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# Wnt mediated protein stabilization ensures proper mitotic microtubule assembly and chromosome segregation

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**Review timeline:**

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## Transaction Report:

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

Editor: Barbara Pauly

1st Editorial Decision 10 September 2014

Thank you very much for the submission of your research manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. We have now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I will only repeat the main points here. While all reviewers agree on the potential interest of the findings, they also pinpoint several aspects that would need to be strengthened before publication of the study can be considered in EMBO reports. For example, referee 1 feels that several apparent inconsistencies, both within the current data set, but also with regard to previous literature would need to be resolved and that in some instances, additional experiments are needed to back up the conclusions, for example with regard to the activity of the wnt pathway under the experimental conditions. This referee also feels that the use of HCT116 cells is not ideal, but upon further discussions with the other reviewers and with an additional advisor we came to the conclusion that you would not need to repeat all experiments in another cell line, as suggested by referee 1. Having said this, showing that the Wnt/STOP pathway is reduced in CIN+ tumor cells would make your study much stronger and in case you have such data we would strongly recommend adding them. The other issues raised by this referee as well as by referees 2 and 3 seem all rather minor and I am sure you will be able to address them rather easily.

Overall, and given the reviewers' constructive comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the reviewers should be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of

 

review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

In their manuscript entitled "Wnt mediated protein stabilization ensures proper mitotic microtubule assembly and chromosomal stability", Ailine Stolz et al. aim to show a rule for Wnt dependent stabilization of microtubules in mitosis. Specifically, they conclude that the recently discovered " Wnt/STOP" process is responsible for the regulation of proper microtubule dynamics in mitosis.

Mitotic abnormalities linked to microtubule polymerisation rate changes in mitosis have been shown before by these authors (Ertych et al., 2014, Nature Cell Biology 8:779). The new data complement the existing study by showing that depleting Lrgp5, Lrp6 and Dvl change microtubule polymerisation rates but this new MS does not provide any novel insights or information about an underlying mechanism. In addition, this manuscript suffers from limitations and contradictions.

There is a fundamental underlying problem with the experimental system used in this manuscript. Specifically, HCT116 cells constitute a poor model because they express a mutant form of b-catenin that is not degraded and thus not sensitive to Wnt signalling. The authors refer to the Wnt signalling state in these cells as basal, which is clearly not the case. In fact, the lack of changes in b-catenin in response to knocking down APC or Axin confirms this.

Figure E1 provides the basis for many of the experiments. This figure reveals inconsistencies that make interpreting the data difficult. For instance, when b-catenin is depleted the levels of Axin1 decrease, the double knockdown of LRP5 and 6 results in poor depletion of Lrp6, and in the b-catenin knockdown LRP6 also decreases. All these changes are predicted to contribute to the reported differences in microtubule dynamics making it difficult to draw clear conclusions. A minor point is the lack of evidence for an effect of the APC siRNA on APC levels.

Throughout the manuscript the authors claim that many of the manipulations they carry out inhibit Wnt signalling; however, they never actually provide data for this.

Figure 1 shows that depleting APC, b-catenin, Axin do not affect MT polymerisation prompting the authors to suggest that these proteins do not contribute to the effects they observe. However, then in Fig 4 they show that depleting these proteins does have an effect and rescues Lrp6 depletion. It is possible that there is a lower limit to the microtubule polymerisation rate and that this prevents any measurable decreases in response to these proteins. However, in their previous paper they show that low levels of taxol can lower polymerisation rate in HCT 116 cells below that in the control situation (Fig 1 D in Ertych et al., 2014) suggesting that lowering polymerisation rates is indeed possible. This suggests that a contribution of APC, b-catenin and/or Axin should be possible to measure if they do indeed contribute as suggested.

The authors conclude that Wnt/STOP, in other words a change in the degradation of specific proteins is the mechanism invoked in increasing the microtubule polymerisation rate. However, this is contradictory to the result showing that Wnt 3a does not produce an effect. The explanation provided is that hyperactivation of Wnt signalling is to blame. However, contradictory data are provided in a recently published (Acebron et al (2014) Mol Cell 54: 663-674; cited in this

 

manuscript) showing that over-activation of Wnt does increase the Wnt/STOP activity.

The statement that WntSTOP is required for proper microtubule assembly is overstated. The only direct evidence for a role of protein degradation in the measured effects is provided by the data in Figure 4 where the effect of MG132 is measured. This is not sufficient proof to conclude that the observed effects are due to WntSTOP, it simply shows that protein degradation by the proteasome is important. In addition, microtubules are clearly assembled properly they just have slightly altered dynamics. One thing that was never discussed is the depolymerisation rate and thus ultimately the parameters of dynamic instability, which together govern microtubule dynamics.

There is no or poor correlation with the measured chromosome defects and the relative protein levels in different clones. For instance, the relative protein level of LRP6 increases from clone 1 to clone 3 (Fig E2). However, the induction of abnormal metaphase spindles is highest for clone 3 and lowest for clone 1 (Fig 2B). Similarly, the lowest level of Dvl were achieved in clone 3 (Fig E2) but microtubule dynamics are the least affected in this clone (Fig 2A). Similarly, microtubule polymerisation rates and chromosome defects also do not correlate.

There are no control experiment for the APC or Axin knockdown experiments to show the link between tubulin dynamics and chromosome mis-segregation. Previous data in the literature has shown chromosomes mis-segregation in APC-depleted cells; however, in those cases direct effects of APC on microtubules were implicated. Here the authors make the link between microtubule dynamics and chromosome defects but they only provide measures of this activity selectively.

