunction with the *in vivo* analysis of Polo and Spindly phosphomimetic mutants provide a compelling demonstration that phosphorylation of Spindly by Polo impairs its binding to Zwilch, hence precluding Dynein-mediated removal of the RZZ complex from kinetochores and consequently delaying the formation of stable end-on attachments. Our findings show that high Polo-kinase activity following mitotic entry directs the RZZ to minimize premature stabilization of erroneous attachments, while a decrease in Polo activity is required during later stages of mitosis to enable the formation of stable amphitelic attachments.
Overall, the findings reported in our manuscript advance the current knowledge in the field by showing that Spindly- Dynein affinity for the RZZ complex at kinetochores is finely-tuned by Polo activity throughout mitotic progression. Importantly, this provides a mechanistic explanation for the previously described, yet molecularly elusive, Polo- destabilizing role on kinetochore-microtubule interactions. This represents important information to better comprehend how cells ensure the fidelity of chromosome segregation and avoid aneuploidy. Thus, our conclusions are also likely to be of interest to a wider community, as Polo/Plk1 is frequently overexpressed in chromosomal instable cancers. For all these reasons, we believe our work will have significant impact and is particularly appropriate for the EMBO Journal.

2nd Editorial Decision 9th June 2019

Thank you again for submitting a new version of your manuscript on Polo-mediated Spindly phosphorylation in kinetochore-microtubule attachment to The EMBO Journal. We have now received comments from three expert referees, copied below for your information. As you will see, these referees are somewhat divided in their opinions - while referee 1 would be supportive of publication pending only minor revisions, referee 3 remains unconvinced that the presented data offer sufficiently decisive support for key conclusions of the study. Referee 2 in general appreciates the potential significance of the study, but also raises a substantive number of important issues that would have to be addressed/clarified in order to make the work sufficiently convincing.

Given the support in principle from two of the three referees, we would like to give you the

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opportunity to respond to the criticisms of the three reviewers through a revised version of this manuscript. While we not all points raised in the reports will in our view necessarily require experimental addressing, we however feel that there are several key concerns that need to be strengthened or clarified through decisive additional data. This particularly includes addition of further controls and statistics (e.g. ref 1 points 2, 3, ref 2 points 4, 8, ref 3 pt 4 re. Polo-T182D), clarification of referee 2's various concerns regarding the fly genetic analyses, and presenting the results of the RNAi suppressor screen data in a more complete and insightful manner (refs 2 & 3). Furthermore, it will be essential to validate that Spindly-S499 phosphorylation occurs in vivo/in cells and in a Polo-dependent fashion, possibly via mass spectrometry if phospho-specific antibodies should not be available (ref 3). We note that various other specific or conceptual concerns may well be clarified through textual changes/discussions and/or inclusion of alternative/additional images/data.

Should you be able to satisfactorily revise the study along these lines, we shall be happy to consider this work further for eventual EMBO Journal publication.

REFEREE REPORTS

Referee #1:

Plk1 plays an important role in cell division, but the function of PLK1 at mitotic kinetochores is not well defined. Conflicting evidence has suggested roles both in the stabilization and destabilization of mitotic kinetochore-microtubule interactions. Here the authors show that PLK1 functions in early prometaphase to destabilize kinetochore-microtubule interactions. As the cell cycle progresses, the dynamic removal of the kinase from metaphase kinetochores allows the stabilization of kinetochore-microtubule interactions and eventual chromosome segregation. Utilizing a constitutively active PLK1 mutant expressed in Drosophila neuroblasts, the authors find that cells overexpressing PLK1 T182D struggle to properly align chromosomes at the metaphase plate and frequently exhibit kinetochores laterally attached to microtubules. These mis-attached kinetochores exhibit increased levels of the RZZ complex components, proteins involved prevention of stable end-on kinetochore-microtubule attachments. Importantly, these phenotypes can be rescued by the depletion of the RZZ component ROD via RNAi. Once chromosomes are properly bi-oriented in metaphase, the RZZ complex is removed from kinetochores via the Spindly/Dynactin/Dynein complex. The authors show that PLK1 mediates this removal of the RZZ complex via the phosphorylation of Spindly at S499. Phosphorylation of Spindly at S499 decreases the affinity of the RZZ complex to the protein, inhibiting its removal. Together this work identifies a novel role for PLK1 in the regulation of the RZZ complex and helps shed light on to the discrepancies on PLK1 function in the regulation of kinetochore-microtubule attachments.

Overall, I am enthusiastic about this manuscript. The separation of the prometaphase and metaphase functions of PLK1 in kinetochore-microtubule attachments will be of great interest to many in the field. The ability to identify a specific target for this regulation with a clear biochemical consequence is also impressive. However, there are several aspects of this manuscript that need to be addressed or clarified. I would recommend this paper for publication in EMBO once these comments are addressed.

Major Concerns:

1. In cells treated with the PLK1 inhibitor BI2536, chromosomes fail to align, often appearing unattached from microtubules. In the proposed model, loss of PLK1 in early prometaphase should increase the appearance of end-on kinetochore-microtubule attachments. How do the authors explain these discrepancies when PLK1 is constitutively expressed/active versus relative to when its catalytic activity is disrupted?

2. The authors demonstrate that knockdown of ROD partially rescues the prometaphase defects observed in PLK1 T182D cells. As their model is that this occurs through loss of the RZZ complex, it would be helpful to show that knockdown of another subunit (i.e., Zw10) is also sufficient to rescue these phenotypes.

3. PLK1 has been shown to modulate AURKB localization and activity at kinetochores in mitosis. Because AURKB is a core regulator of kinetochore-microtubule attachments, it would be helpful for the authors to show that AURKB is not aberrantly regulated in PLK1 T182D cells (testing AURKB localization or the phosphorylation of a downstream substrate).

4. In figure 8, ZW10 does not appear reduced at kinetochores in the Spindly S499A mutant. According to the model, loss of Spindly phosphorylation should increase its affinity for the RZZ complex, thereby stripping the RZZ complex prematurely from kinetochores. The authors highlight this point in figure 9 in which more spindle bound ZW10 is indicated in S499A cells. However, this spindle localization of ZW10 is not visible in the same cells in figure 8.

Minor Concerns

1. Typo. The authors list figure S2A-B after the statement "Accordingly we were able to follow dynein-dependent stripping of ROD-GFP". However, this figure does not depict what is stated.

2. Figure 1G. MAD2 GFP signal is hard to see in these cells. The authors should include a figure with MAD2 localization via antibody to make this clearer as this is an important point.

Referee #2:

Barbosa et al. characterize the role of Polo kinase in the control of the stability of KT attachments to spindle MTs during progression through mitosis in Drosophila (studying neuroblasts in larval brains and S2 cells in culture). The study includes a screen, in which candidate genes were knocked down during Drosophila development (using ey-GAL4 and UAS-RNAi genes) and the modification of effects of a hyper-active Polo kinase variant (Polo[S182D]) by the knockdown was scored. Thereby, knock-down of rod, a component of the RZZ complex, was observed to suppress phenotypes resulting from hyperactive Polo kinase. The RZZ complex is known to be present at kinetochores during prometaphase where it recruits Spindly which in turn recruits Dynein and thereby promotes shedding of RZZ-Spindly-Dynein particles away from the kinetochore by Dynein-mediated transport along kinetochore fibers. The authors have completed a rather comprehensive set of experiments in order to elucidate the functional interactions between Polo kinase, RZZ complex and Spindly. Based on their findings, they present an interesting novel model. Accordingly, Polo phosphorylates Spindly early during prometaphase (on S499) (indicated by in vitro kinase experiments). This inhibits recruitment of Spindly to the kinetochore via binding to the RZZ component Zwilch (indicated by pull down experiments and microscopic quantification of kinetochore signals in various genotypes, including Spindly S499A and S499D expressing cells). Without Spindly, RZZ shedding is prevented and hence RZZ levels at kinetochores remain high (microscopic quantification of kinetochore signals in various genotypes, also shown previously in other studies). At the kinetochore, RZZ appears to inhibit the conversion of lateral to end-on attachments of the kinetochore to spindle MTs (suggested by earlier publications and further confirmed by microscopic characterization of kinetochore and microtubules in various genotypes, including after Ca2+ pretreatments that destabilize non-kinetochore spindle fibers before fixation). After a decrease in Polo activity at the kinetochore (indicated by microscopic quantification), Spindly is proposed to become transformed into the non-phosphorylated state, allowing stable chromosome bi-orientation via end-on attachments of sister kinetochores to spindle microtubules. If Spindly cannot be phosphorylated (S499A), stable attachments appear to develop too early in prometaphase, resulting in frequent merotelic attachments. If Spindly cannot be dephosphorylated (as suggested by the S499D charge mimick mutation and hyperactive Polo[S182T] kinase, stable attachments arise more slowly, delaying the metaphase to anaphase transition.
The manuscript would clearly make a significant novel contribution to our understanding of the mechanisms responsible for a correct integration of chromosomes into the mitotic spindle, if the problems detailed below can be resolved. While many analyses were well done technically, some of the most crucial experiments are not (yet) convincing. Overall, the manuscript is clearly written and includes an intelligent discussion that integrates the proposed model appropriately into the wider context.

