

Manuscript EMBO-2010-74053

Dishevelled, a Wnt signaling component, is involved in mitotic progression in cooperation with Plk1

Koji Kikuchi, Yohei Niikura, Katsumi Kitagawa, and Akira Kikuchi

Corresponding author: Akira Kikuchi, Graduate School of Medicine, Osaka University

Review timeline:

Submission date:	02 March 2010
Editorial Decision:	25 March 2010
Revision received:	06 July 2010
Editorial Decision:	22 July 2010
Revision received:	05 August 2010
Accepted:	06 August 2010

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

25 March 2010

Thank you very much for submitting your paper for consideration to The EMBO Journal editorial office.

As you will see from the actually four reports, both referee's from the Wnt-perspective as well as from the mitosis field find the study of potential interest. However, their individual assessments are rather mixed with some recommending publication conditioned on necessary revisions and others identifying so many technical concerns that they see no chance to address them during a single round of major revisions. Careful reading of their comments reveals that in essence current gain-of-function experiments in multiple cell lines would have to be replaced by loss-of-function/complementation experiments in the most suitable cellular system. Further, the presence of endogenous Dvl1/3 causes a potential redundancy problem that should at least in the crucial experiments be overcome by complementary knockdown. Further, ref#3 relates to the recently established APC/Dvl2 interaction and suggests expansion on their (potential) interplay also during mitosis. In contrast, the results on LRP6/Fz2 though interesting, seem not add too much to the message of the current paper. These altogether, combined with convincing documentation of mitotic phenotypes (as outlined in detail by the experts) sets the necessary framework to reach the rather high expectations according to the aim and scope of our more general and highly competitive journal. All in all, and given the potential interest in your study, we still decided to offer you at least the chance to improve and develop the paper significantly. I therefore urge you to take the very constructive comments of our referees serious but at the same time focus on the essential experiments that I tried to delineate above.

I do understand that this will involve serious and time-consuming additional experimental work with partial uncertain outcome. I would thus understand if you were to take the paper in its current form elsewhere to also avoid later disappointments here. On the other hand, we would be able to grant

additional time upon your request to enable necessary experimental work. In case you were to pursue revisions for the EMBO Journal, I also have to remind you that it is EMBO_J policy to allow a single round of major revisions only. Thus, the final decision on acceptance or rejection entirely depends on the content and strength of the final version of your manuscript!

Thank you for the opportunity to consider your work for publication. I remain with kind regards.

Yours sincerely,

Editor

The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Here, Kikuchi and colleagues report that the pivotal Wnt signaling component, Dishevelled, plays multiple roles in the regulation of the mitotic spindle during mitosis. Although Dvl has previously been implicated in such functions the current study provides a detailed molecular analysis that describes novel interactions (Plk1 and Mps1), phosphorylation events and subcellular localizations that are thorough, convincing and interesting. Of note, the authors also demonstrate that LRP6 is involved in coordinating spindle function

Specific comments:

1. Figure 1: GFP-Dvl2 was used because anti-Dvl2 Ab's were not good enough to reveal endogenous localization during mitosis. Have the authors tried Dvl1/3 Ab's? More generally, since they function redundantly in Wnt signaling, it would be informative to have an idea of the relative expression levels of the three Dvl isoforms in the cell lines used in this study.
2. Figure 2: the authors very nicely show mitotic phosphorylation of Dvl2 but it is not clear if the effect is Wnt dependent. As Wnt signaling is reported to be more active during mitosis, the authors should block this with e.g. Dkk and then ask if mitotic phosphorylation still occurs.
3. Figure 4: TOPFLASH reporter assays should be shown for the various Dvl2 mutants shown in panel E.
4. Figure 6: as for Fig. 2 A, Wnt signaling should be blocked in panel A to see if the LRP6 phosphorylation at mitosis is Wnt dependent.

General comment:

I fear the reader may be confused at times dealing with the various aspects of the reported function of Dvl2 during mitosis. It would therefore be helpful to move the illustration the authors present in the supplement (S5) to the main figures, for easy referral.

Referee #2 (Remarks to the Author):

Summary: Wnt signalling components have been extensively studied for their transcriptional regulatory role. This study brings forth the mitotic role of one more Wnt signalling component, Dishevelled. Recently, mitotic role for other members of Wnt Signalling have become apparent. The study is certainly of interest to a wide audience from both cell cycle and signalling fields. But the claims are unclear and some conclusions are not fully supported. An improved clarity of writing might help the authors. There are some incorrect interpretations of cited literature; its not clear if this is due to compromised clarity of writing or poor understanding of the literature. The manuscript is not ready for publication in the current format.

The authors show that Dishevelled localises to kinetochores and spindles poles and interacts with mitotic kinases, Plk1 and Mps1. Using mutants the authors demonstrate that Plk1 binds to Dvl2 and phosphorylates Dvl2. Significance of Plk1 interaction or phosphorylation is not clearly presented. Depletion of Dvl2 is shown to perturb the stable positioning of the bipolar spindle and spindle

checkpoint proficiency. The authors find reduction in the levels of phosphorylated MPS1 and kinetochore-bound BubR1 and Bub1 in Dvl2 depleted cells indicating that Dvl2 depletion leads to checkpoint loss. Together the experimental findings show that Dvl2 plays a role in mitosis. Other claims for a role of Dvl2 is microtubule dynamics and microtubule attachment at cell cortex or kinetochore are not supported.

Details:

While its an important and interesting area of research, the findings in this manuscript are not fully supported. Areas where new experiments are critical to support the authors claims are listed below, in the order of seriousness of technical flaws.

1. Page 19: Dvl2 plays important role in regulating Mt-kt attachment or dynamics of MT plus-ends. No evidence has been shown to indicate a role for Dvl2 in chromosome attachment or regulation of MT dynamics.
2. Dvl2's role in spindle checkpoint is also novel and interesting but not convincing. If Dvl2's involvement in spindle checkpoint is true, one would expect Dvl2 depleted cells to undergo premature chromosome segregation and accelerated mitotic exit and this is not the case as shown in Figure 4B. The only piece of convincing evidence for supporting Dvl2's checkpoint role with a good rescue plasmid is shown in Fig 5B. Unfortunately even this study has been done in a cell line U20S which is known to have a poor checkpoint response to Nocodazole. The authors will benefit repeating this in HeLa cells since they present a more robust checkpoint response. The authors should explain what relative Mpm2 positive cells mean and also include the data from (-Noc) experiments.
3. The authors claim using Fig 5D that DVL2 is required for activation of MPS1 is not fully supported. This reduced MPS1 activation might be an indirect consequence of Dvl2's loss. As shown in fig 5B if siDVL2 cells show nearly a 50% reduction in mitotic index it's expected that phospho-MPS1 signal would reduce as well. A control for this experiment would be to perform depletion of a known checkpoint component downstream of Mps1 (such as siBUBR1 treatment) and ask if phospho-MPS1 is retained or reduced when the checkpoint is compromised.
4. The finding of Dvl2's localisation at kinetochores and spindle poles is novel. However, localisation of GFP-Dvl2 in live cells is needed to exclude any fixation artifacts. Because the authors have the ability of perform live-cell imaging and have access to the Dvl2-Gfp plasmid, this experiment might easily help strengthen their finding further
5. Using mutant studies they show strong evidence for interaction between Dvl2 and Plk1 however interaction between Mps1 and Dvl2 is not strongly presented. While this might not be important from the scope of the current paper, the authors should at least show a reverse immunoprecipitation of pulling down Mps1 and testing Dvl2's interaction in mitotic cells.
5. Interpretation of inter-kinetochore tension using inter-kinetochore distances measured in fixed cells is not convincing. In fixed cells when the cells are dead there are no active forces to pull and separate the kinetochores for maintaining tension. Therefore using this experimental design to claim the extent of inter-kinetochore tension is incorrect. To quantitate the extent of inter-kinetochore tension the authors need to measure inter-kinetochore distances in live-cells expressing a kinetochore marker such as CenpB-GFP or CenpA-GFP.

Referee #3 (Remarks to the Author):

Components of the Wnt/beta-catenin pathway have been implicated in the proper progression through mitosis. Best characterized among these is Adenomatous Polyposis Coli (APC), an important regulator of beta-catenin degradation, which stabilizes microtubules and clusters at their plus-ends. APC is required for proper spindle orientation during mitosis and also mediates chromosome congression by modulating KT-MT attachment. The Wnt pathway component Dishevelled, a modulator of beta-catenin dependent and independent signaling, has been shown to be required for MT stability in neurons. In addition, recent work by the Kikuchi lab demonstrated a direct interaction between APC and Dishevelled (Matsumoto et al.,

EMBO J 2010), hinting to a shared role in regulating microtubule dynamics, possibly during mitosis as well.

In this manuscript, Kikuchi et al. now describe a role for Dishevelled 2 (Dvl2) and the Wnt pathway components Lrp6 and Fz2 in mitosis. The paper makes four major claims: (A) Dvl2 is required for proper placement of the spindle during mitosis and for proper stability of kinetochore-microtubules. (B) Dvl2 is regulated by Plk1. (C) Dvl2 plays a role in the spindle assembly checkpoint. (D) More Wnt pathway components (Lrp6, Fz2) are involved in mitosis, whereas yet others (Ror2, Wnt5a) are not.

In principle, I think it is interesting to see that Dvl2 also plays a role in mitosis. However, I feel that many of the claims the authors make in the paper are not sufficiently substantiated, as outlined below. Some experiments are not conducted in the most meaningful way and/or are not well controlled. In addition, the authors continuously switch between cell lines, making it hard to interpret the data. In addition, there are a lot of minor weaknesses (unsatisfactory pictures, experiments not well explained, reasons to perform the experiment unclear, imprecise wording, incorrect citations, incomplete experimental procedures). Because of all this, I get the overall feeling that I cannot really 'trust' the data, which considerably dampens my enthusiasm for this potentially interesting story.

Major comments:

1. Given the fact that the APC RNAi (shown previously and here) and Dvl2 RNAi phenotypes are very similar and that the authors have previously reported a direct interaction between the two, I think it would be important to demonstrate whether total cellular levels and/or localization of APC are affected in Dvl2 RNAi cells or vice versa. In addition, the authors should check whether an interaction between APC and Dvl2 can be detected during mitosis, and if so, whether disruption of the interaction yields similar phenotypes. This would greatly help to put the new data in the context of the existing literature on the role of Wnt pathway proteins in mitosis and would help to validate the models that the authors put forward in Figure S5.
2. Can the authors comment on Dvl1 and Dvl3? Have the authors tested whether they have a role in mitosis as well?
3. The authors claim that Dvl2 is required for kinetochore-microtubule attachment (see e.g. abstract). Firstly, I would formulate this more carefully, since in the complete loss of stable attachment, one would expect the chromosomes to scatter much more on and around the spindle (see e.g. DeLuca/Salmon JCB 2002), whereas in Dvl2 RNAi there is relatively good chromosome alignment (see e.g. Figure 4G), indicating that kinetochore-microtubule attachment is still relatively stable. Furthermore, the statement is solely based on Figure 4F, in which the authors only investigate EB1, but not microtubules. Why not looking at tubulin staining, if the authors want to claim that there are less or less stable kinetochore-microtubules? The fact that the authors see less EB1 adjacent to kinetochores under these conditions could also mean that there is a problem with EB1 localization. Therefore, (a) the authors should show microtubule-staining after short nocodazole-treatment (as in Figure 4F), and (b) it would be useful to evaluate EB1 (and APC) localization in Dvl2 RNAi cells.
4. The authors claim that phosphorylation of Dvl2 (by Plk1) is required for spindle orientation and microtubule-kinetochore attachment. The evidence for this is that Dvl2 is phosphorylated on T206 by Plk1 in vitro, that mitotic phosphorylation of Dvl2 is reduced in Plk1 RNAi cells and that overexpression of the Dvl2-T206A mutant causes a spindle orientation defect as well as a reduced inter-KT distance. Additional experiments are required to confirm this. Most importantly, testing of the Dvl2 mutants should be done in an RNAi complementation experiment, which in my opinion can nowadays be considered 'standard'. Instead the authors use an overexpression assay, which is more difficult to interpret. It should be determined whether defective spindle orientation and weak kinetochore-microtubule attachment are seen in Dvl2 RNAi cells, which have been complemented with near-endogenous levels of Dvl2-T206A? What is the localization of Dvl2-T206A in Dvl2 RNAi cells? Does the protein localize to kinetochores? Secondly, one would like to know whether Plk1 RNAi cells actually show a spindle orientation defect. If so, can this be rescued by expression of Dvl2-T206D/E? Does the localization of Dvl2 change in Plk1 RNAi?
5. The authors claim that Dvl2 is required for SAC activation through Mps1 activation. SAC defects are a frequent side effect of RNAi (H, bner et al. 2010). It is therefore essential to perform rescue experiments using ectopically expressed Dvl2 to exclude this possibility. The authors do this for U2OS cells, but it would be good to also show such an experiment for HeLa

cells, with which more of the experiments have been performed.

The claim that Dvl2 acts through Mps1 is based on the author's finding that Mps1 and Dvl2 interact, as judged by co-IP, and that phosphorylation of Mps1 is somewhat reduced after Dvl2 RNAi, although this is not very clear (Figure 5E). The fact that Mps1-Dvl2 interaction is detected in asynchronous cells (Figure 5D) indicates that it might not be checkpoint-activation specific. In addition, the authors are under the assumption (stated in the text) that Mps1 is required for Bub1 and BubR1 localization to kinetochores, which is also impaired in Dvl2 RNAi. However, there is actually agreement in the literature that in human cells (unlike in *Xenopus*), Mps1 is not needed for Bub1 and BubR1 kinetochore localization (Martin-Lluesma/Nigg, *Science* 2002; Liu/Yen, *MBC* 2003; Jelluma/Kops, *Cell* 2008 and citations therein). Thus, even if Mps1 activity is reduced, Dvl2 must act on the checkpoint through some other mechanism.

If the authors want to claim that Mps1 activity depends on Dvl2, this would need to be tested more directly, e.g. by *in vitro* phosphorylation assays (Stucke/Nigg, *Chromosoma* 2004) in the absence or presence of Dvl2 or by looking at CENP-A-phS7, which depends on Aurora kinase activity, which in turn depends on Mps1 activity (Jelluma/Kops, *Cell* 2008). Furthermore: is the interaction between Mps1 and Dvl2 actually needed for checkpoint activity? In the discussion, the authors speculate that Mps1 and Dvl2 interact through the PDZ domain in Dvl2. This could easily be shown, and the Dvl2-delta-PDZ mutant could be tested in an RNAi complementation assay for checkpoint activity.