Specific major concerns:
1. The effects of insc-GAL driven expression of UAS-polo[wt] and UAS-polo[T182D] in larval brain neuroblasts on progression through mitosis are compared (Fig. 1C-H) and different effects were observed. According to the presentation of the authors, the resulting phenotype difference reflects the fact that Polo[T182D] is constitutively active. However, there is a technical problem that questions the validity of the author's interpretation. The two UAS transgenes were made with different vectors (pP{UASp} and pP{Express-UAS}, respectively) with different cis-regulatory elements and they are not inserted at the same chromosomal site. Based on the known properties of these expression vectors, it is very likely that the resulting UAS-polo[wt] expression is considerably lower than the expression of UAS-polo[T182D]. Therefore, it remains a possibility that the different phenotypic consequences reflect simply a difference in expression level and not an effect of the T182D mutation. Similarly all later interpretations from comparison of phenotypic effects after UAS-polo[wt] and UAS-polo[T182D] in larval brain neuroblasts (data in Figs 2, 4 and 5) are compromised by this technical problem.
Ideally the authors should perform the comparison between Polo[wt] and Polo[T182D]by using identical constructs (except for the mutation) inserted into the same chromosomal landing site, i.e., in the current standard manner. It might be argued that this too much of an effort since a large fraction of experiments would have to be repeated for insufficient reasons since the results of analogous experiments with S2 cells that are not affected by this particular technical problem largely concur with the author's interpretation of the neuroblast experiments.
Moreover, Fig S1F presents data of an IB analysis, suggesting that comparable levels of Polo[WT] and Polo[T182D] results after insc-GAL4 driven expression of the different UAS transgenes. However, the presented immunoblot image raises concerns about the technical quality of this important analysis. The anti-Tub bands used for normalization are uneven. While the corresponding figure legend might be read as suggesting that the quantification is based on statistical analysis of independent experiments, no n (number of independent experiments) is given. Perhaps only a single experiment has been done. Please present a clear statistical analysis from multiple experiments.
The differences between the transgenes (UAS-polo[wt] and UAS-polo[T182D]) should be described clearly including comments on potential problems with interpretations.

2. p.7: "Time course analysis shows that these cells undergo a highly asynchronous chromatid migration during anaphase as opposed to the synchrony observed in PoloWT neuroblasts (Fig.2E, G)."
Fig. 2E,G present comparisons between Polo[T182D] and "no transgene" rather than between Polo[T182D] and Polo[WT] as suggested in the text. A careful comparison of Polo[T182D] and Polo[WT] would actually be of interest, as Polo[WT] overexpression in S2 cells has a similar yet milder effect than Polo[T182D] expression concerning the frequency of erroneously attached chromosome in the MG132 (Fig. 2B), while unexpectedly in neuroblasts these two conditions appear to have opposite effects concerning duration NEBD-anaphase onset (Fig. 1D,E); perhaps linked to expression level differences (see 1.).

3. Fig. 2C,D: calcium-stable KT-MT attachments in MG132 arrested S2 metaphase cells were studied, and more frequent lateral attachment (less end-on) of sister KTs after PoloT182D-mRFP compared to PoloWT-mRFP was observed (25% compared to 10% of the metaphase cells, n {greater than or equal to} 58 cells for each condition).
As published recently (Strunov et al. 2018), end-on attachments with some MTs extending laterally beyond the KT appear to be rather common in S2 cells, and the extent of extending MTs is variable. Therefore, how can KT attachments be classified in a binary manner as either end-on or lateral given these gradual differences? Our own research experience would also question the feasibility of such a binary classification. Moreover, can the authors rule out artefactual effects resulting from slight experimental differences of the applied Ca2+ treatment before fixation. How many independent experiments were analyzed? Are all the analyzed cells in Fig. 2D from a single experiment?
The same assay is also used in the experiments presented in Fig. 9D,E and hence the same concerns apply.

4. Fig. 3D-F: In principle, the observed milder mitotic defects resulting from addition of UAS-rod[RNAi] into insc>polo[T182D] might result from a Gal4 protein titration effect by additional UAS[GAL4] binding sites rather than from Rod depletion; as some Gal4 proteins is recruited by UAS-rod[RNAi], there might be less for driving Polo[T182D] expression.

The authors try to rule out this alternative interpretation by the data presented in Fig S1F. As indicated above (see 1.), their analysis does not seem to be of sufficient technical quality to allow firm conclusions. Beyond such a quantitative analysis of expression levels by IB, it would be most convincing if an irrelevant UAS-RNAi gene (for example GFP) was included as a control (comparison insc>UAS-polo[T182D] UAS-GFP[RNAi] with insc>UAS-polo[T182D] UAS-rod[RNAi]).
Note that the authors actually show (Fig S1E) that Rod[RNAi] led to ZW10 depletion at KTs that was greater than when Rod[RNAi] was combined with Polo[T182D] expression. In contrast to their statement (p.8: "an equivalent reduction") a difference is clearly apparent, and this difference might reflect Gal4 titration.

5. The study would certainly gain interest if gene names and potency of the 24 suppressor hits of ey>polo[T182D] was revealed. Table S3 seems useless and perhaps misleading, as the selected and analyzed 222 candidate gene are already enriched for GO terms linked to mitotic functions and the p-values given in Table S3 seem to represent analyses of enrichment compared to the entire Drosophila gene set rather than comparison of enrichment compared to just the 222 candidate gene set.

6. p.11: "... quantified the time from the first lateral contact with the spindle (tilted configuration relative to spindle axis) until biorientation (parallel arrangement relative to spindle axis)": I do not understand how the authors can score reliably the time of the first lateral contact with the spindle. The inter sister KT axis can be tilted relative to the spindle axis also before/without first lateral contact with spindle MTs, and the quality of the MT signals (mostly rather diffuse green except from some more prominent MT bundles) does seem to be far from sufficiently resolved to clearly detect laterally contacting MTs in Fig 5C.

7. Based on the evidence presented in Fig. 7F,G and Movies S19-S21, the authors conclude that "Polo-mediated phosphorylation of Spindly on Ser499 decreases its ability .... to associate with kinetochores at levels required for efficient chromosome congression." (p.14)
The presented evidence seems robust enough for a conclusion concerning the efficiency of chromosome congression; this process seems to be delayed in S2 cells expressing Spindly[S499D]-EGFP instead of Spindly[WT]-EGFP. However, I cannot recognize convincing evidence for the conclusion concerning Spindly association with the KT. Signals of Spindly-EGFP (all versions: wt, S499A and S499D) seem to be so weak that they are often not above background. Moreover, in case of Spindly[499D]-EGFP the normalized MFI at KTs is around 1 at t = 0, as also in case of Spindly[WT]-EGFP (Fig. 7G). (Also in Fig. 8A, the Spindly[499D]-EGFP signals on the unaligned KT does not seem to be any weaker than on case of Spindly[WT]-EGFP).
I might have misunderstood something, but to me this conclusion concerning effects of Polo phosphorylation on Spindly<->KT association seems wrong.

8. In a most crucial comparison (cells expressing Spindly[S499D] without or with ZW10-RNAi) about 4fold less cells were analyzed for the ZW10-RNAi condition (Fig. 8D) and no error bars are present in Fig. 9E. In Fig. 8D, data points in case of the without ZW10-RNAi condition vary over a 4fold range (and also in the other conditions wt and S499A the range of variation is rather large). Why is there far less variation among the far fewer data points in the ZW10-RNAi condition? By chance? Additional data points for this condition should be added. In case of Fig. 9E, data with error bars needs to be presented for the ZW10-RNAi condition.

Minor problems:
- Fig. 7C and 7D: Displaying the schematic illustration of Spindly[1-510] already in panel C is confusing. It would be better to display this in panel D.

- p.7: "... constitutive Polo activation renders KT-MT attachments persistently unstable ...": given the fact that eventually sister kt separation reaches fairly normal values (Fig. 1F) "persistently" seems to strong.

- p.15: "... we treated Spindly transgenes with calcium ..." > the cells expressing different Spindly-EGFP versions were pretreated with Ca^{2+} before fixation ...

- p.16: "... control of the RZZ-Spindly-Dynein module at KTs (Fig.10)." > Fig. 9F

- p. 17: "(Moura and Conde, 2019)" > add into the main reference list

- legend Fig. 4: "... KTs that are non-oriented relative to metaphase axis ..." > sister KTs that are not oriented along the spindle axis ...

- legend Fig. 7G: "... highlighted in D ..." > highlighted in F

- "tips de balance" -> the

- "eclode" > eclose

- "late ecloding flies" -> eclosing

- "Tub-RFP (Mathieu et al, 2011)": please specify the tubulin isoform that was expressed and provide a valid reference.

- "finely-tuned" > fine-tuned

- "occurs at a less extent" > occurs at a lesser extent

- "CuCO4" > CuSO4

Referee #3:

This manuscript by Barbosa and colleagues identifies Polo as a regulator of the interaction of Spindly with the RZZ complex. The study moves from the description of the severe chromosome alignment defects resulting from ectopic expression a constitutively active form of Polo, T182D. The authors carried out a suppressor RNAi screen on a group of candidate target genes, identified various suppressors, and decided to focus on the product of one of them, Rod, a subunit of the RZZ complex. Eventually, the authors focused on Spindly, a binding partner of the RZZ complex, as the crucial focus of Polo regulation. They identify Spindly as a substrate of Polo and propose that Polo represses a conformational change in Spindly required to interact with RZZ. The model is that the constitutively active mutant of Polo locks Spindly in a conformation that cannot interact with the RZZ, leaving the RZZ without an essential partner for its Dynein-mediated removal from kinetochores. The authors refer to previous studies from the Desai group demonstrating that RZZ suppresses end-on kinetochore-microtubule attachment, and that its timely removal is required for end-on conversion of the attachment. Based on these previous observations, the authors argue that failure to remove RZZ from kinetochores is the source of kinetochore-microtubule attachment errors observed in presence of Polo(T182D).

This study clearly represents a major tour de force, especially, but not exclusively, on the imaging side. The authors must be praised for this. On the other hand, I regret having to write that I feel rather unconvinced of the study's main conclusions. In general, I did not understand when and how, in the authors' view, Polo is inactivated to promote the interaction of Spindly with the RZZ. I understand that the authors believe that this is a relatively late event that coincides with the conversion from lateral to end-on attachment. However, I don't see, here or elsewhere, evidence that supports this model. Spindly is clearly visible at kinetochores when microtubules are depolymerized, which is hardly reconcilable with a late recruitment. Under these conditions (colchicine, nocodazole), the authors' hypothesis predicts that Spindly should not be found on kinetochores, but in fact it is there, and apparently in large amounts. Collectively, my enthusiasm is limited because I don't see sufficient evidence in support of the authors' model. I find the model rather convoluted and I am afraid that there likely are alternative explanations for the effects of the Polo(T182D) mutant that are not considered here.

Major point, in no specific order

-The phosphorylation of Spindly at S499 is inferred from an in vitro kinase assay. The existence and regulation of this site in vivo is never tested. The target site is part of a conserved region of Spindly,

but S499 itself is not conserved, implying that the regulation discussed in this manuscript is species-specific.

-The results of the suppressor RNAi screen are presented in Table S3 as a collection of GO terms. None of the other quite numerous suppressors is ever mentioned. This leaves a feeling of incompleteness, and gives the impression that the authors wanted to focus on their preferred hypothesis without spending sufficient time analyzing other hypotheses. This impression is compounded by the problem that the level of suppression observed after Rod RNAi is significant, but largely incomplete (Figure 3). Also, what exactly becomes suppressed when Rod expression is ablated is rather hard to grasp from the presented data (e.g. Figure 3E). At least in part, the effects observed by the authors may result from checkpoint inactivation upon depletion of Rod. The discussion of this possibility is rather anecdotal.

-The analysis of RZZ-Spindly interactions in vitro could be significantly improved. This analysis, in its present form, does not meet the standard for in vitro biochemistry that the field has achieved with other model systems. RZZ and Spindly interact very robustly, and there is no evidence, from previous analyses with C. elegans and human proteins, that phosphorylation is required for their physical interaction. This elevates the burden of the proof in this case.

-Our understanding of the T182D mutant is incomplete. The mutant is presented as "constitutively active", but the level of activity in comparison to the properly phosphorylated Polo kinase is never tested. Clearly no dephophorylation of T182D is possible, but the observed phenotype might also result from incomplete kinase activation with this mutant, a possibility that the authors do not discuss or analyze.

-Figure 6G shows that in presence of Polo T182D, Spindly accumulates at kinetochores and Dynein is required to remove it from there. It is unclear how this can be fitted into the authors' model, as there should be no Spindly on kinetochores.

-It is unclear why the depletion of p50/dynactin (page 11) shows a relatively mild phenotype, if the result of this depletion is ultimately essentially the same caused by expression of Polo(T182D), the retention of RZZ on kinetochores. In the authors' model, this depletion should have effects comparable to those observed when expressing Polo-T182D. Once again, these observations raise the impression that the authors try to support their preferred hypothesis and do not put sufficient weight on evidence that seems to argue against it.

-In general, it is unclear to what extent the persistence of Rod and RZZ on kinetochores in cells expressing Polo(T182D) is a mere consequence of prolonged checkpoint activation or rather of defective release of RZZ.

Minor points
-Please remember to add page numbers to manuscripts! I have considered the front page as page 1 and numbered the rest accordingly.
-In Figure 2E-F, a control with the UAS Polo WT is missing
-Page 3, Introduction: "This Aurora B-independent...": what is the evidence that this regulation is Aurora B independent?
-Page 6, Results: "...which occurs at a less extent...". Replace "at" with "to"

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Response letter

Dear editor,

We would like to thank you for overseeing the review process and the reviewers for their constructive comments on our original work. In this revised version of the manuscript we have carried out additional experiments to address the main points of criticism raised by the reviewers and to strengthen our major conclusions.

Point-by-point response to the editor and reviewers:

Given the support in principle from two of the three referees, we would like to give you the opportunity to respond to the criticisms of the three reviewers through a revised version of this manuscript. While we not all points raised in the reports will in our view necessarily require experimental addressing, we however feel that there are several key concerns that need to be strengthened or clarified through decisive additional data. This particularly includes addition of further controls and statistics (e.g. ref 1 points 2, 3, ref 2 points 4, 8, ref 3 pt 4 re. Polo-T182D), clarification of referee 2's various concerns regarding the fly genetic analyses, and presenting the results of the RNAi suppressor screen data in a more complete and insightful manner (refs 2 & 3). Furthermore, it will be essential to validate that Spindly-S499 phosphorylation occurs in vivo/in cells and in a Polo-dependent fashion, possibly via mass spectrometry if phospho-specific antibodies should not be available (ref 3). We note that various other specific or conceptual concerns may well be clarified through textual changes/discussions and/or inclusion of alternative/additional images/data.

We thank the reviewers for the critical and constructive evaluation of the manuscript. We are pleased that the reviewers recognise the significant interest and potential importance of the work. We found their comments and suggestions very useful and performed additional experiments and analysis in order to:
- provide further controls and statistical analysis,
- clarify concerns regarding the fly genetics data,
- validate in a cellular context the phosphorylation of Spindly S499 by Polo,
- present the results from the genetic screen with gene names and respective potency.
You will also notice that we have slightly modified the title of the manuscript to conform to character limitations.

Referee #1:

Plk1 plays an important role in cell division, but the function of PLK1 at mitotic kinetochores is not well defined. Conflicting evidence has suggested roles both in the stabilization and destabilization of mitotic kinetochore-microtubule interactions. Here the authors show that PLK1 functions in early prometaphase to destabilize kinetochore-microtubule interactions. As the cell cycle progresses, the dynamic removal of the kinase from metaphase kinetochores allows the stabilization of kinetochore-microtubule interactions and eventual chromosome segregation. Utilizing a constitutively active PLK1 mutant expressed in Drosophila neuroblasts, the authors find that cells overexpressing PLK1 T182D struggle to properly align chromosomes at the metaphase plate and frequently exhibit kinetochores laterally attached to microtubules. These mis-attached kinetochores exhibit increased levels of the

RZZ complex components, proteins involved prevention of stable end-on kinetochore-microtubule attachments. Importantly, these phenotypes can be rescued by the depletion of the RZZ component ROD via RNAi. Once chromosomes are properly bi-oriented in metaphase, the RZZ complex is removed from kinetochores via the Spindly/Dynactin/Dynein complex. The authors show that PLK1 mediates this removal of the RZZ complex via the phosphorylation of Spindly at S499. Phosphorylation of Spindly at S499 decreases the affinity of the RZZ complex to the protein, inhibiting its removal. Together this work identifies a novel role for PLK1 in the regulation of the RZZ complex and helps shed light on to the discrepancies on PLK1 function in the regulation of kinetochore-microtubule attachments.

Overall, I am enthusiastic about this manuscript. The separation of the prometaphase and metaphase functions of PLK1 in kinetochore-microtubule attachments will be of great interest to many in the field. The ability to identify a specific target for this regulation with a clear biochemical consequence is also impressive. However, there are several aspects of this manuscript that need to be addressed or clarified. I would recommend this paper for publication in EMBO once these comments are addressed.

Major Concerns:

1. In cells treated with the PLK1 inhibitor BI2536, chromosomes fail to align, often appearing unattached from microtubules. In the proposed model, loss of PLK1 in early prometaphase should increase the appearance of end-on kinetochore-microtubule attachments. How do the authors explain these discrepancies when PLK1 is constitutively expressed/active versus relative to when its catalytic activity is disrupted?