6. For RNAi experiments, the specificity should be tested by using several siRNAs and by showing that the phenotypes can be alleviated by expression of an RNAi-resistant construct. The authors apparently used two different siRNAs for some of the knock-downs (see siRNA list in Supplement; not mentioned in the text), but not for all (e.g. not for Dvl2). In Figure 5B, the authors show rescue of the Dvl2 RNAi-induced checkpoint phenotype by exogenous Dvl2 in U2OS cells, but do not provide such data for any of the other experiments/cell lines. In addition, it is customary, and it would be good if the authors could provide data indicating how efficient their RNAi is (e.g. levels reduced to at least xx %, based on immunoblotting).

Detailed comments:

- Title: 'controls spindle dynamics' is a very fuzzy term and I think the authors should re-think what they actually want to say here
- Abstract and end of introduction: The last sentence '... mediates Wnt-dependent and -independent regulation... through the phosphorylation by Plk1' is extremely confusing and in my opinion incorrect. Please re-phrase
- Several citations are in my opinion not appropriate: (1) Since Plk1 is one of the major topics of this paper, I feel that some primary literature should be cited in addition to the Petronczki et al. *Dev Cell* 2008 review (in particular, since EMBO Journal has the policy to allow as many citations as needed). (2) The authors cite Kang et al. 2007 for the fact that Mps1 is required for SAC activation. This is by no means the first paper showing this and is only one out of several papers that map Mps1 phosphorylation sites. The citation should be changed. (3) The authors cite Abrieu et al. 2001 and Vigneron et al. 2004 for the fact that Bub1, BubR1, Mad1 and Mad2 localization depend on Mps1. However, the work in these papers has been performed in *Xenopus*. In human cells, the localization dependencies are in fact different and this is quite relevant for the arguments in this manuscript. Please correct text and citations. (4) The authors cite three papers for the fact that reduced localization of Bub1 and BubR1 to kinetochores affects kinetochore-microtubule attachment. The first of these citations Jelluma/Kops 2008 is in my opinion not correct. (5) Jelluma/Kops *PLoS one* 2008 should be cited for Mps1 autophosphorylation in addition to Kang et al. and Mattison et al. 2007. (6) A reference should be provided for dominant-negative Tcf4 (TcfDN).
- Figure 1A: An immunoblot for the expression level of GFP-Dvl2 (relative to endogenous Dvl2) should be provided.
- Figure 1C: The upper three pictures seem of extremely low quality. I am not sure what I am supposed to see here. Please replace these pictures. In addition, show whole cells for the staining shown at the bottom (ACA/GFP-Dvl2). See e.g. Zuccolo..Doye *EMBOJ* 2007, Meraldi..Sorgor *EMBOJ* 2005 for examples of kinetochore stainings from nocodazole-arrested cells.
- Figure 1B: In the text, the authors speak of prometa- and metaphase, but it remains unclear which of the pictures represents what. Is there any difference between the upper and lower picture? Is it necessary to show both?
- Figure 1B: I can neither see kinetochore staining in Figure 1A nor in the Supplementary Movie 1. Why is it visible in Figure 1B? Are the cells treated differently? Is the staining extremely weak compared to the spindle signal for Dvl2?
- Supplementary Movie 1: Since GFP-Dvl2 looks quite different in this unfixed cell compared to the

fixed cells (Figure 1A/B), I think it is useful to show this data. However, in my opinion it doesn't make sense to show a movie for a metaphase-'arrested' cell, which does not change in appearance throughout the movie. Please show a single still image from the movie instead, and explain the difference in GFP-Dvl2 signal.

- Figure 2B: Which cells were used?
- Figure 2C: Please show the data for CDK1 and Aurora A, which could serve as a negative control for this experiment.
- Figure 2D: Why are HEK cells used here, and not HeLa cells (as in Figure 2C)?
- Figure 2E: Mutants #5 and #7, which do not show an interaction, have the weakest band in the input, raising the question whether they cannot be detected in the co-IP because of their low abundance. Would it be possible to adjust the amounts? Or show the reciprocal GFP-IP.
- Figure 2E: Is region 354-423 sufficient for binding? Does it contain a motif that fits the sequence that has been reported for Polo-box binding?
- Figure 3A: MBP-Dvl2 - presumably - strongly degrades. How do the authors know that the uppermost band is full length MBP-Dvl2? How do they know that the non-phosphorylated products lack the C-terminus (as stated in the text)? Did they perform an MBP immunoblot?
- Figure 3: It remains unclear whether the C-terminus of Dvl2 could also be phosphorylated by Plk1. I would suggest to additionally express the fragment 434-stop and test it in an in vitro phosphorylation assay as well. Additionally or alternatively, the authors should test whether full length Dvl2-T206A is phosphorylated or not.
- Figure 3: The figure could be shortened. Figure part B, for example, could be left out without loss of clarity.
- Figure 4A: Even when looking at the Materials and Methods section, it remains unclear how this quantification was performed. In the upper part of the Figure, it looks like the boxes are positioned in some angle to the centrosome-centrosome axis, which in my opinion doesn't make sense. Shouldn't they always be aligned with this axis, i.e. directly adjacent to the SPBs? What was the size of the boxes? What kind of projection of the Z-stack (max. intensity? average? sum projection)? How do the authors place the region for the spindle MT area (mentioned in Mat. and Methods)? In addition, the calculation mentioned in Mat. and Methods seems odd. In my opinion the calculation should be: (intensity of astral MT area) / (intensity of astral MT area + intensity of spindle MT area). In addition, why was taxol used in the fixation procedure?
- Figure 4B: The authors state in the text, that one of the daughter cells 'fails to maintain adhesion... after cell division'. However, from the movie, it is clear that these cells eventually establish adhesion. I would therefore rather say that the cells 'fail to immediately gain adhesion to the substratum'. In the movie, it looks like the cells that fail to gain adhesion were overlapping with another adhered cell, whereas the cells that immediately gain adhesion were not. Is this general? Is the same phenotype observed when no other cell is adjacent to the dividing cell? Please also provide some quantification on how often this phenotype was seen (xx out of yy dividing cells).
- Figure 4C/D/E: The authors look at two different spindle orientation phenotypes here, namely spindle rotation and spindle displacement (see e.g. Draviam/Sorger EMBO J 2006). I have the feeling that the authors do not make a clear distinction in the text, which at first confused me. Also, why analyzing all the mutants for one phenotype (spindle rotation), but not the other (displacement). Could the authors provide a quantification for Figure 4D as well?
- Figure 4C and 4E: This is a good example for the use of different cell lines by the authors. Why using HeLa cells in 4C and U2OS cells in 4E? Couldn't this all be done in the same cell line? (In which case, the Figure parts could be combined).
- Figure 4E: Firstly, as mentioned above, this experiment should be performed using RNAi/complementation rather than overexpression. In addition, the authors have to provide data on how well each of the Dvl2 mutants was expressed. It would furthermore be helpful if the authors checked the localization of these mutants.
- Figure 4G: The Dvl2-delta-PDZ mutant (used in Figure 4E) has previously been shown to be deficient in stabilizing microtubules in interphase (Krylova et al. 2001, Ciani et al. 2004). It would be interesting to see whether it has an effect on inter-KT distance.
- Figure 4G: The pictures are not very high quality, and it is unclear to me how the authors can pick kinetochore pairs from these pictures. See e.g. Stumpff/Wordeman Dev Cell 2008, Figure 2, for a similar experiment.
- Text corresponding to Figure 4G: Reduced inter-KT tension may lead to - but does not necessarily lead to - a SAC-dependent mitotic delay. It has recently been shown that reduced 'intra-KT' stretching is more crucial than reduced inter-KT tension for activating the SAC (Maresca/Salmon and Uchida/Hirota, JCB 2009). In my opinion, the text therefore needs to be re-formulated.

- Figure 5: This is a good example where it is extremely confusing that so many different cell lines are used. Figure part A, C, E are HeLa cells, B and F are U2OS and D is HEK293T. These cell lines may very well be different in their checkpoint response (see e.g. Gascoigne/Taylor Cancer Cell 2008) and it would be much better to have all experiments performed in one cell line.
- Figure 5C: An immunoblot is required to demonstrate that the overall levels of Bub1 and BubR1 are still the same after Dvl2 RNAi. Only then, the authors can conclude that there is a localization defect.
- Figure S4C: For the interaction analysis, it would be much better to work with the endogenous proteins rather than with overexpression of tagged versions.
- Figure 5D: Is this immunoprecipitation really done from asynchronous cells (as the legend implies)? Does this mean that the Dvl2 - Mps1 interaction is constitutive and not checkpoint-activation specific? Could the authors provide a negative control for the co-IP? Does APC e.g. associate with Mps1 as well?
- Figure 5D and F: Firstly, it would be much better to test the Dvl2 mutants in an RNAi complementation experiment rather than by overexpression (see above). If in such an experiment the mutants also show different checkpoint responses, it would be very useful to test whether this correlates with their ability to associate with Mps1. With such data, the authors could make the point that Dvl2-Mps1 interaction is indeed needed for checkpoint activity. With the current data, it is not possible to make this link.
- Figure 5E: Was the mitotic index the same in the control and the Dvl2 RNAi cells? This is important to assess whether there really is a difference in Mps1 phosphorylation. It seems Mps1 is overall less abundant in the Dvl2 RNAi sample. How is the 'relative phosphorylation' indicated at the bottom determined?
- Figure 6: The whole paper is, I assume, also meant to be read by people from the mitosis field. For them, it will be really hard to understand the meaning of this figure. I think it would help to add a little schematic that shows the proteins that are examined here together with the pathways, in which they are supposed to play a role. I imagine something like a reduced version of Figure 1 from the Kikuchi et al. review in Trends Cell Biol 2009.
- Figure 6A: There is no immunoblot for Fz2. In addition, it would be useful to provide an immunoblot for Dvl2. Are the levels unaffected by Lrp6 or Fz2 RNAi?
- Figure 6B: Show a separate picture for Lrp6 pS1490 for interphase as well (is the signal gone or just reduced?).
- Figure 6F: The experiment needs a control for the mitotic index in control versus Dvl2 RNAi cells. Plk1 is not a particularly good control, since it is already expressed during G2 (see e.g. Sumara/Peters Curr Biol 2004).
- Figure 6G: The authors state that 'depletion of Lrp6 or Fz2 did not affect MT-KT attachment'. However, the pictures in this figure imply that the RNAi cells do have a problem with chromosome alignment, which could be indicative of an attachment defect. Please provide a quantification, which percentage of mitotic cells had aligned versus non-aligned chromosomes (analogous to Fig. S3F). In addition, I recommend checking for cold-resistant or low conc. of nocodazole-resistant microtubules (using staining for microtubules) in both Dvl2 as well as Lrp6 and Fz2 RNAi.
- Figure S1: Similar interphase pictures are already published in Matsumoto et al., EMBO J 2010. Figure S1A therefore seems unnecessary.
- The introduction states that the kinase activities of Bub1, BubR1, Mps1 and Aurora B are necessary for the spindle assembly checkpoint (SAC). For both Bub1 as well as Aurora B this is controversial (see e.g. Klebig/Meraldi JCB 2009; Maresca/Salmon J Cell Sci 2010). BubR1 kinase activity seems only partially required for the SAC (e.g. Malureanu/van Deursen Dev Cell 2009). Mps1 is the only of these kinases for which it is very clear that its kinase activity is required for the SAC. Please re-formulate.
- Text page 8: The argument that phosphorylation of Dvl2 in mitosis was checked because the protein has potential phosphorylation sites is a bit odd - I assume that almost every protein has potential phosphorylation sites.
- Text page 12 'Dvl2 might be required for... but not for the centrosomal functions'. What are the 'centrosomal functions'? I would rather say: 'required for spindle orientation, but not for bipolar spindle formation'.
- Discussion: The discussion starts with an extensive summary, which in my opinion could be shortened.
- Material and Methods 'Reagents and antibodies': I don't think that phalloidin was used in this study. MG132 etc. are mentioned twice.
- Material and Methods 'Cell culture...'. Please indicate which protocol was used for which figures.

The thymidine/MG132 protocol (Figure S4) seems to be missing.

- Material and Methods: Image analysis for the quantification of Bub1 and BubR1 intensities is not described, I think.
- Material and Methods 'Immunoblotting...': Please indicate which protease and phosphatase inhibitors were used. Cell number? Volume of lysis buffer?
- Material and Methods 'Segregation axis analysis': Wouldn't it make more sense to call this 'spindle axis analysis'? It is not mentioned how the measurement was performed. (Text only says '... axis was measured by taking Z-stack images...'). This is the first step in the procedure, but one would really like to know how the authors proceeded.
- Figure legend to Figure 5A fails to indicate in which way the MPM2-stained cells were analyzed. FACS? Immunofluorescence microscopy? Also in several other figure legends additional information is needed (which antibody was used for detection, which cell cycle stage was analyzed, which cell type was used).
- Figure S3D and legend to Figure S3D: should say 'spindle pole distance' rather than 'spindle distance'. Legend says that measurements were in mm, which I assume should be μm . In addition, please provide an example picture for the quantification of EB1 intensity.
- In addition, there are several typos and imprecise wording throughout the text.

Referee #4 (Remarks to the Author):

In this paper, the authors addressed the mitotic roles of Dvl2 in mammalian cells. They found that Dvl2 is involved in three processes in mitosis; a) spindle orientation, b) microtubule-kinetochore attachment, and c) the spindle assembly checkpoint. Among the roles of Dvl2, phosphorylation by Plk1 is required for a) and b), while it is dispensable for c). In addition, a) is dependent on Wnt signaling, while b) and c) are Wnt-independent. These results clearly demonstrate the novel aspects of Dvl2 as a mitotic regulator like other molecules of the Wnt/ -catenin pathway. Although the experiments were well organized to dissect the roles of Dvl2 to draw the elegant model as shown in Fig. S5, there are several concerns on the interpretation of the data.

Major criticism

1. To determine the requirement of each region of Dvl2 or phosphorylation by Plk1 on spindle orientation or the spindle assembly checkpoint (Figure 4E, 5F, 6C), the authors observed the effect of ectopic expression of each mutant in the presence of endogenous Dvl2. These assays could detect the phenotype of each mutant only when it had the dominant negative effect. For example, in Figure 4E, DIX and PDZ did not show spindle misorientation, but they might show defects when they are replaced with endogenous Dvl2. Ideally, these assays need to be done in siDVL2 cells expressing RNAi-resistant constructs, and at the moment we are not sure whether Plk1-dependent and independent roles can be clearly distinguished. Therefore, authors need to attenuate current conclusion if replacement experiments are technically difficult.

2. Concerning the role of Dvl2 on microtubule-kinetochore attachment (Figure 4F), it is better to observe chromosome misalignment in siDVL2 cells in the presence of MG132, a proteasome inhibitor, considering that Dvl2 is also involved in the SAC. If Dvl2 is involved in MT-KT attachment through kinetochore localization of Bub1 and BubR1, siDVL2 cells will show massive chromosome misalignment in the presence of MG132. I also wonder whether Lrp6 and Fz2 are really dispensable for MT-KT attachment, because in Figure 6G, siLRP6 and siFZ2 cells showed chromosome misalignment as siDVL2 cells in the presence of low dose nocodazole, which was not seen in control cells (Figure 4F). Authors should comment on the issue.

3. Kinetochore localization of Dvl2 was not as clear as localization on spindle poles (Figure 1). If the roles of Dvl2 on MT-KT attachment and the SAC depend on kinetochore localization of Dvl2, and different domains of Dvl2 are responsible for respective roles, kinetochore localization domain is supposed to reside on domains other than these domains. This would be addressed by observing the kinetochore localization of GFP-Dvl2 constructs schematically shown in Figure 2E.

Minor criticism

1. For general readers, domain names of Dvl2 (DIX, PDZ, and DEP) may not be familiar and need explanation.

2. As there are three highly homologous Dsh homologues in human cells (Dvl1-3), comments on the functional redundancy among them on mitotic regulation would be helpful to readers.
3. Concerning Figure 5C, expression level of Bub1 and BubR1 in siDVL2 should be checked before concluding that Dvl2 is required for the recruitment of these proteins to kinetochores.

1st Revision - Authors' Response

06 July 2010

To all referees

We had many critiques from four referees and tried to respond to all of them. As a result, the revised manuscript has been changed. The most important change is that we reexamined most experiments using HeLaS3 cells, because the Editor also recommended that we should replace the original experiments with loss-of-function/complementation experiments using the same cell line. The functions of Dvl in mitosis were examined in Dvl2-depleted HeLaS3 cells. We also showed the roles of Dvl in mitosis using U2OS cells, because we would like to prove that our findings are not specific to HeLaS3 cells. However, because overexpression of Dvl deletion mutants caused HeLaS3 cells to die, the effects of Dvl deletion mutants on mitotic events in HeLaS3 cells could not be examined. Therefore, experiments with overexpression of deletion mutants of Dvl2 in U2OS cells were removed so that experiments were performed in the same cell line.

Another important change is the complex formation between Dvl2 and Mps1 in mitosis. Because original experiments were done in asynchronous HEK293T cells, the binding of Dvl2 and Mps1 was examined in asynchronous and mitotic HeLaS3 cells in the revised manuscript. As compared with asynchronous culture, the complex between Dvl2 and Mps1 was significantly reduced in mitosis. These results suggest that Mps1 forms a complex with Dvl2 in interphase and that they are dissociated in mitosis. It is likely that the binding of Dvl2 and Mps1 is not necessary for the activation of Mps1. Therefore, we have taken off our original statement that Dvl2 formed a complex with Mps1 and was involved in its activation in mitosis. In addition, the complex formation of Dvl2 with Bub1 and BubR1 was examined in asynchronous HEK293T cells in the original manuscript. These were experiments with overexpressed proteins, we removed them in revised version.

I hope that our responses including these changes are acceptable.

Referee #1

Here, Kikuchi and colleagues report that the pivotal Wnt signaling component, Dishevelled, plays multiple roles in the regulation of the mitotic spindle during mitosis. Although Dvl has previously been implicated in such functions the current study provides a detailed molecular analysis that describes novel interactions (Plk1 and Mps1), phosphorylation events and subcellular localizations that are thorough, convincing and interesting. Of note, the authors also demonstrate that LRP6 is involved in coordinating spindle function

Specific comments:

1. Figure 1: GFP-Dvl2 was used because anti-Dvl2 Ab's were not good enough to reveal endogenous localization during mitosis. Have the authors tried Dvl1/3 Ab's? More generally, since they function redundantly in Wnt signaling, it would be informative to have an idea of the relative expression levels of the three Dvl isoforms in the cell lines used in this study.

HeLaS3 cells expressed three Dvls. Because the sensitivity of each antibody seemed to be different, it is difficult to say which Dvl is abundant mostly in these cells (Supplementary Figure S14A). Depletion of either Dvl1 or Dvl3 also showed the phenotypes similar to that by deletion of Dvl2,

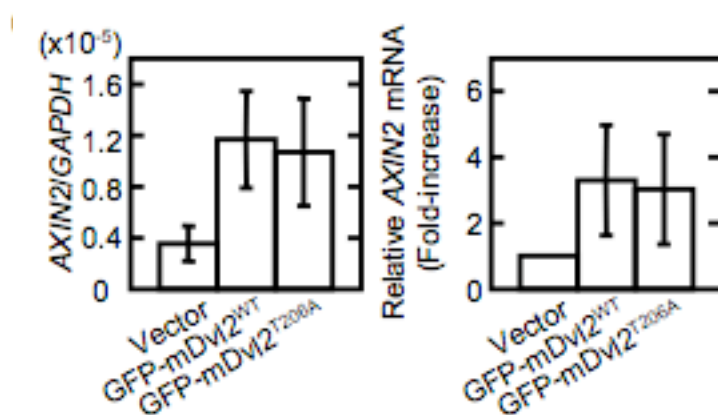
and the extent of the phenotypes by depletion of all of Dvl1, Dvl2, and Dvl3 were similar to that by depletion of Dvl2 (Supplementary Figure S14B and C). Because the efficiency of the depletion of Dvl1, Dvl2, and Dvl3 was more than 90%, we believe that Dvls do not act redundantly in mitosis. Therefore, Dvls may function as a heterotrimer, because it was shown that Wnt3a-dependent transcription is suppressed by depletion of either of Dvl1, Dvl2, or Dvl3 and that Dvl1 and Dvl3 are found in the Dvl2 immune complex (Cell. Signal., 20, 413-452, 2008). These are described in the text (page 14, lines 6 through 16).

2. Figure 2: the authors very nicely show mitotic phosphorylation of Dvl2 but it is not clear if the effect is Wnt dependent. As Wnt signaling is reported to be more active during mitosis, the authors should block this with e.g. Dkk and then ask if mitotic phosphorylation still occurs.

According to the referee's comment, we treated the cells with recombinant Dkk1 and examine the phosphorylation states of Dvl2. Dkk1 did not affect mitotic phosphorylation of Dvl2. Therefore, the phosphorylation of Dvl2 might be due to Plk1 but not to Wnt stimulation. However, because the phosphorylation was assessed by a mobility shift on an SDS-gel, if Wnt-dependent phosphorylation of Dvl occurs without its mobility shift, we can not exclude the possibility that Wnt induces the phosphorylation of Dvl, which is not indicated as a mobility shift. The results are shown in Supplementary Figure S2C and described in the text (page 5, the bottom through page 6, line 4, page 16, line 3 from the bottom through page 17, line 3).

3. Figure 4: TOPFLASH reporter assays should be shown for the various Dvl2 mutants shown in panel E.

As described above, experiments with overexpression of deletion mutants of Dvl2 in U2OS cells were removed. Therefore, the experiments that the referee requested are not essential in the revised version. If the referee does not mind, I would not like to show the results. Instead, effects of Dvl2^{WT} and Dvl2^{T206A} on the expression of endogenous *AXIN2* mRNA were examined in HeLaS3 cells. The results are shown below.



4. Figure 6: as for Fig. 2 A, Wnt signaling should be blocked in panel A to see if the LRP6 phosphorylation at mitosis is Wnt dependent.

It has been reported that LRP6 is phosphorylated by Cyclin Y/Pftk in mitosis (Dev. Cell, 17, 788-799, 2009). We confirmed these observations as shown in Figure 6A. Although Dkk1 has been shown to inhibit GSK-3-mediated LRP6 phosphorylation at Ser1490 in response to Wnt3a, our results showed that Dkk1 does not affect the LRP6 phosphorylation in double thymidine-MG132-blocked mitotic cells. The reason for that Dkk1 cannot inhibit the LRP6 phosphorylation might be due to that Ser1490 of LRP6 is phosphorylated by Cyclin Y/Pftk more dominantly than a Wnt signal. However, it is still possible that Wnt induces the phosphorylation of LRP6 in mitosis, because LRP6 is known to be phosphorylated other threonine and serine residues in response to Wnt

signaling. They are shown in Supplementary Figure S12B and described in the text (page 12, lines 11 through 13, page 17, lines 4 through 13).

General comment:

I fear the reader may be confused at times dealing with the various aspects of the reported function of Dvl2 during mitosis. It would therefore be helpful to move the illustration the authors present in the supplement (S5) to the main figures, for easy referral.

According to the referee's suggestion, Supplementary Figure S5 in the original manuscript was moved to Figure 7 in the revised manuscript.

Referee #2

Summary: Wnt signalling components have been extensively studied for their transcriptional regulatory role. This study brings forth the mitotic role of one more Wnt signalling component, Dishevelled. Recently, mitotic role for other members of Wnt Signalling have become apparent. The study is certainly of interest to a wide audience from both cell cycle and signalling fields. But the claims are unclear and some conclusions are not fully supported. An improved clarity of writing might help the authors. There are some incorrect interpretations of cited literature; its not clear if this is due to compromised clarity of writing or poor understanding of the literature. The manuscript is not ready for publication in the current format.

The authors show that Dishevelled localises to kinetochores and spindles poles and interacts with mitotic kinases, Plk1 and Mps1. Using mutants the authors demonstrate that Plk1 binds to Dvl2 and phosphorylates Dvl2. Significance of Plk1 interaction or phosphorylation is not clearly presented. Depletion of Dvl2 is shown to perturb the stable positioning of the bipolar spindle and spindle checkpoint proficiency. The authors find reduction in the levels of phosphorylated MPS1 and kinetochore-bound BubR1 and Bub1 in Dvl2 depleted cells indicating that Dvl2 depletion leads to checkpoint loss. Together the experimental findings show that Dvl2 plays a role in mitosis. Other claims for a role of Dvl2 is microtubule dynamics and microtubule attachment at cell cortex or kinetochore are not supported.

Details:

While its an important and interesting area of research, the findings in this manuscript are not fully supported. Areas where new experiments are critical to support the authors claims are listed below, in the order of seriousness of technical flaws.

1. Page 19: Dvl2 plays important role in regulating Mt-kt attachment or dynamics of MT plus-ends. No evidence has been shown to indicate a role for Dvl2 in chromosome attachment or regulation of MT dynamics.

We previously demonstrated the involvement of Dvl2 in the dynamics of MT in interphase (EMBO J. 29, 1192-1204, 2010). As shown in Figure 4A, EB1 signals at the plus-ends of spindle MTs were associated with the centromeres (ACA staining) on metaphase chromosomes in control HeLaS3 cells. A low concentration of nocodazole, which is known to reduce the dynamics of MTs, induced chromosome misalignment, but the MT-KT attachment was not affected in control cells. Chromosomes were misaligned on a metaphase plate with a failure of chromosome congression in Dvl2-depleted cells. In these cells, the EB1 signals were slightly reduced, but still associated with KTs. Nocodazole enhanced a failure of chromosome congression and caused the dissociation of MTs from KTs in Dvl2-depleted cells. We also observed MTs using an anti-b-tubulin antibody in the presence of a low concentration of nocodazole, because mature MT-KT attachments stabilize MTs called kinetochore-fibers (K-fibers). K-fibers disappeared in Dvl2-depleted cells treated with a low concentration of nocodazole, whereas K-fibers in control cells was resistant to reduction of spindle dynamics by nocodazole treatment. However, depletion of Dvl2 did not affect the amount of spindle MTs (by assessment of b-tubulin staining), spindle pole distance (by assessment of g-

tubulin staining), and MT nucleation activity (by assessment of EB1 staining). The results suggested that Dvl2 is involved in the MT-KT attachment. The results are shown in Figure 4A and B and Supplementary Figure 7A-C and described in the text (page 8, line 4 from the bottom through page 9, line 14).

2. Dvl2's role in spindle checkpoint is also novel and interesting but not convincing. If Dvl2's involvement in spindle checkpoint is true, one would expect Dvl2 depleted cells to undergo premature chromosome segregation and accelerated mitotic exit and this is not the case as shown in Figure 4B. The only piece of convincing evidence for supporting Dvl2's checkpoint role with a good rescue plasmid is shown in Fig 5B. Unfortunately even this study has been done in a cell line U2OS which is known to have a poor checkpoint response to Nocodazole. The authors will benefit repeating this in HeLa cells since they present a more robust checkpoint response. The authors should explain what relative Mpm2 positive cells mean and also include the data from (-Noc) experiments.

When cells were treated with nocodazole to disrupt MTs, GFP-Dvl2 clearly accumulated at KTs. In addition, depletion of Dvl2 reduced the numbers of mitotic cells increased by the treatment with nocodazole or taxol. If the SAC is intact, mitotic index must be increased. These are the results that we speculated the involvement of Dvl in SAC. According to the referee's comment, we performed rescue experiments in Dvl2-depleted HeLaS3 and U2OS cells treated with nocodazole. Expression of GFP-mouse Dvl2^{WT} rescued the phenotype of Dvl2-depleted cells. The results were shown in Figure 5A and Supplementary Figure 10B and described in the text (page 10, lines 2 through 9 from the bottom).

Relative Mpm2 positive cells meant the ratio of Mpm2 positive cells in Dvl2-depleted cells to those in control cells in the original manuscript. However, because the results were obtained in U2OS cells, we removed them in the revised manuscript.

3. The authors claim using Fig 5D that DVL2 is required for activation of MPS1 is not fully supported. This reduced MPS1 activation might be an indirect consequence of Dvl2's loss. As shown in fig 5B if siDVL2 cells show nearly a 50% reduction in mitotic index it's expected that phospho-MPS1 signal would reduce as well. A control for this experiment would be to perform depletion of a known checkpoint component downstream of Mps1 (such as siBUBR1 treatment) and ask if phospho-MPS1 is retained or reduced when the checkpoint is compromised.