The observation that the expression of constitutively active Polo delays the formation of stable end-on attachments is actually in perfect agreement with our model and with previous work from our group (Moutinho-Santos et al 2012 - PMID:22389397) where we have shown that depletion or inhibition of Polo in S2 cells results in the hyperstabilization of KT-MT interactions and in the formation of syntelic attachments. This is adressed in the discussion section of the manuscript.

2. The authors demonstrate that knockdown of ROD partially rescues the prometaphase defects observed in PLK1 T182D cells. As their model is that this occurs through loss of the RZZ complex, it would be helpful to show that knockdown of another subunit (i.e., Zw10) is also sufficient to rescue these phenotypes.

Following the reviewer suggestion, we depleted Zw10 from S2 cells expressing PoloT182D. As observed for Rod-depleted cells, knockdown of Zw10 equally rescues the congression phenotype caused by constitutively active Polo, thus supporting it occurs through loss of the RZZ complex. We have included these new data in Figure S4 of the revised manuscript.

3. PLK1 has been shown to modulate AURKB localization and activity at kinetochores in mitosis. Because AURKB is a core regulator of kinetochore-microtubule attachments, it would be helpful for the authors to show that AURKB is not aberrantly regulated in PLK1 T182D cells (testing AURKB localization or the phosphorylation of a downstream substrate).

As suggested, we assessed the phosphorylation *status* of Spc105 Ser35 (Ser60 in human KNL1), a well described substrate of Aurora B (Welburn et al 2010 - PMID:20471944; Bajaj et al 2018 - PMID:30100357). The result is presented in Figure S1C,D of the revised

manuscript and demonstrates that Aurora B activity is not significantly altered by the expression of PoloT182D.

4. In figure 8, ZW10 does not appear reduced at kinetochores in the Spindly S499A mutant. According to the model, loss of Spindly phosphorylation should increase its affinity for the RZZ complex, thereby stripping the RZZ complex prematurely from kinetochores. The authors highlight this point in figure 9 in which more spindle bound ZW10 is indicated in S499A cells. However, this spindle localization of ZW10 is not visible in the same cells in figure 8.

We have replaced the original Fig.8A and included in the revised version of the manuscript a new Fig.9A that better represents the streaming of Zw10 in cells expressing the Spindly S499A transgene**.**

Minor Concerns

1. Typo. The authors list figure S2A-B after the statement "Accordingly we were able to follow dynein-dependent stripping of ROD-GFP". However, this figure does not depict what is stated.

Figure S2A-B of the original manuscript (now Fig.S3A-B in the revised version) depicts the streaming of ROD-GFP in PoloWT neuroblasts and its impairment when PoloT182D is expressed or when Dynein KT function is disrupted.

2. Figure 1G. MAD2 GFP signal is hard to see in these cells. The authors should include a figure with MAD2 localization via antibody to make this clearer as this is an important point.

We tried to follow the reviewer suggestion, but unfortunately all Mad2 antibodies that we tested failed to work for immunofluorescence analysis in neuroblasts. As an equally valid alternative, we have used an antibody against Mad1 (Conde et al 2013 - PMID:23685359), which confirms that more KTs are accumulate Mad1 when PoloT182D is expressed. This new result is present in Figure S1A,B of the revised manuscript.

Referee #2:

Barbosa et al. characterize the role of Polo kinase in the control of the stability of KT attachments to spindle MTs during progression through mitosis in Drosophila (studying neuroblasts in larval brains and S2 cells in culture). The study includes a screen, in which candidate genes were knocked down during Drosophila development (using ey-GAL4 and UAS-RNAi genes) and the modification of effects of a hyper-active Polo kinase variant (Polo[S182D]) by the knockdown was scored. Thereby, knock-down of rod, a component of the RZZ complex, was observed to suppress phenotypes resulting from hyperactive Polo kinase. The RZZ complex is known to be present at kinetochores during prometaphase where it recruits Spindly which in turn recruits Dynein and thereby promotes shedding of RZZ-Spindly-Dynein particles away from the kinetochore by Dynein-mediated transport along kinetochore fibers. The authors have completed a rather comprehensive set of experiments in order to elucidate the functional interactions between Polo kinase, RZZ complex and Spindly. Based on their findings, they present an interesting novel model. Accordingly, Polo phosphorylates Spindly early during prometaphase (on S499) (indicated by in vitro kinase

experiments). This inhibits recruitment of Spindly to the kinetochore via binding to the RZZ component Zwilch (indicated by pull down experiments and microscopic quantification of kinetochore signals in various genotypes, including Spindly S499A and S499D expressing cells). Without Spindly, RZZ shedding is prevented and hence RZZ levels at kinetochores remain high (microscopic quantification of kinetochore signals in various genotypes, also shown previously in other studies). At the kinetochore, RZZ appears to inhibit the conversion of lateral to end-on attachments of the kinetochore to spindle MTs (suggested by earlier publications and further confirmed by microscopic characterization of kinetochore and microtubules in various genotypes, including after Ca2+ pretreatments that destabilize non-kinetochore spindle fibers before fixation). After a decrease in Polo activity at the kinetochore (indicated by microscopic quantification), Spindly is proposed to become transformed into the non-phosphorylated state, allowing stable chromosome bi-orientation via end-on attachments of sister kinetochores to spindle microtubules. If Spindly cannot be phosphorylated (S499A), stable attachments appear to develop too early in prometaphase, resulting in frequent merotelic attachments. If Spindly cannot be dephosphorylated (as suggested by the S499D charge mimick mutation and hyperactive Polo[S182T] kinase, stable attachments arise more slowly, delaying the metaphase to anaphase transition.
The manuscript would clearly make a significant novel contribution to our understanding of the mechanisms responsible for a correct integration of chromosomes into the mitotic spindle, if the problems detailed below can be resolved. While many analyses were well done technically, some of the most crucial experiments are not (yet) convincing. Overall, the manuscript is clearly written and includes an intelligent discussion that integrates the proposed model appropriately into the wider context.

Specific major concerns:
1. The effects of insc-GAL driven expression of UAS-polo[wt] and UAS-polo[T182D] in larval brain neuroblasts on progression through mitosis are compared (Fig. 1C-H) and different effects were observed. According to the presentation of the authors, the resulting phenotype difference reflects the fact that Polo[T182D] is constitutively active. However, there is a technical problem that questions the validity of the author's interpretation. The two UAS transgenes were made with different vectors (pP{UASp} and pP{Express-UAS}, respectively) with different cis-regulatory elements and they are not inserted at the same chromosomal site. Based on the known properties of these expression vectors, it is very likely that the resulting UAS-polo[wt] expression is considerably lower than the expression of UAS-polo[T182D]. Therefore, it remains a possibility that the different phenotypic consequences reflect simply a difference in expression level and not an effect of the T182D mutation. Similarly all later interpretations from comparison of phenotypic effects after UAS-polo[wt] and UAS-polo[T182D] in larval brain neuroblasts (data in Figs 2, 4 and 5) are compromised by this technical problem.
Ideally the authors should perform the comparison between Polo[wt] and Polo[T182D]by using identical constructs (except for the mutation) inserted into the same chromosomal landing site, i.e., in the current standard manner. It might be argued that this too much of an effort since a large fraction of experiments would have to be repeated for insufficient reasons since the results of analogous experiments with S2 cells that are not affected by this particular technical problem largely concur with the author's interpretation of the neuroblast experiments.
Moreover, Fig S1F presents data of an IB analysis, suggesting that comparable levels of Polo[WT] and Polo[T182D] results after insc-GAL4 driven expression of the different UAS transgenes. However, the presented immunoblot image raises concerns about the technical quality of this important analysis. The anti-Tub bands used for normalization are uneven.

While the corresponding figure legend might be read as suggesting that the quantification is based on statistical analysis of independent experiments, no n (number of independent experiments) is given. Perhaps only a single experiment has been done. Please present a clear statistical analysis from multiple experiments.
The differences between the transgenes (UAS-polo[wt] and UAS-polo[T182D]) should be described clearly including comments on potential problems with interpretations.

The reviewer is correct: the expression of UAS-polo[WT] in larval brain neuroblasts is considerably lower than the expression of UAS-polo[T182D]. We have reassessed Polo levels by immunoblotting, which we now present as Figure S2F,G in the revised version of the manuscript. The difference in protein levels are now evident and following the reviewer suggestion we now provide the quantifications from 3 independent experiments (new Figure S2G).
It is our opinion however, that this difference in Polo expression does not challenge the major conclusions of our study. Firstly, the expression of PoloT182D was used as a mean to aberrantly increase Polo activity and evaluate the impact of this deregulation on KT-MT attachments and on chromosome congression, while at the same time enabling us to screen for suppressers of its phenotype as potential new Polo targets. This strategy allowed us to identify Spindly S499 as new Polo substrate and subsequent biochemical and cellular characterization of this phosphorylation led us to a new regulatory model, whose details were uncovered without the interference of differential Polo expression.
Secondly, despite higher expression of UAS-polo[T182D], this is unlikely to be the major underlying cause of the different phenotypes observed in neuroblasts. As acknowledged by the reviewer, equivalent experiments in S2 with comparable levels of PoloWT and PoloT182D (see Fig.S6A) demonstrate an obvious accumulation of congression and attachment defects specifically when the later transgene is expressed (Fig.2C, D). Even though this largely concurs with our interpretations of the neuroblasts data, the differences between the transgenes UAS-polo[WT] and UAS-polo[T182D] are now clearly described and discussed in the revised version of the manuscript.

2. p.7: "Time course analysis shows that these cells undergo a highly asynchronous chromatid migration during anaphase as opposed to the synchrony observed in PoloWT neuroblasts (Fig.2E, G)."
Fig. 2E,G present comparisons between Polo[T182D] and "no transgene" rather than between Polo[T182D] and Polo[WT] as suggested in the text. A careful comparison of Polo[T182D] and Polo[WT] would actually be of interest, as Polo[WT] overexpression in S2 cells has a similar yet milder effect than Polo[T182D] expression concerning the frequency of erroneously attached chromosome in the MG132 (Fig. 2B), while unexpectedly in neuroblasts these two conditions appear to have opposite effects concerning duration NEBD-anaphase onset (Fig. 1D,E); perhaps linked to expression level differences (see 1.).

We have now modified Fig.2 to show in panels E and G that the expression of PoloWT has a very minor effect on chromosome segregation while expression of PoloT182D has a significant impact on the accuracy of chromosome segregation and consequently on genome stability.

3. Fig. 2C,D: calcium-stable KT-MT attachments in MG132 arrested S2 metaphase cells were studied, and more frequent lateral attachment (less end-on) of sister KTs after PoloT182D-mRFP compared to PoloWT-mRFP was observed (25% compared to 10% of the metaphase cells, n {greater than or equal to} 58 cells for each condition).

As published recently (Strunov et al. 2018), end-on attachments with some MTs extending laterally beyond the KT appear to be rather common in S2 cells, and the extent of extending MTs is variable. Therefore, how can KT attachments be classified in a binary manner as either end-on or lateral given these gradual differences? Our own research experience would also question the feasibility of such a binary classification. Moreover, can the authors rule out artefactual effects resulting from slight experimental differences of the applied Ca2+ treatment before fixation. How many independent experiments were analyzed? Are all the analyzed cells in Fig. 2D from a single experiment?
The same assay is also used in the experiments presented in Fig. 9D,E and hence the same concerns apply.

These experiments were repeated at least two times in order to account for experimental differences. The quantifications shown in Fig.2C, D and in the new Fig.10D, E, correspond to KTs from at least 58 and 44 cells, respectively.
To evaluate the attachment configuration, cells where observed at multiple Z planes of 280nm and each KT was evaluated throughout the Z-stack. Given that the Calcium treatment removes most of the microtubules that are not attached to KTs we were only able to accurately visualize KT fibers that were attached to KTs in an “end on” or lateral configuration.

4. Fig. 3D-F: In principle, the observed milder mitotic defects resulting from addition of UAS-rod[RNAi] into insc>polo[T182D] might result from a Gal4 protein titration effect by additional UAS[GAL4] binding sites rather than from Rod depletion; as some Gal4 proteins is recruited by UAS-rod[RNAi], there might be less for driving Polo[T182D] expression. The authors try to rule out this alternative interpretation by the data presented in Fig S1F. As indicated above (see 1.), their analysis does not seem to be of sufficient technical quality to allow firm conclusions. Beyond such a quantitative analysis of expression levels by IB, it would be most convincing if an irrelevant UAS-RNAi gene (for example GFP) was included as a control (comparison insc>UAS-polo[T182D] UAS-GFP[RNAi] with insc>UAS-polo[T182D] UAS-rod[RNAi]).
Note that the authors actually show (Fig S1E) that Rod[RNAi] led to ZW10 depletion at KTs that was greater than when Rod[RNAi] was combined with Polo[T182D] expression. In contrast to their statement (p.8: "an equivalent reduction") a difference is clearly apparent, and this difference might reflect Gal4 titration.

To examine whether the suppression of PoloT182D phenotype in neuroblasts is rescued by RNAi-mediated downregulation of Rod simply due to titration of the GAL4 transcription factor, we have conducted an additional control in which UASlacZ is co-expressed together with UASPolo[T182D] (New Fig.3D). The results show that the presence of an additional UAS site driving lacZ expression does not rescue PoloT182D phenotype, hence arguing against Gal4 titration as the underlying cause for the suppression phenotype that is observed when UAS-Rod[RNAi] is co-expressed.
As for Zw10 levels at unattached KTs in neuroblasts depleted of ROD vs neuroblasts depleted of ROD in a PoloT182D background the mean values only differ in 10%, which in our view is unlikely to reflect biological significance.

5. The study would certainly gain interest if gene names and potency of the 24 suppressor hits of ey>polo[T182D] was revealed. Table S3 seems useless and perhaps misleading, as the selected and analyzed 222 candidate gene are already enriched for GO terms linked to mitotic functions and the p-values given in Table S3 seem to represent analyses of enrichment

compared to the entire Drosophila gene set rather than comparison of enrichment compared to just the 222 candidate gene set.

Following the reviewer suggestion, we have included the identity and the potency of the suppressor genes in the revised version of the manuscript (new Table S3).

6. p.11: "... quantified the time from the first lateral contact with the spindle (tilted configuration relative to spindle axis) until biorientation (parallel arrangement relative to spindle axis)": I do not understand how the authors can score reliably the time of the first lateral contact with the spindle. The inter sister KT axis can be tilted relative to the spindle axis also before/without first lateral contact with spindle MTs, and the quality of the MT signals (mostly rather diffuse green except from some more prominent MT bundles) does seem to be far from sufficiently resolved to clearly detect laterally contacting MTs in Fig 5C.

To make sure we could score the first lateral KT-MT contact by live-microscopy, image acquisition of neuroblasts was initiated prior to nuclear envelope breakdown. Each cell was recorded at multiple time points and with z plane stacks of 500 nm. This allowed us to clearly visualize the overlap of KT and tubulin signals and confidently determine when a lateral interaction was first established (t=0s).

7. Based on the evidence presented in Fig. 7F,G and Movies S19-S21, the authors conclude that "Polo-mediated phosphorylation of Spindly on Ser499 decreases its ability .... to associate with kinetochores at levels required for efficient chromosome congression." (p.14) The presented evidence seems robust enough for a conclusion concerning the efficiency of chromosome congression; this process seems to be delayed in S2 cells expressing Spindly[S499D]-EGFP instead of Spindly[WT]-EGFP. However, I cannot recognize convincing evidence for the conclusion concerning Spindly association with the KT. Signals of Spindly-EGFP (all versions: wt, S499A and S499D) seem to be so weak that they are often not above background. Moreover, in case of Spindly[499D]-EGFP the normalized MFI at KTs is around 1 at t = 0, as also in case of Spindly[WT]-EGFP (Fig. 7G). (Also in Fig. 8A, the Spindly[499D]-EGFP signals on the unaligned KT does not seem to be any weaker than on case of Spindly[WT]-EGFP).
I might have misunderstood something, but to me this conclusion concerning effects of Polo phosphorylation on Spindly<->KT association seems wrong.

We do agree with the reviewer that the differences in the levels of Spindly[WT]-EGFP and Spindly[S499D]-EGFP at late congressing KTs are not striking. However, the main point from the results presented in Fig.7F, G (now Fig. 8D, E) was to demonstrate that Spindly[S499D]-EGFP requires longer time to accumulate at KTs (which does eventually occur and to similar levels as Spindly[WT]-EGFP). The graph from Fig. 7G (new Fig 8E) quantitatively represents the slower KT recruitment of Spindly[S499D]-EGFP throughout chromosome congression. t=0 is a reference point and represents the first attempt that a KT pair moving towards the pole shifts to an anti-poleward direction and starts to congress to the metaphase plate. Although we agree that the overall signal intensities of Spindly-EGFP are rather low, careful quantification does allow us to detect important differences between Spindly[WT]-EGFP and Spindly[S499D]-EGFP. Both Fig. 6D (PoloWT expressing cell) and Fig. 7G (new Fig. 8E) show that Spindly[WT]-EGFP accumulates around 120seconds after the directional shift (t=0), which coincides with stable congression of the KT to the cell equator. On the other hand, KT accumulation of either Spindly[WT]-EGFP in PoloT182D expressing cells (Fig.6D) or Spindly[S499D]-EGFP (new Fig. 8D) is significantly delayed

and this correlates with an increase in the time required to initiate KT congression. At t=0, both SpindlyEGFP transgenes localize at KTs to similar amounts (similar MFI). However, Spindly[WT]-EGFP is able to stably accumulate at KTs shortly after (120 sec), while Spindly[S499D]-EGFP takes longer to accumulate (400 sec). Therefore, it is the delay in KT accumulation that underlies the delay in chromosome congression, which eventually occurs when Spindly[S499D]-EGFP reaches levels that are (probably) sufficiently elevated to compensate its reduced affinity towards the RZZ. We modified the text in the current version of the manuscript to better describe the effect that S499 phosphorylation has on Spindly association with KTs.