We carefully repeated this experiment. The upper band (phosphorylated active form) of Mps1 was reduced, and the lower band (unphosphorylated inactive form) of Mps1 was increased in Dvl2-depleted cells as compared with control cells, while depletion of Dvl2 did not affect the total amount of Mps1. Consistent with this result, the activation of Mps1 monitored by the phosphorylation of Cenp-A at Ser7 was decreased in the Dvl2-depleted cells. The results are shown in Figure 5B and C and described in the text (page 11, lines 5 through 8).

According to the referee's comment, we also examined effects of depletion of BubR1 on the Mps1 phosphorylation. Depletion of BubR1 did not affect the level of phosphorylated (the upper band of Mps1) Mps1, rather slightly increased the total amount of Mps1 (the sum of upper and lower bands of Mps1) slightly. Therefore, phosphorylated Mps1 was reduced specifically in Dvl2-depleted cells and it is likely that Mps1 acts downstream of Dvl2 and independent or parallel of BubR1. These results are shown in Figure 5B and Supplementary Figure 11A and described in the text (page 11, lines 8 through 12).

4. The finding of Dvl2's localisation at kinetochores and spindle poles is novel. However, localisation of GFP-Dvl2 in live cells is needed to exclude any fixation artifacts. Because the authors have the ability of perform live-cell imaging and have access to the Dvl2-Gfp plasmid, this experiment might easily help strengthen their finding further

A live-imaging experiment of the subcellular localization of GFP-Dvl2 in mitosis was performed. GFP-Dvl2 was visible at the spindle poles in living cells. The localization of GFP-Dvl2 to KTs in

living cells was difficult to be detected. Even in fixed cells, GFP-Dvl2 was detected on KT's clearly when the cells were treated with nocodazole to activate SAC as shown in Figure 1C. The results are shown in Supplementary Movie 1 and Supplementary Figure S1B and described in the text (page 5, lines 8 through 12 from the bottom).

5. Using mutant studies they show strong evidence for interaction between Dvl2 and Plk1 however interaction between Mps1 and Dvl2 is not strongly presented. While this might not be important from the scope of the current paper, the authors should at least show a reverse immunoprecipitation of pulling down Mps1 and testing Dvl2's interaction in mitotic cells.

Because original experiments were done in asynchronous HEK293T cells, the binding of Dvl2 and Mps1 was examined in asynchronous and nocodazole-arrested HeLaS3 cells. As compared with asynchronous culture, Mps1 immunoprecipitated with Dvl2 was reduced in nocodazole-arrested cells. In addition, Mps1 did not immunoprecipitate Dvl2 in a reciprocal experiment. These results suggest that Mps1 forms a complex with Dvl2 in interphase but that they may be dissociated in mitosis. Therefore, it is unlikely that the binding of Dvl2 and Mps1 is necessary for the activation of Mps1. Dvl2 dissociated from Mps1 may attach to KT and be involved in the recruitment of Bub1 and BubR1 to KT. The results are shown in Figure 5E and described in the text (page 11, lines 5 through 7 from the bottom, page 15, lines 7 through 10 from the bottom).

6. Interpretation of inter-kinetochore tension using inter-kinetochore distances measured in fixed cells is not convincing. In fixed cells when the cells are dead there are no active forces to pull and separate the kinetochores for maintaining tension. Therefore using this experimental design to claim the extent of inter-kinetochore tension is incorrect. To quantitate the extent of inter-kinetochore tension the authors need to measure inter-kinetochore distances in live-cells expressing a kinetochore marker such as CenpB-GFP or CenpA-GFP.

According to the referee's comment, we measured the inter-KT distance using live-HeLa cells stably expressing GFP-Cenp-A. The results are shown in Figure 4C and Supplementary movies 6 and 7 and described in the text (page 9, lines 7 through 12 from the bottom).

Referee #3 (Remarks to the Author):

Components of the Wnt/beta-catenin pathway have been implicated in the proper progression through mitosis. Best characterized among these is Adenomatous Polyposis Coli (APC), an important regulator of beta-catenin degradation, which stabilizes microtubules and clusters at their plus-ends. APC is required for proper spindle orientation during mitosis and also mediates chromosome congression by modulating KT-MT attachment.

The Wnt pathway component Dishevelled, a modulator of beta-catenin dependent and independent signaling, has been shown to be required for MT stability in neurons. In addition, recent work by the Kikuchi lab demonstrated a direct interaction between APC and Dishevelled (Matsumoto et al., EMBO J 2010), hinting to a shared role in regulating microtubule dynamics, possibly during mitosis as well.

In this manuscript, Kikuchi et al. now describe a role for Dishevelled 2 (Dvl2) and the Wnt pathway components Lrp6 and Fz2 in mitosis. The paper makes four major claims: (A) Dvl2 is required for proper placement of the spindle during mitosis and for proper stability of kinetochore-microtubules. (B) Dvl2 is regulated by Plk1. (C) Dvl2 plays a role in the spindle assembly checkpoint. (D) More Wnt pathway components (Lrp6, Fz2) are involved in mitosis, whereas yet others (Ror2, Wnt5a) are not.

In principle, I think it is interesting to see that Dvl2 also plays a role in mitosis. However, I feel that many of the claims the authors make in the paper are not sufficiently substantiated, as outlined below. Some experiments are not conducted in the most meaningful way and/or are not well controlled. In addition, the authors continuously switch between cell lines, making it hard to interpret the data. In addition, there are a lot of minor weaknesses (unsatisfactory pictures, experiments not well explained, reasons to perform the experiment unclear, imprecise wording,

incorrect citations, incomplete experimental procedures). Because of all this, I get the overall feeling that I cannot really 'trust' the data, which considerably dampens my enthusiasm for this potentially interesting story.

Major comments:

1. Given the fact that the APC RNAi (shown previously and here) and Dvl2 RNAi phenotypes are very similar and that the authors have previously reported a direct interaction between the two, I think it would be important to demonstrate whether total cellular levels and/or localization of APC are affected in Dvl2 RNAi cells or vice versa. In addition, the authors should check whether an interaction between APC and Dvl2 can be detected during mitosis, and if so, whether disruption of the interaction yields similar phenotypes. This would greatly help to put the new data in the context of the existing literature on the role of Wnt pathway proteins in mitosis and would help to validate the models that the authors put forward in Figure S5.

We have already shown that the protein levels of APC are not affected in Dvl2-depleted cells (EMBO J. 29, 1192-1204, 2010, Supplementary Figure S6A). The reviewer probably knows that it is difficult to see endogenous APC in mitotic cells by a immunocytochemical study at present. Whether the binding of endogenous Dvl and APC affects their subcellular localization in mitosis will be clarified in the future. The biochemical interaction between APC and Dvl2 was observed in mitotic HeLaS3 cells. The results are shown in Figure 4E and described in the text (page 10, lines 6 and 7).

To disrupt the interaction of APC with Dvl2 at the endogenous level, the armadillo domain of APC (APC^{Arm+}), which interacts with Dvl and inhibits the binding of APC and Dvl, was expressed in HeLaS3 cells and its effect on the spindle rotation and the inter-KT distance was examined. Expression of APC^{Arm+} increased the spindle angle and reduced the inter-KT distance, suggesting that the binding of Dvl2 and APC is necessary for the correct spindle dynamics. The results are shown in Figures 3C and 4D and described in the text (page 10, lines 8 through 10).

2. Can the authors comment on Dvl1 and Dvl3? Have the authors tested whether they have a role in mitosis as well?

HeLaS3 cells expressed three Dvls. Because the sensitivity of each antibody seemed to be different, it is difficult to say which Dvl is abundant mostly in these cells. Depletion of either Dvl1 or Dvl3 also showed the phenotypes similar to that of Dvl2, and the extent of the phenotypes by depletion of all of Dvl1, Dvl2, and Dvl3 were similar to that by the depletion of Dvl2. Because the efficiency of the depletion of Dvl1, Dvl2, and Dvl3 was more than 90%, we believe that Dvls do not act redundantly in mitosis. Therefore, Dvls may function as a heterotrimer, because it was shown that Wnt3a-dependent transcription is suppressed by depletion of either of Dvl1, Dvl2, or Dvl3 and that Dvl1 and Dvl3 are found in the Dvl2 immune complex (Cell. Signal., 20, 443-452, 2008). The results are shown in Supplementary Figure S14A, B, and C are described in the text (page 14, lines 6 through 16).

3. The authors claim that Dvl2 is required for kinetochore-microtubule attachment (see e.g. abstract). Firstly, I would formulate this more carefully, since in the complete loss of stable attachment, one would expect the chromosomes to scatter much more on and around the spindle (see e.g. DeLuca/Salmon JCB 2002), whereas in Dvl2 RNAi there is relatively good chromosome alignment (see e.g. Figure 4G), indicating that kinetochore-microtubule attachment is still relatively stable. Furthermore, the statement is solely based on Figure 4F, in which the authors only investigate EB1, but not microtubules. Why not looking at tubulin staining, if the authors want to claim that there are less or less stable kinetochore-microtubules? The fact that the authors see less EB1 adjacent to kinetochores under these conditions could also mean that there is a problem with EB1 localization. Therefore, (a) the authors should show microtubule-staining after short nocodazole-treatment (as in Figure 4F), and (b) it would be useful to evaluate EB1 (and APC) localization in Dvl2 RNAi cells.

EB1 signals at the plus-ends of spindle MTs were associated with the centromeres (ACA staining) on metaphase chromosomes in control HeLaS3 cells. The low concentration of nocodazole induced chromosome misalignment, but the MT-KT attachment was not affected in control cells. As the referee said, MT-KT attachment might not be disrupted completely by depletion of Dvl2, because EB1 signals at the plus-ends of spindle MTs were slightly reduced, but still associated with the KTs in Dvl2-depleted cells in the absence of a low concentration of nocodazole as shown in Figure 4A in the revised manuscript. However, chromosome alignment (congression) was immature in Dvl2-depleted cells, because the depletion of Dvl2 increased prometaphase cells, in which chromosome alignment is immature, and reduced metaphase cells, in which chromosomes are completely aligned at metaphase plate as shown in Supplementary Figure S9. This suggested that MT-KT attachment might be less stable in Dvl2-depleted cells. To confirm this, original experiments in Figure 4F (Figure 4A in the revised manuscript) were done. The results are shown in Figure 4A and Supplementary Figure S9 and described in the text (page 8, line 4 from the bottom through page 9, line 4, page 10, lines 13 and 14 from the bottom).

The reason why we used anti-EB1 antibody in this experiment was that the EB1 staining renders the MT-KT attachment visible clearly because EB1 tends to be concentrated to the plus-end of MTs. According to the referee's suggestion, we also stained the cells with an anti- β -tubulin antibody in the presence a low concentration of nocodazole. We found that K-fibers in Dvl2-depleted cells were dramatically reduced in the presence of a low concentration of nocodazole. We think that this result strengthened a role of Dvl2 in MT-KT attachment. The results are shown in Figure 4B and described in the text (page 9, line 5 through 9).

As I already respond to comment 1, we would like to examine the subcellular localization of endogenous APC and EB1 as the future study.

4. The authors claim that phosphorylation of Dvl2 (by Plk1) is required for spindle orientation and microtubule-kinetochore attachment. The evidence for this is that Dvl2 is phosphorylated on T206 by Plk1 in vitro, that mitotic phosphorylation of Dvl2 is reduced in Plk1 RNAi cells and that overexpression of the Dvl2-T206A mutant causes a spindle orientation defect as well as a reduced inter-KT distance. Additional experiments are required to confirm this. Most importantly, testing of the Dvl2 mutants should be done in an RNAi complementation experiment, which in my opinion can nowadays be considered 'standard'. Instead the authors use an overexpression assay, which is more difficult to interpret. It should be determined whether defective spindle orientation and weak kinetochore-microtubule attachment are seen in Dvl2 RNAi cells, which have been complemented with near-endogenous levels of Dvl2-T206A? What is the localization of Dvl2-T206A in Dvl2 RNAi cells? Does the protein localize to kinetochores?

We agree to the referee's comment and expressed Dvl2^{WT} or Dvl2^{T206A} in Dvl2-depleted cells. Exogenous GFP-mouse (m) Dvl2^{WT} and GFP-mDvl2^{T206A}, which are resistant to siRNA for human Dvl2, were expressed in Dvl2-depleted HeLaS3 cells. GFP-mDvl2^{WT} rescued spindle misrotation and reduction in the inter-KT distance induced by depletion of Dvl2, but GFP-mDvl2^{T206A} could not rescue them. The results are shown in Figures 3C and 4D and Supplementary Figure S6A and described in the text (page 8, lines 2 through 6, page 9, lines 4 through 6 from the bottom).

GFP-mDvl2^{T206A} localized to spindles and spindle poles in Dvl2-depleted cells. When cells were treated with nocodazole to activate SAC, GFP-mDvl2^{T206A} localized to KTs in Dvl2-depleted cells as well as GFP-mDvl2^{WT}. The results are shown in Supplementary Figures S6B and C and S10A and described in the text (page 8, lines 6 through 8, page 10, lines 3 and 4 from the bottom).

Secondly, one would like to know whether Plk1 RNAi cells actually show a spindle orientation defect. If so, can this be rescued by expression of Dvl2-T206D/E? Does the localization of Dvl2 change in Plk1 RNAi?

Plk1 is known to be required for the recruitment of γ -tubulin to mitotic centrosomes and for the formation of functional bipolar spindles (van Vugt *et al.*, J Biol Chem., 279, 36841-36854, 2004). Inhibition of Plk1 causes defective centrosome and monopolar spindles. As shown in Figures 3A, 4A, and 4D, bipolar spindles were observed in Dvl2-depleted cells. Therefore, the phenotype

induced by the depletion of Plk1 must be more severe than that induced by the depletion of Dvl2. Therefore, we do not think that Dvl2 can rescue spindle orientation defect in Plk1-depleted cells. Further, the localization of Dvl2 could be changed in Plk1-depleted cells. As shown in Figure 3C, Dvl2^{T206A} could not rescue the spindle rotation in Dvl2-depleted cells. It is possible that Dvl2 mediates Plk1 functions partly in spindle assembly. We would like to examine whether the phosphorylation of Dvl2 affects its localization in mitotic cells as a separate study.

5. The authors claim that Dvl2 is required for SAC activation through Mps1 activation. SAC defects are a frequent side effect of RNAi (Hü bner et al. 2010). It is therefore essential to perform rescue experiments using ectopically expressed Dvl2 to exclude this possibility. The authors do this for U2OS cells, but it would be good to also show such an experiment for HeLa cells, with which more of the experiments have been performed.