8. In a most crucial comparison (cells expressing Spindly[S499D] without or with ZW10-RNAi) about 4fold less cells were analyzed for the ZW10-RNAi condition (Fig. 8D) and no error bars are present in Fig. 9E. In Fig. 8D, data points in case of the without ZW10-RNAi condition vary over a 4fold range (and also in the other conditions wt and S499A the range of variation is rather large). Why is there far less variation among the far fewer data points in the ZW10-RNAi condition? By chance? Additional data points for this condition should be added. In case of Fig. 9E, data with error bars needs to be presented for the ZW10-RNAi condition.

As suggested, we increased the number of Spindly[S449D]-EGFP cells depleted of Zw10 in the analysis of the prometaphase duration. The results are now presented in Figure 9C, D of the revised manuscript and are consistent with our previous analysis. Interestingly, the range of variation in the Zw10 RNAi remains lower than in all the other conditions. The underlying reason for this effect remains however unknown.
As for Fig. 9E (now Fig 10E in the revised manuscript), we have included more data corresponding to an additional independent experiment and error bars are now presented.

Minor problems:
- Fig. 7C and 7D: Displaying the schematic illustration of Spindly[1-510] already in panel C is confusing. It would be better to display this in panel D.

In the revised version of the manuscript, the schematic illustration of Spindly[FL] and Spindly[1-510] are displayed separately in Fig. 8A and Fig. 8B.

- p.7: "... constitutive Polo activation renders KT-MT attachments persistently unstable ...": given the fact that eventually sister kt separation reaches fairly normal values (Fig. 1F) "persistently" seems to strong.

We have removed “persistently” from the text.

- p.15: "... we treated Spindly transgenes with calcium ..." > the cells expressing different Spindly-EGFP versions were pretreated with Ca^{2+} before fixation ...

We have modified the text accordingly

- p.16: "... control of the RZZ-Spindly-Dynein module at KTs (Fig.10)." > Fig. 9F

We thank the reviewer for noticing this error. We have corrected it in the revised version of the manuscript.

- p. 17: "(Moura and Conde, 2019)" > add into the main reference list

The reference has been added to the main reference list.

- legend Fig. 4: "... KTs that are non-oriented relative to metaphase axis ..." > sister KTs that are not oriented along the spindle axis ...

We have modified the text accordingly.

- legend Fig. 7G: "... highlighted in D ..." > highlighted in F

We thank the reviewer for noticing this error. We have corrected it in the revised version of the manuscript.

- "tips de balance" -> the

We thank the reviewer for pointing this error, which we have now corrected in the revised manuscript.

- "eclode" > eclose

We have modified the text accordingly

- "late ecloding flies" -> eclosing

We have modified the text accordingly

- "Tub-RFP (Mathieu et al, 2011)": please specify the tubulin isoform that was expressed and provide a valid reference.

α-Tubulin-RFP was expressed. This has been specified in the revised version of the manuscript.

- "finely-tuned" > fine-tuned

We have modified the text accordingly

- "occurs at a less extent" > occurs at a lesser extent

We have modified the text accordingly

- "CuCO4" > CuSO4

We have modified the text accordingly

Referee #3:

This manuscript by Barbosa and colleagues identifies Polo as a regulator of the interaction

of Spindly with the RZZ complex. The study moves from the description of the severe chromosome alignment defects resulting from ectopic expression a constitutively active form of Polo, T182D. The authors carried out a suppressor RNAi screen on a group of candidate target genes, identified various suppressors, and decided to focus on the product of one of them, Rod, a subunit of the RZZ complex. Eventually, the authors focused on Spindly, a binding partner of the RZZ complex, as the crucial focus of Polo regulation. They identify Spindly as a substrate of Polo and propose that Polo represses a conformational change in Spindly required to interact with RZZ. The model is that the constitutively active mutant of Polo locks Spindly in a conformation that cannot interact with the RZZ, leaving the RZZ without an essential partner for its Dynein-mediated removal from kinetochores. The authors refer to previous studies from the Desai group demonstrating that RZZ suppresses end-on kinetochore-microtubule attachment, and that its timely removal is required for end-on conversion of the attachment. Based on these previous observations, the authors argue that failure to remove RZZ from kinetochores is the source of kinetochore-microtubule attachment errors observed in presence of Polo(T182D).

This study clearly represents a major tour de force, especially, but not exclusively, on the imaging side. The authors must be praised for this. On the other hand, I regret having to write that I feel rather unconvinced of the study's main conclusions. In general, I did not understand when and how, in the authors' view, Polo is inactivated to promote the interaction of Spindly with the RZZ. I understand that the authors believe that this is a relatively late event that coincides with the conversion from lateral to end-on attachment. However, I don't see, here or elsewhere, evidence that supports this model.

Understanding how and when Polo is inactivated is obviously an important question, which by itself certainty merits to be described in an additional paper. We have discussed in the manuscript possible mechanisms that might contribute to decrease Polo activity and Spindly S499 phosphorylation *status* as chromosomes become bioriented (please see Discussion). However, directly and mechanistically assessing these hypotheses in the present article would represent a tremendous undertaking and diverge from our main findings: Polo-mediated phosphorylation of Spindly precludes premature Dynein-mediated removal of the RZZ complex when initial lateral contacts are established and consequently prevents precocious stabilization of erroneous attachments.
Nevertheless, we have now monitored phosphorylation of Spindly S499 by Polo throughout mitosis using a phospho-specific antibody. The results are presented in Figure 7 of the revised manuscript and clearly demonstrate that phosphorylation of Spindly S499 is elevated at unaligned KTs and virtually undetected at aligned KTs, hence further supporting the regulatory model that we propose.

Spindly is clearly visible at kinetochores when microtubules are depolymerized, which is hardly reconcilable with a late recruitment. Under these conditions (colchicine, nocodazole), the authors' hypothesis predicts that Spindly should not be found on kinetochores, but in fact it is there, and apparently in large amounts.

Spindly is visible at KTs when microtubules are depolymerized because, as we have mentioned in the manuscript, it is recruited to unattached KTs through interactions with the RZZ complex that remain unaffected by Polo activity. It is important to highlight here that our model does not predict an impairment in the recruitment of Spindly to KTs when Polo activity is elevated, but rather an increase of its stripping along microtubules mediated by Dynein (please see Fig.6 and Fig.8). Therefore, because Polo activity does not prevent

Spindly recruitment but instead promotes its stripping, the colchicine results actually support our model.

Collectively, my enthusiasm is limited because I don't see sufficient evidence in support of the authors' model. I find the model rather convoluted and I am afraid that there likely are alternative explanations for the effects of the Polo(T182D) mutant that are not considered here.

Major point, in no specific order

-The phosphorylation of Spindly at S499 is inferred from an in vitro kinase assay. The existence and regulation of this site in vivo is never tested. The target site is part of a conserved region of Spindly, but S499 itself is not conserved, implying that the regulation discussed in this manuscript is species-specific.

We have raised an antibody that specifically recognizes Spindly phosphorylation on S499. Our analysis clearly demonstrates that phosphorylation of this residue by Polo also occurs in a cellular context. The results show that depletion or inhibition of Polo abolishes the KT staining corresponding to S499 phosphorylation, as does the depletion of Spindly or expression of Spindly[S499A]. Moreover, we could observe that S499 phosphorylation is predominantly elevated on unaligned KTs and virtually absent from KTs of aligned chromosomes. This phosphorylation pattern is in perfect agreement with our model: Polo-mediated phosphorylation of Spindly S499 following mitotic entry directs the RZZ to minimize premature stabilization of erroneous attachments, while a decrease of Spindly S499 phosphorylation during later stages of mitosis allows the formation of stable amphitelic attachments. This new set of results is presented in Figure 7 and Figure S7 of the revised manuscript.
Although the phosphorylation site identified in *Drosophila* Spindly does not seem to be conserved in vertebrates, additional residues conforming to Polo/Plk1 recognition motif are present within the same domain, hinting that an analogous regulatory mechanism may take place in these organisms.

-The results of the suppressor RNAi screen are presented in Table S3 as a collection of GO terms. None of the other quite numerous suppressors is ever mentioned. This leaves a feeling of incompleteness, and gives the impression that the authors wanted to focus on their preferred hypothesis without spending sufficient time analyzing other hypotheses. This impression is compounded by the problem that the level of suppression observed after Rod RNAi is significant, but largely incomplete (Figure 3). Also, what exactly becomes suppressed when Rod expression is ablated is rather hard to grasp from the presented data (e.g. Figure 3E). At least in part, the effects observed by the authors may result from checkpoint inactivation upon depletion of Rod. The discussion of this possibility is rather anecdotal.

We have now included the names of all the suppressor genes in the new Table S3. We selected Rod for further studies because we aimed to uncover how Polo activity destabilizes KT-MT attachments. Given that previous studies had shown that Rod interacts with Ndc80 to prevent the formation of stable end-on attachments (Cheerambathur et al, 2013), we focused on Rod as this was the suppressor hit that could more plausibly act as an attachment destabilizer.

Concerning the issue of what becomes suppressed when Rod expression is ablated, our data strongly points towards a suppression of KT-MT instability (Fig. 3; Fig.10 D,E and Fig.S4) and aneuploidy (Fig.3F). As shown by our data, depletion of Rod partially restores the mitotic fidelity, which most likely underlies the observed rescue in fly viability (Fig. 3). The rescue is unlikely to result from checkpoint inactivation, since depletion of Mad2 failed to rescue mitotic fidelity in a PoloT182D background (Fig. S4). This is now discussed in the revised version of the manuscript.

-The analysis of RZZ-Spindly interactions in vitro could be significantly improved. This analysis, in its present form, does not meet the standard for in vitro biochemistry that the field has achieved with other model systems. RZZ and Spindly interact very robustly, and there is no evidence, from previous analyses with C. elegans and human proteins, that phosphorylation is required for their physical interaction. This elevates the burden of the proof in this case.

We agree with the reviewer that the *in vitro* interaction between Spindly and Zwilch is not particularly striking. The outcome of a pull-down assay is affected by innumerous parameters, which include the concentrations of protein, salt and detergent present in the reaction mixture. We could certainly increase the robustness of the interaction by increasing the quantity of protein or by decreasing the concentration of salt or detergent. However, by performing the pull-down under more stringent conditions (pull-down buffer supplemented with 200 mM NaCl + Tween 0.05%) we were able to clearly demonstrate that phosphorylation of Spindly on its S499 negatively impacts the physical interaction of the full-length protein with Zwilch. We have confirmed the Spindly-Zwilch interaction in a reciprocal pull-down experiment (new Fig. S6B).

-Our understanding of the T182D mutant is incomplete. The mutant is presented as "constitutively active", but the level of activity in comparison to the properly phosphorylated Polo kinase is never tested. Clearly no dephophorylation of T182D is possible, but the observed phenotype might also result from incomplete kinase activation with this mutant, a possibility that the authors do not discuss or analyze.

To confirm that PoloT182D mutant is constitutively active we assessed its capacity to phosphorylate Sas-4 during interphase. Sas-4 is a centrosomal protein phosphorylated by Polo/Plk1 during mitosis (Ramani et al 2018 - PMID:30590037). However, using a phospho-specific antibody, we were able to detect phosphorylation of Sas-4 (phSas4) already during interphase when PoloT182D-EGFP was expressed. In contrast, no phSas4 could be detected at centrosomes of interphase cells expressing the PoloWT-EGFP transgene. This result demonstrates that the T182D renders Polo kinase active, an observation that has already been extensively reported (Deming et al. 2002; Fu et al. 2008; Kishi et al. 2009; Li et al. 2010; Lindon and Pines 2004; Loncarek et al. 2010; Macurek et al. 2008; Peschiaroli et al. 2006; Smits et al. 2000; van de Weerdt et al. 2005; van Vugt et al. 2004; Yamaguchi et al. 2005; Zhang et al. 2005; Zhou et al. 2003). Given the confirmatory character of this result, its inclusion in the manuscript would be, in our view, rather superfluous. We opted to present it in this letter as Figure R1.

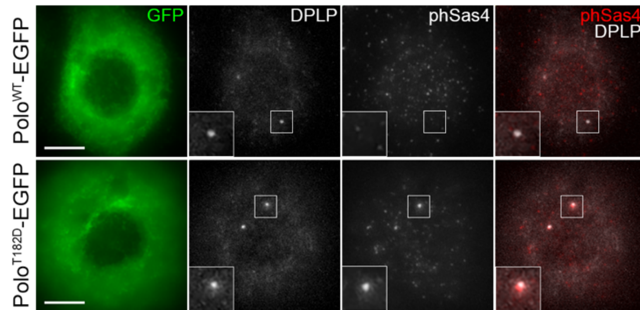

Figure R1. Representative immunofluorescence images of Polo-dependent phosphorylated Sas-4 in Drosophila S2 cells expressing either Polo^{WT}-EGFP or Polo^{T182D}-EGFP. Insets show magnifications of the outlined region, which highlight a centrosome. DPLP was used as a centrosome reference. Scale bar: 5um.

-Figure 6G shows that in presence of Polo T182D, Spindly accumulates at kinetochores and Dynein is required to remove it from there. It is unclear how this can be fitted into the authors' model, as there should be no Spindly on kinetochores.

As mentioned above, our model does not predict an impairment in the recruitment of Spindly to KTs when Polo activity is elevated, but rather an increase of its stripping along microtubules mediated by Dynein. If the model is correct, in the absence of Dynein, Spindly interaction with its KT receptor RZZ is no longer challenged and Spindly can accumulate at KTs even in the presence of high Polo activity.

-It is unclear why the depletion of p50/dynactin (page 11) shows a relatively mild phenotype, if the result of this depletion is ultimately essentially the same caused by expression of Polo(T182D), the retention of RZZ on kinetochores. In the authors' model, this depletion should have effects comparable to those observed when expressing Polo-T182D. Once again, these observations raise the impression that the authors try to support their preferred hypothesis and do not put sufficient weight on evidence that seems to argue against it.

The mild phenotype resulting from depletion of p50/dynactin could simply result from a lower efficiency/expression of this particular RNAi. We were not biased towards a favorite hypothesis. We conducted our line of inquiry throughout this work based on the most solid and robust results. In that respect, Rod and Spindly came out as key players involved in the PoloT182D phenotype and for that reason were studied in more detail. Moreover, expression of PoloT182D or depletion of Spindly in the larval brain affected adult viability, whereas down-regulation of p50/Dynactin allowed flies to eclose. This, and additional data that is described throughout the manuscript, suggested us that Polo activity was affecting Dynein-mediated removal of KT RZZ through Spindly.

-In general, it is unclear to what extent the persistence of Rod and RZZ on kinetochores in cells expressing Polo(T182D) is a mere consequence of prolonged checkpoint activation or rather of defective release of RZZ.

We have shown that RZZ accumulation at late congressing KTs is increased in PoloT182D expressing cells. Rather than a persistent RZZ localization, we show that there is in fact higher levels of RZZ at these KTs (Fig.4A-D and Fig.5A, B). This is not the case for the SAC protein Mad2, which accumulates to the same levels at late congressing KTs in either PoloWT or PoloT182D expressing neuroblasts (data not shown). Moreover, by depleting

either ZW10 or Mad2, we established a correlation between RZZ KT localization and efficiency of chromosome congression, which is not observed for Mad2 (Fig. S4).

Minor points

-Please remember to add page numbers to manuscripts! I have considered the front page as page 1 and numbered the rest accordingly.

We apologize for this inconvenience. Following the reviewer suggestion, we added page numbers to the revised version of the manuscript.

-*In Figure 2E-F, a control with the UAS Polo WT is missing*

This has been included in the revised version of the manuscript.

-Page 3, Introduction: "This Aurora B-independent...": what is the evidence that this regulation is Aurora B independent?

Destabilization of KT-MT attachments has been shown to occur through Rod interaction with Ndc80 N-terminal tail (Cheerambathur *et al*, 2013). The authors propose that this mechanism occurs in an Aurora B independent manner. This was based on the *in vivo* observation that depletion of the RZZ complex was sufficient to rescue the ability of the Ndc80 to mediate KT-MT interactions even when Ndc80 N-terminus tail is phosphomimetic for Aurora B sites**.**

-Page 6, Results: "...which occurs at a less extent...". Replace "at" with "to"

We have modified the text accordingly.

3rd Editorial Decision 19th November 2019

Thank you for submitting a revised version of your manuscript on Spindly control by Polo. All three original referees have now looked at it again, and I am pleased to say that they consider the paper substantially improved and have no more objections against publication in The EMBO Journal. However, referee 3 still notes several caveats that in my view warrant your consideration and response, together with respective changes to the manuscript text as appropriate.

In addition, there are also a number of editorial points that should be addressed at this point.

REFEREE REPORTS

Referee #1:

For this revised version, that authors have conducted an impressive range of additional experiments and made a variety of changes to the text and figures. These additions have nicely addressed my prior comments, and also appear to have done an excellent job of addressing the comments from the other reviewers. This paper should be accepted with congratulations to the authors for the beautiful work.