According to the referee's comment, rescue experiments were performed in Dvl2-depleted HeLaS3 cells. Expression of Dvl2^{WT} increased mitotic index that was reduced in nocodazole-treated Dvl2-depleted cells. The similar results were also observed using U2OS cells. The results are shown in Figure 5A and Supplementary Figure S10B and described in the text (page 10, lines 2 through 9 from the bottom).

The claim that Dvl2 acts through Mps1 is based on the author's finding that Mps1 and Dvl2 interact, as judged by co-IP, and that phosphorylation of Mps1 is somewhat reduced after Dvl2 RNAi, although this is not very clear (Figure 5E). The fact that Mps1-Dvl2 interaction is detected in asynchronous cells (Figure 5D) indicates that it might not be checkpoint-activation specific. In addition, the authors are under the assumption (stated in the text) that Mps1 is required for Bub1 and BubR1 localization to kinetochores, which is also impaired in Dvl2 RNAi. However, there is actually agreement in the literature that in human cells (unlike in Xenopus), Mps1 is not needed for Bub1 and BubR1 kinetochore localization (Martin-Lluesma/Nigg, Science 2002; Liu/Yen, MBC 2003; Jelluma/Kops, Cell 2008 and citations therein). Thus, even if Mps1 activity is reduced, Dvl2 must act on the checkpoint through some other mechanism.

If the authors want to claim that Mps1 activity depends on Dvl2, this would need to be tested more directly, e.g. by in vitro phosphorylation assays (Stucke/Nigg, Chromosoma 2004) in the absence or presence of Dvl2 or by looking at CENP-A-phS7, which depends on Aurora kinase activity, which in turn depends on Mps1 activity (Jelluma/Kops, Cell 2008). Furthermore: is the interaction between Mps1 and Dvl2 actually needed for checkpoint activity? In the discussion, the authors speculate that Mps1 and Dvl2 interact through the PDZ domain in Dvl2. This could easily be shown, and the Dvl2-delta-PDZ mutant could be tested in an RNAi complementation assay for checkpoint activity.

We carefully repeated the experiments in original Figure 5D and E. The upper band of Mps1 was reduced, and the lower band of Mps1 was increased in Dvl2-depleted cells as compared with control cells. Depletion of Mps1 did not affect the total amount of Mps1. Consistent with this result, the activation of Mps1 monitored by the phosphorylation of Cenp-A at Ser7 was decreased in the Dvl2-depleted cells. The results are shown in Figure 5B and C and described in the text (page 11, lines 5 through 8).

Because original experiments were done in asynchronous HEK293T cells, the binding of Dvl2 and Mps1 was examined in asynchronous and mitotic HeLaS3 cells. As compared with asynchronous culture, Mps1 immunoprecipitated with Dvl2 was reduced in mitosis. In addition, Mps1 did not immunoprecipitate Dvl2 in a reciprocal experiment. These results suggest that Mps1 forms a complex with Dvl2 in interphase and that they are dissociated in mitosis. It is unlikely that the binding of Dvl2 and Mps1 is necessary for the activation of Mps1. The results are shown in Figure 5E and described in the text (page 11, lines 5 through 7 from the bottom).

Although some papers indeed suggested that Mps1 is not needed for Bub1 and BubR1 kinetochore localization, it has also been reported that Mps1 is required for the kinetochore localization of BubR1 in human cells (PNAS, 104, 20232-20237, 2007). Taken together, Dvl2 appears to be involved in the activation of Mps1 and the localization of Bub1 and BubR1 to the KTs, but it is unlikely that Dvl2 functions as a scaffold protein to recruit Mps1, Bub1, or BubR1. In addition, Dvl2 dissociated from Mps1 may attach to the KTs and be involved in the recruitment of

Bub1 and BubR1 to the KTs. These are described in the text (page 15, lines 11 through 14 from the bottom, page 15, lines 1 through 3 from the bottom).

6. For RNAi experiments, the specificity should be tested by using several siRNAs and by showing that the phenotypes can be alleviated by expression of an RNAi-resistant construct. The authors apparently used two different siRNAs for some of the knock-downs (see siRNA list in Supplement; not mentioned in the text), but not for all (e.g. not for Dvl2). In Figure 5B, the authors show rescue of the Dvl2 RNAi-induced checkpoint phenotype by exogenous Dvl2 in U2OS cells, but do not provide such data for any of the other experiments/cell lines. In addition, it is customary, and it would be good if the authors could provide data indicating how efficient their RNAi is (e.g. levels reduced to at least xx %, based on immunoblotting).

We expressed GFP-mDvl2^{WT} in Dvl2-depleted HeLaS3 cells and examined whether they can rescue the phenotypes (spindle orientation, inter-KT distance, and mitotic index) induced by depletion of Dvl2. GFP-mDvl2^{WT} could rescue all the phenotypes induced by depletion of Dvl2. The results are shown in Figures 3C, 4D, and 5A and described in the text (page 8, lines 2 through 4, page 9, lines 5 and 6 from the bottom, page 10, lines 5 through 7 from the bottom).

siRNA for Dvl2 reduced the expression levels of Dvl2 to less than 10% in HeLaS3 cells. The results are shown in Supplementary Figure S14A.

Detailed comments:

- Title: 'controls spindle dynamics' is a very fuzzy term and I think the authors should re-think what they actually want to say here

We changed the title to “Dishevelled, a Wnt signaling component, is involved in mitotic progression in cooperation with Plk1.”

- Abstract and end of introduction: The last sentence '... mediates Wnt-dependent and -independent regulation... through the phosphorylation by Plk1' is extremely confusing and in my opinion incorrect. Please re-phrase

We changed the last sentence of abstract and introduction.

- Several citations are in my opinion not appropriate: (1) Since Plk1 is one of the major topics of this paper, I feel that some primary literature should be cited in addition to the Petronczki et al. Dev Cell 2008 review (in particular, since EMBO Journal has the policy to allow as many citations as needed).

We cited the following papers concerning Plk1 (Arnaud *et al.*, Chromosoma, 107 424-429, 1998; Sumara *et al.*, Mol. Cell, 9, 515-525, 2002; Elowe *et al.*, Genes Dev., 21, 2205-2219, 2007; van Vugt *et al.*, J Biol Chem., 279, 36841-36854., 2004).

(2) The authors cite Kang et al. 2007 for the fact that Mps1 is required for SAC activation. This is by no means the first paper showing this and is only one out of several papers that map Mps1 phosphorylation sites. The citation should be changed.

We cited the following papers concerning Mps1 (Abrieu, A. *et al.*, Cell, 106, 83-93, 2001; Stucke, VM. *et al.*, EMBO J., 21, 1723-1732, 2002; Liu, S. *et al.*, Mol. Biol. Cell, 14, 1638-1651, 2003).

(3) The authors cite Abrieu et al. 2001 and Vigneron et al. 2004 for the fact that Bub1, BubR1, Mad1 and Mad2 localization depend on Mps1. However, the work in these papers has been performed in Xenopus. In human cells, the localization dependencies are in fact different and this is quite relevant for the arguments in this manuscript. Please correct text and citations.

Although Liu et al. (Mol. Biol. Cell, 14, 1638-1651, 2003) indeed suggested that Mps1 is not needed for the localization of Bub1 and BubR1 to KT, it has also been reported that Mps1 is required for the localization of BubR1 to KT in human cells (Kang, J. et al. PNAS, 104, 20232-20237, 2007). We cited both sides of papers concerning a role of Mps1 in the recruitment of KT proteins (page 15, lines 11 through 14 from the bottom).

(4) The authors cite three papers for the fact that reduced localization of Bub1 and BubR1 to kinetochores affects kinetochore-microtubule attachment. The first of these citations Jelluma/Kops 2008 is in my opinion not correct.

We removed the paper.

(5) Jelluma/Kops PLoS one 2008 should be cited for Mps1 autophosphorylation in addition to Kang et al. and Mattison et al. 2007.

We cited the paper that the referee pointed out.

(6) A reference should be provided for dominant-negative Tcf4 (TcfDN).

We cited a paper describing a dominant-negative Tcf4 (Tetsu, O, and McCormick, F., Nature, 398, 422-426, 1999).

- Figure 1A: An immunoblot for the expression level of GFP-Dvl2 (relative to endogenous Dvl2) should be provided.

We showed the expression level of GFP-Dvl2 in HeLaS3 and U2OS cells in Supplementary Figure S1A. The results are described in the text (page 5, lines 6 and 7, page 5, lines 8 and 9 from the bottom).

- Figure 1C: The upper three pictures seem of extremely low quality. I am not sure what I am supposed to see here. Please replace these pictures. In addition, show whole cells for the staining shown at the bottom (ACA/GFP-Dvl2). See e.g. Zuccolo..Doye EMBOJ 2007, Meraldi..Sorger EMBOJ 2005 for examples of kinetochore stainings from nocodazole-arrested cells.

We replaced the original pictures with better pictures observed in HeLaS3 cells, and showed whole cells for the staining for centromere (ACA) and GFP-Dvl2.

- Figure 1B: In the text, the authors speak of prometa- and metaphase, but it remains unclear which of the pictures represents what. Is there any difference between the upper and lower picture? Is it necessary to show both?

We removed this figure in the revised manuscript.

Figure 1B: I can neither see kinetochore staining in Figure 1A nor in the Supplementary Movie 1. Why is it visible in Figure 1B? Are the cells treated differently? Is the staining extremely weak compared to the spindle signal for Dvl2?

As the referee said, the staining signals of GFP-Dvl2 for spindle poles and spindles were strong. It was difficult to detect the localization of GFP-Dvl2 to KTs in small pictures such as Figure 1A. According to the referee's comment, we replaced the original pictures with better pictures observed in HeLaS3 cells in Figure 1B.

- Supplementary Movie 1: Since GFP-Dvl2 looks quite different in this unfixed cell compared to the fixed cells (Figure 1A/B), I think it is useful to show this data. However, in my opinion it doesn't make sense to show a movie for a metaphase-'arrested' cell, which does not change in appearance throughout the movie. Please show a single still image from the movie instead, and explain the difference in GFP-Dvl2 signal.

According to the referee's suggestion, a single still image from the movie is shown in Supplementary Figure S1B. Live-image analysis showed that GFP-Dvl2 was observed to localize to the spindle poles mainly, but it was hard to see the localization of GFP-Dvl2 to the KTs. This appearance of GFP-Dvl2 in the unfixed cells was different from that of the fixed cells. These statements are described in the text (page 5, lines 10 through 12 from the bottom).

- Figure 2B: Which cells were used?

We used HeLaS3 cells.

- Figure 2C: Please show the data for CDK1 and Aurora A, which could serve as a negative control for this experiment.

We showed Dvl2 forms a complex with Plk1 at endogenous levels in mitotic HeLaS3 cells (Figure 2C). We also demonstrated that Dvl2 did not form a complex with CDK1 or Aurora A in HEK293 cells even though they were overexpressed. The results are shown in Supplementary Figure S2D and described in the text (page 6, lines 7 and 8).

- Figure 2D: Why are HEK cells used here, and not HeLa cells (as in Figure 2C)?

We first showed that Dvl2 interacts with Plk1 at endogenous level in HeLaS3 cells. Original experiments in Figure 2D and E were performed to examine which regions of Dvl2 and Plk1 are required for their binding. Because overexpression of Dvl deletion mutants caused cell death of HeLaS3 cells, HEK293 cells were used in this experiment. These figures were moved to Supplementary Figure 3A and B.

- Figure 2E: Mutants #5 and #7, which do not show an interaction, have the weakest band in the input, raising the question whether they cannot be detected in the co-IP because of their low abundance. Would it be possible to adjust the amounts? Or show the reciprocal GFP-IP.

It was difficult to adjust the expression levels of all of different GFP-Dvl2 deletion mutants with HA-Plk1. The expression levels of # 5 and #7 were similar to that of #2. Under the conditions, only #2 could associate with Plk1.

Figure 2E: Is region 354-423 sufficient for binding? Does it contain a motif that fits the sequence that has been reported for Polo-box binding?

In this study we did not analyze the functional significance of the binding between Dvl2 and Plk1. Therefore, whether the region aa 354-423 of Dvl2 is sufficient for the binding to Plk1 was not examined.

Dvl2(354-423) does not contain the typical consensus sequence, [Pro/Phe]-[F/Pro]-[F/Ala/Gln]-[Thr/Gln/His/Met]-Ser-[pThr/pSer]-[Pro/X], but has Ser-Ser-Ser, Ser-Ser-Met, and Ser-Ser-Leu. This statement is described in the text (page 6, lines 13 through 15)

- Figure 3A: MBP-Dvl2 - presumably - strongly degrades. How do the authors know that the uppermost band is full length MBP-Dvl2? How do they know that the non-phosphorylated products lack the C-terminus (as stated in the text)? Did they perform an MBP immunoblot?

So far we have used this construct in various experiments. From the molecular weight, the uppermost band is likely to be full length MBP-Dvl2. Of course, when it lacks one or two amino acids in the C-terminal, it is difficult to know that the uppermost band is a full length protein. MBP was fused to the N-terminus of Dvl2 in this construct. Because Coomassie Brilliant Blue staining bands were recognized by an anti-MBP antibody (data not shown), we thought degradation products lack the C-terminal region.

- Figure 3: It remains unclear whether the C-terminus of Dvl2 could also be phosphorylated by Plk1. I would suggest to additionally express the fragment 434-stop and test it in an in vitro phosphorylation assay as well. Additionally or alternatively, the authors should test whether full length Dvl2-T206A is phosphorylated or not.

As pointed out by the referee, we could not exclude the possibility that the C-terminal region of Dvl2 is phosphorylated. The important finding in the present study is that T206 of Dvl2 is one of the phosphorylation sites by Plk1 and that Dvl2^{T206A} does not rescue the spindle rotation and the inter-KT distance but do the SAC in Dvl2-depleted cells as shown in Figures 3C, 4D, and 5A. We will find additional Plk1-dependent phosphorylation sites in the future study.

- Figure 3: The figure could be shortened. Figure part B, for example, could be left out without loss of clarity.

According to the referee's comment, Figure 3B and D is moved to Supplementary Figure S4. Furthermore, Figure 2D and E was moved to Supplementary Figure S3. The remaining pictures in original Figures 2 and 3 were combined into revised Figure 2

- Figure 4A: Even when looking at the Materials and Methods section, it remains unclear how this quantification was performed. In the upper part of the Figure, it looks like the boxes are positioned in some angle to the centrosome-centrosome axis, which in my opinion doesn't make sense. Shouldn't they always be aligned with this axis, i.e. directly adjacent to the SPBs? What was the size of the boxes? What kind of projection of the Z-stack (max. intensity? average? sum projection?)? How do the authors place the region for the spindle MT area (mentioned in Mat. and Methods)? In addition, the calculation mentioned in Mat. and Methods seems odd. In my opinion the calculation should be: (intensity of astral MT area) / (intensity of astral MT area + intensity of spindle MT area). In addition, why was taxol used in the fixation procedure?