Referee #2:

The revisions made by Barbosa et al. have eliminated previous problems. The additional experimental evidence, including analyses with anti-phosphoS499-Spindly indicating that this phosphorylation occurs and behaves in vivo as predicted by the original version, has further strengthened their arguments. I consider the revised version to be acceptable.

Referee #3:

The authors have put al lot of effort in this manuscript and its revision. While I remain unconvinced, I support publication, making confidence in future studies to clear my doubts.

Nonetheless, even after reading their rebuttal, I would like to raise the warning that the authors' model is puzzling and I would advice them to make an effort and be more clear about it. Specifically, in the rebuttal, they write:

"It is important to highlight here that our model does not predict an impairment in the recruitment of Spindly to KTs when Polo activity is elevated, but rather an increase of its stripping along microtubules mediated by Dynein (please see Fig.6 and Fig.8)"

And a few lines later they write

"...we were able to clearly demonstrate that phosphorylation of Spindly on its S499 negatively impacts the physical interaction of the full-length protein with Zwilch.

And also in the Abstract

"We find that Polo phosphorylates Spindly and impairs its ability to bind to Zwilch."

I am puzzled because Spindly is recruited to kinetochores through its interaction with RZZ and therefore a reduced interaction with RZZ predicts lower kinetochore levels. If the problem is only in the stripping phase, kinetochore levels should be identical or higher because the interaction with RZZ ought to remain equally strong and no stripping means higher levels. Therefore, I don't see how these statements go together. In one case, the authors claim that phosphorylation affects stripping but does not impair the recruitment of Spindly, and in two other cases they claim that the interaction with RZZ (known to be important for Spindly recruitment) is affected.

Also, I apologise for not making my point about PoloT182D sufficiently clear. The question I asked is whether this constitutively active mutant is more active than wild type Polo in mitosis, which personally I think is highly unlikely. It is clear that the phosphorylation of a Polo substrate at a time when Polo is hardly any active will be enhanced in presence of the PoloT182D mutant, as the latter is a non-cell-cycle regulatable allele. This is what the authors show in their rebuttal, but it is not relevant to the point I am raising. The authors are looking at a mitotic phenotype, when wild type Polo is being actively phosphorylated on its activation loop and is therefore highly active. Here the fair comparison is between wild type Polo and mutant Polo. Given the established role of activation loop phosphorylation on kinase activation, I think it is reasonable to assume that wild type Polo in mitosis, which will be phosphorylated on the activation loop, will be as active as, and likely significantly more active than, PoloT182D (the latter having a single charge). Whether the activity of Polo (the state of its activation loop phosphorylation) is dynamically controlled during mitosis is an open question, as far as I can tell, and I don't see that the study here adds to it. Therefore, just to be as clear as possible: As far as I am concerned, I would not rule out that the phenotype the authors observe is due to INSUFFICIENT Polo activity, rather than overexpression.

Finally, as already remarked in my original review, the authors deserve praise for the body of experiments they present, but sometimes less is more. In this revision, they even include results with an antibody against Spindly Phospho-S499. With all due respect, while it is likely that this antibody recognises a Polo substrate, I don't see strong evidence that the shown kinetochore signal corresponds to Phospho-S499 Spindly

Point-by-point response to the editor and reviewers:

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We thank the reviewer for his comments.

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We thank the reviewer for recognizing our efforts into this work and for supporting its publication. In this new re-revised version of the manuscript we attempted to describe our model in a clearer manner (Fig.10F). We certainly agree with the reviewer that a reduced interaction of Spindly with the RZZ (detected in vitro) would in principle predict lower levels of Spindly at kinetochores regardless of attachment status or Dynein activity. However, our data does not fit with this prediction, which can be plausibly explained if we consider that:
(i) Spindly is most likely recruited to the RZZ in its unphosphorylated form. Only then becomes phosphorylated on S499 by kinetochore-localized Polo.
(ii) Although S499 phosphorylation decreases Spindly affinity to the RZZ (Fig.8A-C), this is not sufficient (in cells) for Spindly to fall of kinetochores in the absence of Dynein or microtubules (Fig.6E-H). This suggests that in the intricate and complex environment of the kinetochore, additional factors may contribute to maintain Spindly bound to the RZZ, even though its affinity towards Zwilch is reduced.
(iii) On the other hand, if Dynein is allowed to function normally, it will readily overcome these factors and strip Spindly, which in its phosphorylated form can easily disengage from the RZZ leaving it behind.

In conclusion, our data supports a model, in which Polo-mediated phosphorylation of Spindly on S499 provides a mechanism to uncouple the recruitment of Dynein-Spindly to the RZZ from its stripping. This ensures that the RZZ can recruit Dynein-Spindly to kinetochores (S499 arrives unphosphorylated) without itself being removed (S499 leaves phosphorylated). This is important to maintain high levels of RZZ on prometaphase kinetochores, which is required to prevent premature stabilization of merotelic attachments and support SAC signaling (Fig.10F).

Also, I apologise for not making my point about PoloT182D sufficiently clear. The question I asked is whether this constitutively active mutant is more active than wild type Polo in mitosis, which personally I think is highly unlikely. It is clear that the phosphorylation of a Polo substrate at a time when Polo is hardly any active will be enhanced in presence of the PoloT182D mutant, as the latter is a non-cell-cycle regulatable allele. This is what the authors show in their rebuttal, but it is not relevant to the point I am raising. The authors are looking at a mitotic phenotype, when wild type Polo is being actively phosphorylated on its activation loop and is therefore highly active. Here the fair comparison is between wild type Polo and mutant Polo. Given the established role of activation loop phosphorylation on kinase activation, I think it is reasonable to assume that wild type Polo in mitosis, which will be phosphorylated on the activation loop, will be as active as, and likely significantly more active than, PoloT182D (the latter having a single charge). Whether the activity of Polo (the state of its activation loop phosphorylation) is dynamically controlled during mitosis is an open question, as far as I can tell, and I don't see that the study here adds to it. Therefore, just to be as clear as possible: As far as I am concerned, I would not rule out that the

phenotype the authors observe is due to INSUFFICIENT Polo activity, rather than overexpression.

We understand the reviewer's point and agree that our data does not directly show that PoloT182D is more active than wild type Polo in mitosis, nor we think it is expected to be. Our claim here, which has been extensively demonstrated by previous studies, is that PoloT182D remains constitutively active throughout mitosis, whereas PoloWT activation status decreases as cells progress from prometaphase to metaphase (Liu D, Davydenko O, Lampson MA. Polo-like kinase-1 regulates kinetochore-microtubule dynamics and spindle checkpoint silencing. J Cell Biol 2012)

The reviewer raises the possibility that PoloT182D may be less active than PoloWT. However, it has been shown *in vitro* that the kinase activity was not affected by the phosphomimetic mutation and was comparable to the activity of wild type PLK1 isolated from prometaphase cells, when it is maximal (Paschal CR, Maciejowski J, Jallepalli PV. A stringent requirement for Plk1 T210 phosphorylation during K-fiber assembly and chromosome congression. Chromosoma 2012). In line with this, mutant PoloT182D (T1210D in human PLK1) has been extensively used as a means to express a constitutively active kinase (see reference list on previous rebuttal letter).

In our previous rebuttal letter, we have shown that T182D mutation is enough to activate Polo to phosphorylate a mitotic substrate during interphase, a cell cycle stage where the kinase should not be active. In our view, such an observation would not fit a scenario where Polo kinase activity is precluded by the T182D mutation. Furthermore, if this phosphomimetic alteration was to cause insufficient Polo activity, we would not expect a dominant negative effect of the transgene, as endogenous Polo is still present in these mitotic cells. Instead, we support the idea that PoloT182D is active (similar to wild type Polo) during mitosis, but lacks the autoinhibitory mechanism that is dependent on T-loop dephosphorylation. We show that, in control cells, Polo T-loop phosphorylation decreases as cells progress to metaphase (Fig.1A, B) and propose that this is important to decrease the kinase activity towards Spindly.

Finally, as already remarked in my original review, the authors deserve praise for the body of experiments they present, but sometimes less is more. In this revision, they even include results with an antibody against Spindly Phospho-S499. With all due respect, while it is likely that this antibody recognises a Polo substrate, I don't see strong evidence that the shown kinetochore signal corresponds to Phospho-S499 Spindly

The experiments with the antibody against Spindly phospho-S499 were an attempt to answer the reviewer's comment on the putative *in vivo* significance of this particular phosphorylation event. In the revised manuscript, we show that the antibody signal is lost from kinetochores in cells depleted of Polo kinase or treated with Polo inhibitor BI2536 (Fig.7D, E). This would only suggest that the antibody was recognizing a Polo substrate, with no direct evidence that it corresponded to Spindly. However, we also show that the antibody signal significantly decreases in cells depleted of Spindly and is no longer able to recognize an epitope dependent on Ser499 when this residue is mutated to an alanine (S499A) (new Fig.EV4A-D). We believe that these observations validate the specificity of the antibody.

Accepted 2nd December 2019

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.