To exclude the effects of spindle angle on MT intensity, we measured the intensities of the total and spindle MT areas as shown in Figure 3A and calculated (intensity of the total MT area – intensity of

the spindle MT area)/intensity of the MT spindle area as the relative astral MT intensity. The original experimental procedures came from the following paper (Thoma et al., Nat. Cell Biol., 11, 994-1001, 2009).

Taxol was used to protect astral MTs from depolymerization during pre-extraction step, because astral MTs are relatively unstable and invisible compared with spindle MTs. The original experimental procedures also came from Thoma et al., Nat. Cell Biol., 11, 994 -1001, 2009.

- Figure 4B: The authors state in the text, that one of the daughter cells 'fails to maintain adhesion... after cell division'. However, from the movie, it is clear that these cells eventually establish adhesion. I would therefore rather say that the cells 'fail to immediately gain adhesion to the substratum'. In the movie, it looks like the cells that fail to gain adhesion were overlapping with another adhered cell, whereas the cells that immediately gain adhesion were not. Is this general? Is the same phenotype observed when no other cell is adjacent to the dividing cell? Please also provide some quantification on how often this phenotype was seen (xx out of yy dividing cells).

We changed the statement to “the cells fail to immediately gain adhesion to the substratum”. The phenotype pointed out by the referee was quantified. Approximately 37% of Dvl2-depleted dividing cells (10/27) failed to gain adhesion and were overlapping with another adhered cell, whereas only 10% of control cells showed the phenotypes. The results are shown in Figure 3B and described in the text (page 7, lines 9 through 13 from the bottom).

When no other cell was adjacent to the dividing cell, one of the daughter cell did not maintain adhesion. The results are shown in Supplementary Figure 5B and described in the text (page 7, lines 8 and 9 from the bottom).

- Figure 4C/D/E: The authors look at two different spindle orientation phenotypes here, namely spindle rotation and spindle displacement (see e.g. Draviam/Sorger EMBO J 2006). I have the feeling that the authors do not make a clear distinction in the text, which at first confused me. Also, why analyzing all the mutants for one phenotype (spindle rotation), but not the other (displacement). Could the authors provide a quantification for Figure 4D as well?

We used a “word” spindle misorientation as the mean of rotation of the spindle axis within the cells. According to the referee’s comment, the spindle displacement in living cells was also quantified. The results are shown in Figure 3D and Supplementary movies 4 and 5 and described in the text (page 7, line 4 through 7 from the bottom, page 8, lines 10 through 15).

- Figure 4C and 4E: This is a good example for the use of different cell lines by the authors. Why using HeLa cells in 4C and U2OS cells in 4E? Couldn't this all be done in the same cell line? (In which case, the Figure parts could be combined).

The reason why we used U2OS cells in original Figure 4E is that overexpression of Dvl deletion mutants in HeLaS3 cells induces cell death but not in U2OS cells. Expression of a Dvl mutant with substitution of a single amino acid (Dvl2^{T206A}) did not affect cell growth of HeLaS3 cells. Therefore, we performed rescue experiments with GFP-mDvl2^{WT} and GFP-mDvl2^{T206A} in Dvl2-depleted HeLaS3 cells. GFP-mDvl2^{WT} rescued the spindle rotation but GFP-mDvl2^{T206A} did not. The results are shown in Figure 3C and described in the text (page 8, lines 2 through 4).

We removed experiments with overexpression of Dvl2 deletion mutants in U2OS cells so that we would like to emphasize the role of the phosphorylation of Dvl2 by Plk1 in spindle rotation.

- Figure 4E: Firstly, as mentioned above, this experiment should be performed using RNAi/complementation rather than overexpression. In addition, the authors have to provide data on how well each of the Dvl2 mutants was expressed. It would furthermore be helpful if the authors checked the localization of these mutants.

To do all the experiments in HeLaS3 cells, we removed the experiments with overexpression of Dvl2 mutants in U2OS cells. The localization of GFP-mDvl2^{T206A} was shown in Supplementary Figures S6C and S10A.

- Figure 4G: The Dvl2-delta-PDZ mutant (used in Figure 4E) has previously been shown to be deficient in stabilizing microtubules in interphase (Krylova et al. 2001, Ciani et al. 2004). It would be interesting to see whether it has an effect on inter-KT distance.

As describe above, it was difficult to express Dvl2DPDZ in HeLaS3 cells because it caused cell death. Since we are also interested in the effects of this mutant in the inter-KT distance, we will check it in appropriate cells as a future study.

- Figure 4G: The pictures are not very high quality, and it is unclear to me how the authors can pick kinetochore pairs from these pictures. See e.g. Stumpff/Wordeman Dev Cell 2008, Figure 2, for a similar experiment.

We replaced the original pictures with better ones. We carefully identified each KT pair from Z-stack images from 0.2 mm-thick sections of a cell. To support this result, we added new data using living HeLa cells stably expressing GFP-Cenp-A. The results are shown in Figure 4C and D and described in the text (page 9, lines 7 through 12 from the bottom).

- Text corresponding to Figure 4G: Reduced inter-KT tension may lead to - but does not necessarily lead to - a SAC-dependent mitotic delay. It has recently been shown that reduced 'intra-KT' stretching is more crucial than reduced inter-KT tension for activating the SAC (Maresca/Salmon and Uchida/Hirota, JCB 2009). In my opinion, the text therefore needs to be re-formulated.

We re-formulated the sentences. We mentioned that the inter-KT distance was measured to quantify inter-KT-tension (page 9, lines 10 through 12 from the bottom).

- Figure 5: This is a good example where it is extremely confusing that so many different cell lines are used. Figure part A, C, E are HeLa cells, B and F are U2OS and D is HEK293T. These cell lines may very well be different in their checkpoint response (see e.g. Gascoigne/Taylor Cancer Cell 2008) and it would be much better to have all experiments performed in one cell line.

In the revised manuscript we used HeLaS3 cells in most experiments. U2OS cells were used in some experiments to demonstrate that our findings are not specific for one cell line. We showed that GFP-mDvl2 rescues the SAC defect induced by depletion of Dvl2 in HeLaS3 and U2OS cells in Figure 5A and Supplementary Figure 10B. We also showed effects of depletion of Dvl2 on Mps1 activity and the localization of Bub1 and BubR1 to KTs in HeLaS3 cells in Figure 5B-D. These results are described in the text (page 10, lines 4 through 9 from the bottom, page 11, lines 5 through 8, page 11, lines 14 through 17).

In original manuscript we used U2OS cells for the experiments to see the effects of deletion mutants of Dvl2 on the SAC due to the reason that expression of Dvl2 deletion mutants induced cell death of HeLaS3 cells. Because the reviewer claimed that it is confusing to use different cell lines, we removed the experiments using deletion mutants of Dvl2 in U2OS cells.

- Figure 5C: An immunoblot is required to demonstrate that the overall levels of Bub1 and BubR1 are still the same after Dvl2 RNAi. Only then, the authors can conclude that there is a localization defect.

According to the referee's suggestion, the levels of Bub1 and BubR1 in Dvl2-depleted cells were shown in Supplementary Figures S11B and 14A and described in the text (page 11, lines 14 and 15).

- Figure S4C: For the interaction analysis, it would be much better to work with the endogenous proteins rather than with overexpression of tagged versions.

We examined whether Dvl2 forms a complex with Mps1 or BubR1 at endogenous levels in asynchronous or nocodazole-arrested HeLaS3 cells. The results were shown in Figure 5E and Supplementary Figure S11D and described in the text (page 11, lines 4 through 7 from the bottom).

- Figure 5D: Is this immunoprecipitation really done from asynchronous cells (as the legend implies)? Does this mean that the Dvl2 - Mps1 interaction is constitutive and not checkpoint-activation specific? Could the authors provide a negative control for the co-IP? Does APC e.g. associate with Mps1 as well?

Because original experiments were done in asynchronous HEK293T cells, the binding of Dvl2 and Mps1 was examined in asynchronous and nocodazole-arrested HeLaS3 cells. As compared with asynchronous culture, Mps1 immunoprecipitated with Dvl2 was reduced in nocodazole-arrested cells. A reciprocal experiment also showed the same results. These results suggested that Mps1 forms a complex with Dvl2 in interphase, but they may be dissociated in mitosis.

According to the referee's comment, the association between APC and Mps1 was also examined. APC did not precipitate Mps1 in asynchronous and nocodazole-arrested HeLaS3 cells. The results are shown in Figure 5E and Supplementary Figure S11E and described in the text (page 11, lines 3 through 7 from the bottom).

- Figure 5D and F: Firstly, it would be much better to test the Dvl2 mutants in an RNAi complementation experiment rather than by overexpression (see above). If in such an experiment the mutants also show different checkpoint responses, it would be very useful to test whether this correlates with their ability to associate with Mps1. With such data, the authors could make the point that Dvl2-Mps1 interaction is indeed needed for checkpoint activity. With the current data, it is not possible to make this link.

As described above, the reason why we used U2OS cells for the experiments to see the effects of deletion mutants of Dvl2 on the SAC is that expression of Dvl2 deletion mutants induced cell death of HeLaS3 cells. However, the reviewer claimed that it is confusing to use different cell lines. Therefore, we removed experiments with overexpression of Dvl2 deletion mutants in U2OS cells.

The results in Figure 5E suggested that the binding to Dvl2 is not necessary for the activation of Mps1.

- Figure 5E: Was the mitotic index the same in the control and the Dvl2 RNAi cells? This is important to assess whether there really is a difference in Mps1 phosphorylation. It seems Mps1 is overall less abundant in the Dvl2 RNAi sample. How is the 'relative phosphorylation' indicated at the bottom determined?

The mitotic index was the same in control and Dvl2-depleted cells. After treatment with MG132, we collected mitotic cells only by mechanically shake-off and confirmed that an amount of Aurora A in Dvl2-depleted cells was the same as that in control cells. We carefully repeated this experiment and confirmed that the upper band of Mps1 was reduced and the lower band of Mps1 was increased in Dvl2-depleted cells as compared with control cells, while depletion of Dvl2 did not affect the total amount of Mps1. These results are shown in Figure 5B and described in the text (page 11, lines 5 and 6).

Relative phosphorylation was calculated by intensity of upper Mps1 band/ intensity of upper Mps1 band + intensity of lower Mps1 band.

- Figure 6: The whole paper is, I assume, also meant to be read by people from the mitosis field. For them, it will be really hard to understand the meaning of this figure. I think it would help to add a little schematic that shows the proteins that are examined here together with the pathways, in which they are supposed to play a role. I imagine something like a reduced version of Figure 1 from the Kikuchi et al. review in Trends Cell Biol 2009.

The outline of Wnt signaling pathway is added in Figure 6A.

- Figure 6A: There is no immunoblot for Fz2. In addition, it would be useful to provide an immunoblot for Dvl2. Are the levels unaffected by Lrp6 or Fz2 RNAi?

An anti-Fz2 antibody recognizing endogenous Fz2 is not available. Instead, it was already shown in our previous paper (Sato, A. et al., EMBO J., 2010, 29, 41-54) that siRNA for Fz2 decreases mRNA levels of Fz2 and shows no off target effects on LRP6 or Ror2.

The level of Dvl2 was not affected by siRNA of LRP6 or Fz2. The results are shown in Supplementary Figure S12C.

- Figure 6B: Show a separate picture for Lrp6 pS1490 for interphase as well (is the signal gone or just reduced?).

LRP6 was also observed cell surface membrane in interphase, but it was not phosphorylated at Ser1490. The results are shown in Supplementary Figure S12A and described in the text (page 12, lines 3 through 5).

Figure 6F: The experiment needs a control for the mitotic index in control versus Dvl2 RNAi cells. Plk1 is not a particularly good control, since it is already expressed during G2 (see e.g. Sumara/Peters Curr Biol 2004).

After treatment with MG132, we collected mitotic cells only by mechanically shake-off and also confirmed that an amount of Aurora A in Dvl2-depleted cells was the same as that in control cells. We added western blotting of Aurora A as a mitotic marker in Figure 6C.

- Figure 6G: The authors state that 'depletion of Lrp6 or Fz2 did not affect MT-KT attachment'. However, the pictures in this figure imply that the RNAi cells do have a problem with chromosome alignment, which could be indicative of an attachment defect. Please provide a quantification, which percentage of mitotic cells had aligned versus non-aligned chromosomes (analogous to Fig. S3F). In addition, I recommend checking for cold-resistant or low conc. of nocodazole-resistant microtubules (using staining for microtubules) in both Dvl2 as well as Lrp6 and Fz2 RNAi.

We compared the effects of depletion of LRP6 or Fz2 on chromosome alignment (congression) and MT-KT attachment in the presence or absence of a low concentration of nocodazole. These experiments were done in the presence of MG132. According to the referee's comment, we quantified which percentage of mitotic cells had aligned versus non-aligned chromosomes in control, LRP6-, or Fz2-depleted cells. Nocodazole treatment induced chromosome misalignment in 79% of control cells. Depletion of LRP6 or Fz2 did enhance chromosome misalignment to 86% in the presence of a low concentration of nocodazole. However MT-KT attachment was not affected. In addition, K-fibers were resistant to reduction of spindle dynamics by the treatment with a low concentration of nocodazole in LRP6- or Fz2-depleted cells. Therefore, it is conceivable that MT-KT attachment was not affected by depletion of these receptors. The results are shown in Figure 6G and Supplementary Figure S13 A and B and described in the text (page 13, lines 5 though 14).

- *Figure S1: Similar interphase pictures are already published in Matsumoto et al., EMBO J 2010. Figure S1A therefore seems unnecessary.*

Figure S1A was removed.

- *The introduction states that the kinase activities of Bub1, BubR1, Mps1 and Aurora B are necessary for the spindle assembly checkpoint (SAC). For both Bub1 as well as Aurora B this is controversial (see e.g. Klebig/Meraldi JCB 2009; Maresca/Salmon J Cell Sci 2010). BubR1 kinase activity seems only partially required for the SAC (e.g. Malureanu/van Deursen Dev Cell 2009). Mps1 is the only of these kinases for which it is very clear that its kinase activity is required for the SAC. Please re-formulate.*

The regulation of the SAC signal is complicated. We re-formulated introduction and did not mention their kinase activity (page 4, lines 10 through 19)

- *Text page 8: The argument that phosphorylation of Dvl2 in mitosis was checked because the protein has potential phosphorylation sites is a bit odd - I assume that almost every protein has potential phosphorylation sites.*

We deleted the sentence.

Text page 12 'Dvl2 might be required for... but not for the centrosomal functions'. What are the 'centrosomal functions'? I would rather say: 'required for spindle orientation, but not for bipolar spindle formation'.

We changed to “but not for bipolar spindle formation” (page 8, lines 14 and 15).

- *Discussion: The discussion starts with an extensive summary, which in my opinion could be shortened.*

The first paragraph of Discussion was shortened.

- *Material and Methods 'Reagents and antibodies': I don't think that phalloidin was used in this study. MG132 etc. are mentioned twice.*

We corrected our careless mistakes.

- *Material and Methods 'Cell culture...'. Please indicate which protocol was used for which figures. The thymidine/MG132 protocol (Figure S4) seems to be missing.*

We indicated which protocols were used for which figures.

- *Material and Methods: Image analysis for the quantification of Bub1 and BubR1 intensities is not described, I think.*

Quantification of Bub1 and BubR1 intensities was described in legend to Figure 5D.

- *Material and Methods 'Immunoblotting...': Please indicate which protease and phosphatase inhibitors were used. Cell number? Volume of lysis buffer?*

The detailed information of protease and phosphatase inhibitors were described. However, I have never been requested to describe cell numbers and volume of cell lysis buffer for immunoblotting recently.

- *Material and Methods 'Segregation axis analysis': Wouldn't it make more sense to call this 'spindle axis analysis'? It is not mentioned how the measurement was performed. (Text only says '... axis was measured by taking Z-stack images...'. This is the first step in the procedure, but one would really like to know how the authors proceeded.*

We described detailed protocol for this assay.

- *Figure legend to Figure 5A fails to indicate in which way the MPM2-stained cells were analyzed. FACS? Immunofluorescence microscopy? Also in several other figure legends additional information is needed (which antibody was used for detection, which cell cycle stage was analyzed, which cell type was used).*

We removed this figure, because U2OS cells were used in this experiment.

- *Figure S3D and legend to Figure S3D: should say 'spindle pole distance' rather than 'spindle distance'. Legend says that measurements were in mm, which I assume should be $\times 00B5m$. In addition, please provide an example picture for the quantification of EB1 intensity.*

We corrected mistakes and added an example picture for the quantification of EB1 intensity in Supplementary Figure S7C.

- *In addition, there are several typos and imprecise wording throughout the text.*

We corrected careless mistakes and asked the native speakers to edit the whole manuscript.

Referee #4:

In this paper, the authors addressed the mitotic roles of Dvl2 in mammalian cells. They found that Dvl2 is involved in three processes in mitosis; a) spindle orientation, b) microtubule-kinetochore attachment, and c) the spindle assembly checkpoint. Among the roles of Dvl2, phosphorylation by Plk1 is required for a) and b), while it is dispensable for c). In addition, a) is dependent on Wnt signaling, while b) and c) are Wnt-independent. These results clearly demonstrate the novel aspects of Dvl2 as a mitotic regulator like other molecules of the Wnt/b-catenin pathway. Although the experiments were well organized to dissect the roles of Dvl2 to draw the elegant model as shown in Fig. S5, there are several concerns on the interpretation of the data.

Major criticism

1. To determine the requirement of each region of Dvl2 or phosphorylation by Plk1 on spindle orientation or the spindle assembly checkpoint (Figure 4E, 5F, 6C), the authors observed the effect of ectopic expression of each mutant in the presence of endogenous Dvl2. These assays could detect the phenotype of each mutant only when it had the dominant negative effect. For example, in Figure

4E, DDIX and DPDZ did not show spindle misorientation, but they might show defects when they are replaced with endogenous Dvl2. Ideally, these assays need to be done in siDVL2 cells expressing RNAi-resistant constructs, and at the moment we are not sure whether Plk1-dependent and independent roles can be clearly distinguished. Therefore, authors need to attenuate current conclusion if replacement experiments are technically difficult.

We agreed to the referee's comment that functions of Dvl2 mutants must be examined in Dvl2-depleted cells. Dvl2^{WT} but not Dvl2^{T206A} rescued spindle misorientation and inter-KT distance in Dvl2-depleted HeLaS3 cell. However, Dvl2^{T206A} rescued the SAC defect in Dvl2-depleted cells as well as Dvl2^{WT}. The results are shown in Figures 3C, 4D, and 5A and described in the text (page 8, lines 2 through 4, page 9, lines 4 through 6 from the bottom, page 10, lines 4 through 7 from the bottom).

However, because expression of Dvl2 deletion mutants in HeLaS3 cells caused cell death, rescue experiments with Dvl2 deletion mutants could not be succeeded. The roles of Dvl2 deletion mutants on mitotic progression are not essential in the current study, and we focused on the functions of Dvl2 phosphorylation by Plk1. Therefore, the results using overexpression of Dvl2 deletion mutants were removed. We believe that the changes do not affect our conclusion in this study.

2. Concerning the role of Dvl2 on microtubule-kinetochore attachment (Figure 4F), it is better to observe chromosome misalignment in siDVL2 cells in the presence of MG132, a proteasome inhibitor, considering that Dvl2 is also involved in the SAC. If Dvl2 is involved in MT-KT attachment through kinetochore localization of Bub1 and BubR1, siDVL2 cells will show massive chromosome misalignment in the presence of MG132. I also wonder whether Lrp6 and Fz2 are really dispensable for MT-KT attachment, because in Figure 6G, siLRP6 and siFZ2 cells showed chromosome misalignment as siDVL2 cells in the presence of low dose nocodazole, which was not seen in control cells (Figure 4F). Authors should comment on the issue.

Original experiments in Figure 4F (Figure 4A in revised version) were done in the presence of MG132 and we compared the effects of depletion of Dvl2 on chromosome alignment (congression) in the presence or absence of a low concentration of nocodazole, which is known to reduce the spindle dynamics.

EB1 signals at the plus-ends of spindle MTs were associated with the centromeres (ACA staining) on metaphase chromosomes in control HeLaS3 cells. The low concentration of nocodazole induced chromosome misalignment, but the MT-KT attachment was not affected in control cells. Chromosomes were misaligned on a metaphase plate with a failure of chromosome congression in Dvl2-depleted cells. In these cells, the EB1 signals were slightly reduced, but still associated with KTs. Nocodazole treatment enhanced a failure of chromosome congression and caused the dissociation of MTs from the KTs in Dvl2-depleted cells. Consistent with these results, depletion of Dvl2 increased prometaphase cells, in which chromosome alignment is immature, and reduced metaphase cells, in which chromosomes are completely aligned at metaphase plate. The results are shown in Figure 4A and Supplementary Figure S9 and described in the text (page 8, line 4 from the bottom through page 9, line 4, page 10, lines 13 and 14 from the bottom).

We compared the effects of depletion of LRP6 or Fz2 on chromosome alignment and MT-KT attachment in the presence or absence of the low concentration of nocodazole. We quantified which percentage of mitotic cells had aligned versus non-aligned chromosomes in control, LRP6-, or Fz2-depleted cells. The low concentration of nocodazole induced chromosome misalignment in 79% of control cells. Although depletion of LRP6 or Fz2 enhanced chromosome misalignment in the presence of the low concentration of nocodazole, it did not affect MT-KT attachment. In addition, K-fibers in LRP6- or Fz2-depletion cells were resistant to reduction of spindle dynamics by a low concentration of nocodazole. Therefore, it is conceivable that the attachment of MT to KT was not affected by depletion of these receptors. The results are shown in Figure 6G and Supplementary Figure S13A and B and described in the text (page 13, line 5 through 14).

3. Kinetochore localization of Dvl2 was not as clear as localization on spindle poles (Figure 1). If the roles of Dvl2 on MT-KT attachment and the SAC depend on kinetochore localization of Dvl2, and different domains of Dvl2 are responsible for respective roles, kinetochore localization domain

is supposed to reside on domains other than these domains. This would be addressed by observing the kinetochore localization of GFP-Dvl2 constructs schematically shown in Figure 2E.

As the referee said, the staining signals of GFP-Dvl2 for spindle poles were strong. It was difficult to detect the localization of GFP-Dvl2 to KTs in small pictures such as Figure 1A. According to the referee's comment, we replaced the original pictures with better pictures observed in HeLaS3 cells. Dvl2 localized to clearly the KTs when cells were treated with nocodazole to activate the SAC. The results are shown in Figure 1B and C and described in the text (page 5, lines 11 through 14).

Major critiques from other reviewers and the Editor were that we should use the same cell line in all experiments. In addition, because U2OS cells have a poor checkpoint response to MT poisons, to use HeLa cells was recommended. Therefore, we analyzed HeLa cells basically in all experiments of the revised manuscript and used U2OS cells to confirm the findings in HeLa cells. However, because overexpression of Dvl2 deletion mutants in HeLaS3 cells caused cell death, we could not succeed to observe their intracellular localization. Since we are interested in which domain of Dvl is responsible for the subcellular localization and functions in mitosis, we would like to study how Dvl2 localizes to the KTs as a separate project and to clarify this point in the near future.

Minor criticism

1. For general readers, domain names of Dvl2 (DIX, PDZ, and DEP) may not be familiar and need explanation.

We explained the DIX, PDZ, and DEP domains in the text (page 6, lines 11 through 13).

2. As there are three highly homologous Dsh homologues in human cells (Dvl1-3), comments on the functional redundancy among them on mitotic regulation would be helpful to readers.

HeLaS3 cells expressed three Dvls. Because the sensitivity of each antibody seemed to be different, it is difficult to say which Dvl is abundant mostly in these cells. Depletion of either Dvl1 or Dvl3 also showed the phenotypes (spindle angles and mitotic index) similar to that by the depletion of Dvl2, and the extent of the phenotypes by depletion of all of Dvl1, Dvl2, and Dvl3 were similar to that by depletion of Dvl2. Because the efficiency of the depletion of Dvl1, Dvl2, and Dvl3 was more than 90%, we believe that Dvls do not act redundantly in mitosis. Therefore, Dvls may function as a heterotrimer, because it was shown that Wnt3a-dependent transcription was suppressed by depletion of either of Dvl1, Dvl2, or Dvl3 and that Dvl1 and Dvl3 are found in the Dvl2 immune complex (Cell. Signal., 20, 413-452, 2008). These results are shown in Supplementary Figure S14A, B, and C and described in the text (page 14, lines 6 through 16).

3. Concerning Figure 5C, expression level of Bub1 and BubR1 in siDVL2 should be checked before concluding that Dvl2 is required for the recruitment of these proteins to kinetochores.

Expression levels of Bub1 and BubR1 were examined in Dvl2-depleted cells. Depletion of Dvl2 did not affect their expression levels. The results were shown in Supplementary Figures S11B and S14A and described in the text (page 11, lines 14 and 15).

Your revised manuscript has now been re-assessed by two of the original referees whose comments you will find enclosed.

As you will see both referees appreciate the amount of changes that certainly improved the quality of the paper. Though, ref#3 raises still additional concerns, there seems in light of the overwhelming support from the other referees no major conceptual reason to further delay efficient proceedings of the paper. I thus recommend to consider the mostly textual changes and attend to the experimental points only in case if not too extensive further analysis would be needed. All in all, I am looking

forward to receive your ultimate, modified version for final acceptance as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors have done significant new work to address points raised by all reviewers and the overall conclusions of the story has not significantly changed.

I think this is an important new insight into the function of Dvl (and Wnt signaling) with respect to mitotic function.

There are, of course, more points that can be more fully addressed in this study but this is beyond the scope of the current ms and it should be published to stimulate new research in this area.

Referee #3 (Remarks to the Author):

Kikuchi and colleagues have submitted a revised version of their manuscript on the role of Dishevelled 2 (Dvl2) in mitosis. The authors addressed many of the points that had been raised. I find the experimental part greatly improved (better pictures, clearer figures, very useful RNAi rescue experiments). I also appreciate that the data on Dvl1 and Dvl3 is now mentioned, and I generally acknowledge that a considerable amount of work has been put into this manuscript. Unfortunately, I find the data still poorly presented and the text in large parts unsatisfactory (see detailed comments below).

Comments for the experimental part:

A) The title now states that Dvl acts 'in cooperation with Plk1', which I find a fuzzy expression that (at least to me) implies that the interaction between the two is needed for their function, which the authors do not show. I would also have appreciated if the authors had at least attempted to partially rescue the Plk1 RNAi phenotype by expression of a phospho-mimicking mutant of Dvl2-T206. In the comments to the reviewers, the authors argue that Plk1 RNAi cells often have monopolar spindles, and therefore cannot be examined for spindle orientation or inter-KT distance. However, as far as I can judge from the literature, a considerable number of Plk1 RNAi cells does have bipolar spindles, and it should therefore be possible to examine these phenotypes and also to check whether a phospho-mimicking mutation at Dvl2-T206 partially rescues the phenotype, which would strengthen the link between Plk1 and Dvl2.

B) Abstract, discussion and Figure 7 mention that Wnt receptors are not involved in the spindle assembly checkpoint. Where is this shown?

C) Concerning the role of Dvl2 in the spindle assembly checkpoint: the data would be even more convincing if the authors showed that Bub1/BubR1 localization and CENP-A Ser7 phosphorylation can also be rescued by expression of mDvl2, which should be easy to do.

D) On page 8/9, the authors cite Figure 4A for a chromosome congression defect in Dvl2-depleted cells. This is not visible in the Figure and, as far as I can see, there is no quantification provided from which this can be deduced.

While the experimental part is now quite clear, the text is still not well-structured, is often imprecise in the wording and does not always discuss the existing literature appropriately. Some examples:

A) The title puts an emphasis on Plk1, but the introduction mentions Plk1 only with a single sentence and does not even explain which spindle phenotypes have been observed after knock-down or inhibition of Plk1. In addition, the known link between Plk1 and BubR1 (e.g. Elowe GenesDev 2007, Matsumura JBC 2007, Huang JCB 2008), which I find relevant for this paper, is not discussed.

B) The introduction on the spindle assembly checkpoint is very basic and is not focussed on the knowledge that is relevant for the experiments in the paper.

C) With respect to the role of Dvl2 in regulating the mitotic spindle, the word 'spindle

dynamics' is still frequently used when describing spindle positioning/orientation defects. This is problematic because the question of whether microtubule dynamics are impaired was not addressed, probably leading to false assumptions on the reader's side. Why not say 'proper spindle positioning' instead?

D) I would also prefer if primary literature was cited rather than a review, where this is easily possible (e.g. on page 16: Petronczki 2008 review is cited for active SAC in Plk1-depleted cells). Another example: Two reviews are cited for 'The presence of SAC kinases Bub1 and BubR1 at the KT was shown to be essential for the SAC.' Firstly, Bub1 and BubR1 are certainly required for the SAC, but to my knowledge there is no formal proof that their localization at the KT is needed for the SAC. (The experiments that come closest to showing this are in Kiyomitsu et al, Dev Cell 2007, which is not cited.) Secondly, it would in any case be more appropriate to cite primary literature.

E) The discussion is often imprecise, lacks some important information and is not particularly well assembled. For example, when elaborating on the importance of Dvl2 in the SAC, the existing literature is still not appropriately discussed. The interaction between Dvl2 and APC is discussed, but the experiments using the APC armadillo domain are not mentioned. The part on 'Wnt signaling and mitosis' could be formulated more smoothly.

F) In addition, some smaller mistakes in the text and figure legends (imprecise wording, missing quantifications, unclear how experiment was performed) still make the manuscript hard to read.

Minor comments and inaccuracies:

a) In the Material and Methods section, 'aprotinin' is mentioned under 'phosphatase inhibitors', but is a protease inhibitor.

b) The legend to Figure 1A states that GFP-Dvl2 is shown in green (which I would find logical), but in the figure GFP-Dvl2 is in red.

c) Figure 2F: '(in Figure 3D)' should be deleted

d) Legend to Figure 3A: square bracket missing. It is also still unclear to me how the authors decide where to place the borders of the 'total MT' or 'spindle' area. Is it just determined by eye? This is equally unclear in the Thoma et al. 2009 paper, which is given as reference.

e) Figure 3D: It remains unclear how pictures were selected for measurement. Only one timepoint from one cell? Several timepoints? How many cells?

f) When a low concentration of nocodazole was used, it would be better to write '+ low Noc.' rather than '+Noc.'. The latter always implies that microtubules are gone.

g) Were cells in Figure 5D also treated with MG132? Since Dvl2-depleted cells should generally escape the arrest induced by nocodazole, how were mitotic cells selected? Is it a mitotic shake-off? Or are mitotic cells identified by condensed DNA?

h) Legend to Figure 7A: Sentence is misleading. Better: '... depends on Plk1-dependent phosphorylation of Dvl2 and on Wnt receptors.'

i) Supplementary Movie 1 can be omitted without loss of information.

j) Abstract, '... was required for ... microtubule (MT)-KT attachment': Better: '... was required for ... stable (MT)-KT attachment', since some attachment is clearly present.

k) Page 6 '... did not form a complex... even though they were overexpressed'. In my opinion, one cannot generally say that interactions are easier to detect when the candidate interaction partners are overexpressed. For example, if the interaction is indirect, it might be observable with the endogenous proteins, but not with overexpressed proteins. I would therefore rather say '... when these were overexpressed'.

l) Several typos are still present throughout the manuscript. In one case T216 is written instead of T206.

m) Page 8 'Because it was difficult to observe astral MTs practically...'. What do the authors mean? They were able to measure astral MTs (Figure 3A), so why can't they observe them?

n) Page 11 'exhibits opposing effects on spindle dynamics...'. I would find it much better to conclude that '... it is likely that Plk1-mediated phosphorylation of Dvl2 is required for proper spindle positioning and MT-KT attachment, but not SAC activation.'

o) Page 13 '... did not affect MT-KT attachment'. Since there is a chromosome congression defect, it is likely that MT-KT attachment is affected. I think the authors want to say that MT-KT attachment is not abolished (rather than not affected).

p) Discussion, page 14 '... in the Dvl2 immunocomplex'. I think the authors mean '... in a complex with Dvl2' or '... in a Dvl2 immunoprecipitation'.

q) Discussion: '... the SAC is not active in Dvl2-depleted cells'. The mitotic index is still increased to 10 - 40 % in Dvl2 RNAi cells treated with nocodazole or taxol (Figure 5A). Wouldn't it

therefore be better to say '... is only partially active...'?

r) Discussion 'Dvl2 was found to form a complex with Mps1 in the asynchronous state'. I think the authors mean '... was found to form a complex... in interphase' or '... in cells of an asynchronously growing culture'

2nd Revision - Authors' Response

05 August 2010

Referee #3

Comments for the experimental part:

A) The title now states that Dvl acts 'in cooperation with Plk1', which I find a fuzzy expression that (at least to me) implies that the interaction between the two is needed for their function, which the authors do not show. I would also have appreciated if the authors had at least attempted to partially rescue the Plk1 RNAi phenotype by expression of a phospho-mimicking mutant of Dvl2-T206. In the comments to the reviewers, the authors argue that Plk1 RNAi cells often have monopolar spindles, and therefore cannot be examined for spindle orientation or inter-KT distance. However, as far as I can judge from the literature, a considerable number of Plk1 RNAi cells does have bipolar spindles, and it should therefore be possible to examine these phenotypes and also to check whether a phospho-mimicking mutation at Dvl2-T206 partially rescues the phenotype, which would strengthen the link between Plk1 and Dvl2.

The referee requested to show whether a Dvl2-T206E mutant (pospho mimicking mutation) rescues the phenotypes induced by deletion of Plk1. I believe that this experiment is important, but it will take at least one month because we do not have the Dvl2 mutant and have to make the construct. Because we have a plan to make an anti-phospho-Dvl2 T206 antibody to analyze the temporary and special regulation of the phosphorylation in intact cells, we would like to clarify the functions of the phosphorylation of Dvl2 using the Dvl2 mutant and the anti-phospho Dvl2 T206 antibody as a next study.

B) Abstract, discussion and Figure 7 mention that Wnt receptors are not involved in the spindle assembly checkpoint. Where is this shown?

Our results showed the number of mitotic cells increased following nocodazole treatment in LRP6- or Fz2-depleted cells as well as control cells. These results suggest that depletion of LRP6 and Fz2 do not affect the SAC. Therefore, we mentioned that the Wnt receptors are not involved in the SAC. These are described in the text (page 14, lines 1 through 4 from the bottom).

C) Concerning the role of Dvl2 in the spindle assembly checkpoint: the data would be even more convincing if the authors showed that Bub1/BubR1 localization and CENP-A Ser7 phosphorylation can also be rescued by expression of mDvl2, which should be easy to do.

The referee suggested that we should examine whether the expression of mDvl2 rescues the localization of Bub1 and BubR1 to KTs in Dvl2-depleted cells. According to the referee's comment, we performed the experiment. As expected, Bub1 and BubR1 were localized to the KTs as observed by the expression of GFP-mDvl2 in Dvl2-depleted cells treated with nocodazole. The results were shown in Figure 5D and described in the text (page 12, line 11 and 12 from the bottom).

The referee also asked whether GFP-mDvl2 rescues the phosphorylation of CENP-A-Ser7 in Dvl2-depleted cells. Both the antibodies for phosphor-CENP-A-Ser7 and GFP are rabbit polyclonal antibodies, and their mouse antibodies are not available at present. I do not know how long it takes to get their other species' antibodies. In addition, because we performed several rescue experiments by the expression of GFP-mDvl2 in Dvl2-depleted cells, I do not think that this experiment is essential for the current manuscript.

D) On page 8/9, the authors cite Figure 4A for a chromosome congression defect in Dvl2-depleted cells. This is not visible in the Figure and, as far as I can see, there is no quantification provided from which this can be deduced.

Because confocal microscope strictly cuts off unfocused signals, we carefully observed chromosome congression in control or Dvl2-depleted cells by sectioning Z-axis. We assembled the sections for 3D-projection, and pictures of 3D-projection are shown in 'referee-only' supplementary information. The pictures represent a chromosome congression defect in Dvl2-depleted cells. In addition, we showed that depletion of Dvl2 increases the numbers of prometaphase cells, in which chromosome alignment is immature, and reduces the numbers of metaphase cells, in which chromosomes are completely aligned at metaphase plate. The results are described in the text (page 11, lines 12 and 13) and shown in Supplementary Figure S9.

While the experimental part is now quite clear, the text is still not well-structured, is often imprecise in the wording and does not always discuss the existing literature appropriately. Some examples: A) The title puts an emphasis on Plk1, but the introduction mentions Plk1 only with a single sentence and does not even explain which spindle phenotypes have been observed after knock-down or inhibition of Plk1. In addition, the known link between Plk1 and BubR1 (e.g. Elowe GenesDev 2007, Matsumura JBC 2007, Huang JCB 2008), which I find relevant for this paper, is not discussed.

According to the referee's comment, we described the information about Plk1 in the text (page 4, lines 1 through 6 from the bottom; page 15, lines 1 and 2 from the bottom).

B) The introduction on the spindle assembly checkpoint is very basic and is not focussed on the knowledge that is relevant for the experiments in the paper.

We added further explanation for the SAC in the text (page 4, lines 9 through 12 from the bottom).

C) With respect to the role of Dvl2 in regulating the mitotic spindle, the word 'spindle dynamics' is still frequently used when describing spindle positioning/orientation defects. This is problematic because the question of whether microtubule dynamics are impaired was not addressed, probably leading to false assumptions on the reader's side. Why not say 'proper spindle positioning' instead?

According to the referee's comment, "proper spindle positioning" was use at the appropriate sites instead of "spindle dynamics".

D) I would also prefer if primary literature was cited rather than a review, where this is easily possible (e.g. on page 16: Petronczki 2008 review is cited for active SAC in Plk1-depleted cells). Another example: Two reviews are cited for 'The presence of SAC kinases Bub1 and BubR1 at the KTs was shown to be essential for the SAC.' Firstly, Bub1 and BubR1 are certainly required for the SAC, but to my knowledge there is no formal proof that their localization at the KT is needed for the SAC. (The experiments that come closest to showing this are in Kiyomitsu et al, Dev Cell 2007, which is not cited.) Secondly, it would in any case be more appropriate to cite primary literature.

We cited primary literatures as much as possible. Examples are page 12, lines 11 through 13 and page 17, lines 4 and 5.

E) The discussion is often imprecise, lacks some important information and is not particularly well assembled. For example, when elaborating on the importance of Dvl2 in the SAC, the existing

literature is still not appropriately discussed. The interaction between Dvl2 and APC is discussed, but the experiments using the APC armadillo domain are not mentioned. The part on 'Wnt signaling and mitosis' could be formulated more smoothly.

We rewrote the Discussion part (page 15, lines 2 from the bottom through page 16, line 4; page 16, lines 14 and 15).

F) In addition, some smaller mistakes in the text and figure legends (imprecise wording, missing quantifications, unclear how experiment was performed) still make the manuscript hard to read.

We checked careless mistakes and improved figure legends

Minor comments and inaccuracies:

a) In the Material and Methods section, 'aprotinin' is mentioned under 'phosphatase inhibitors', but is a protease inhibitor.

We appreciate that the referee finds our careless mistake.

b) The legend to Figure 1A states that GFP-Dvl2 is shown in green (which I would find logical), but in the figure GFP-Dvl2 is in red.

c) Figure 2F: '(in Figure 3D)' should be deleted

d) Legend to Figure 3A: square bracket missing. It is also still unclear to me how the authors decide where to place the borders of the 'total MT' or 'spindle' area. Is it just determined by eye? This is equally unclear in the Thoma et al. 2009 paper, which is given as reference.

e) Figure 3D: It remains unclear how pictures were selected for measurement. Only one timepoint from one cell? Several timepoints? How many cells?

f) When a low concentration of nocodazole was used, it would be better to write '+ low Noc.' rather than '+Noc.'. The latter always implies that microtubules are gone.

g) Were cells in Figure 5D also treated with MG132? Since Dvl2-depleted cells should generally escape the arrest induced by nocodazole, how were mitotic cells selected? Is it a mitotic shake-off? Or are mitotic cells identified by condensed DNA?

h) Legend to Figure 7A: Sentence is misleading. Better: '... depends on Plk1-dependent phosphorylation of Dvl2 and on Wnt receptors.'

We changed our mistakes and added new description in legends to Figures 1A, 3A, 3D, 4A, 5D, and 7A. We also changed Figures 2F, 4A, and 6G.

i) Supplementary Movie 1 can be omitted without loss of information.

We omitted it.

j) Abstract, '... was required for ... microtubule (MT)-KT attachment': Better: '... was required for ... stable (MT)-KT attachment', since some attachment is clearly present.

We changed the sentence (page 2, line 8).

k) Page 6 '... did not form a complex... even though they were overexpressed'. In my opinion, one cannot generally say that interactions are easier to detect when the candidate interaction partners are overexpressed. For example, if the interaction is indirect, it might be observable with the

endogenous proteins, but not with overexpressed proteins. I would therefore rather say '... when these were overexpressed'.

We changed the sentence (page 7, line 8).

l) Several typos are still present throughout the manuscript. In one case T216 is written instead of T206.

We checked typos and changed them.

m) Page 8 'Because it was difficult to observe astral MTs practically...'. What do the authors mean? They were able to measure astral MTs (Figure 3A), so why can't they observe them?

We removed the sentences and wrote new sentences (page 9, line 11 from the bottom).

n) Page 11 'exhibits opposing effects on spindle dynamics...'. I would find it much better to conclude that '... it is likely that Plk1-mediated phosphorylation of Dvl2 is required for proper spindle positioning and MT-KT attachment, but not SAC activation.'

o) Page 13 '... did not affect MT-KT attachment'. Since there is a chromosome congression defect, it is likely that MT-KT attachment is affected. I think the authors want to say that MT-KT attachment is not abolished (rather than not affected).

p) Discussion, page 14 '... in the Dvl2 immunocomplex'. I think the authors mean '... in a complex with Dvl2' or '... in a Dvl2 immunoprecipitation'.

q) Discussion: '... the SAC is not active in Dvl2-depleted cells'. The mitotic index is still increased to 10 - 40 % in Dvl2 RNAi cells treated with nocodazole or taxol (Figure 5A). Wouldn't it therefore be better to say '... is only partially active...'?

r) Discussion 'Dvl2 was found to form a complex with Mps1 in the asynchronous state'. I think the authors mean '... was found to form a complex... in interphase' or '... in cells of an asynchronously growing culture'

We changed these sentences as the referee suggested.

Additional Supporting Material