There is no evidence provided for a functional relationship between mutations in the investigated proteins and mutations or defects in cancer. In fact, colorectal cancer invariably carries APC mutations, which are associated with elevated Wnt signalling. In that scenario, mutations that lower Wnt signalling (i.e. defective LRP 5/6) should restore normal microtubule polymerisation rates and rescue any associated phenotype. Furthermore, in their previous publication the authors state that "Reconstitution of proper microtubule assembly rates by chemical or genetic means suppresses CIN and thereby, unexpectedly, accelerates tumour growth in vitro and in vivo" (Ertych et al., 2014). Together these considerations question the relevance of the new findings in the context of cancer. They also indicate the likely significant contribution of Wnt-independent functions of APC, Dvl, etc. particularly their direct effect on microtubules and microtubule dynamics in mitosis, which are only mentioned in passing in the discussion.

Another consideration is that the microtubule polymerisation rates differ by 25-30% in different controls making the 25-30 % difference in the selected clones relatively minor changes.

Referee #2:

Review of manuscript for EMBO Reports - EMBOR-2014-39410V1
Wnt mediated protein stabilization ensures proper mitotic microtubule assembly and chromosomal stability
By Stolz et al (Bastians laboratory)
The reviewed manuscripts describes a previously unrecognized role of Wnt signaling in regulating microtubule assembly rates and thereby affecting mitotic spindle formation and proper chromosome segregation. The authors provide solid, although preliminary evidence that the recently described Wnt-dependent stabilization of proteins (Wnt/STOP) during mitosis likely affects the regulation of microtubule assembly and spindle formation during mitosis in human cells.
This is a nice and relevant study that brings to our attention the previously unappreciated, β-catenin independent functions of Wnt signaling during mitosis. Given the frequent chromosomal instability and mitotic errors in cancer cells and the fact that Wnt-signaling is often affected in cancer, it is of a broad biological significance and interest of scientific community to explore the possible links between Wnt signaling and mitosis. The experiments are carefully done, with appropriate controls, and they are logically linked together. The authors do a nice work in testing the possible involvement of the novel Wnt/STOP pathway, thereby mapping new directions for the future

 

research. The article is appropriate for publishing in EMBO Reports and will be of interest to broad scientific community.
Before publishing, several minor issues should be addressed:
1. The Wnt signaling pathway is arguably rather complex. Therefore, the authors should pay more attention to the description of the Wnt pathway in the introduction. A little bit more explanations and clarifications of the pathway and the individual players would be very useful.
2. The language of the manuscript should be extensively edited. The sentences are often very long, complex and rather difficult to navigate. Some expressions are unnecessary complicated and rather confusing, e.g. ". . .increased rates of microtubule assembly rates . . ." (page 5) or ". . . abnormal metaphase spindle structures . . ." (page 9) - why not abnormal metaphase spindles?
3. Some details about the experiment with MG132 should be presented already in the text. MG132 has strong and multifaceted effects on cells and therefore specifying that the cells were arrested in mitosis and treated only for one hour would improve the understanding for the reader (everything is correctly explained in Material and Methods, which are by the way very clearly and neatly written).
4. Page 11, the authors show the percentage of cells deviating from modal number, but do not state whether the modal number changes during the culturing. This would be useful information and the modal number should be added to the figure or to the text. Of course, the data is available in the supplementary data (Figure E2), but just a simple statement that the modal number remained 45 in all cases would make the reading easier.
5. Finally, it would be useful to provide a simple schematic model of the findings.

Referee #3:

EMBOR-2014-39410V1

Here, Stolz et al. analyzed the potential effects of mitotic Wnt signaling (referred to as Wnt/STOP pathway) on mitosis progression. In a HCT116 colon cancer cell line, the authors investigated microtubule assembly rate upon perturbing Wnt pathway components. Their main finding is that endogenous Wnt signaling during mitosis is required to maintain the proper microtubule assembly rate, and that LRP6 and Dvl are essential components while β-Catenin, Axin and APC are dispensable for this process. An accelerated microtubule assembly rate upon loss of LRP6 or Dvl leads to abnormal mitotic spindle formation and chromosome missegregation that ultimately results in aneuploidy, a hallmark of human cancer. Those are interesting and novel findings. A very novel point is the authors' proposal that Wnt/STOP pathway, a branch of the Wnt pathway conferring protein stabilization during mitosis, is a pathway responsible for the +end microtubule dynamics required for proper microtubule assembly, microtubule-kinetochore (MT-KT) attachment and proper segregation of daughter chromosomes during mitosis.
This manuscript reports a novel involvement of the Wnt/STOP pathway in the process of mitosis and will be of general interest to the cancer biology field since chromosome instability is a hallmark of cancer and the Wnt pathway is one of the main signaling pathways involved in cancer development. Publication is recommended after revisions that probably will not require new experiments but rather a more detailed discussion according to the points listed below.

Major points

1. There is a previous paper by Kikuchi et al. (reference #13 of the current manuscript), indicating that endogenous Wnt signaling through Wnt receptors (both Fz and LRP6) and Dvl accounts only for spindle orientation but not for MT-KT attachment, while Dvl is required for MT-KT attachment in a Wnt-independent way. This discrepancy, probably due to cell lines used, merits discussion in the current manuscript.

2. The mechanistic basis of Wnt/STOP pathway proposed by Acebron et al. (reference #10 of current manuscript) is an earlier finding that Wnt induces GSK3 sequestration inside multivesicular bodies (Taelman et al., 2010, Reference #8). Endosomal sequestration of Axin and GSK3 is not only regulating β-Catenin stability but also causes global protein stabilization (Taelman et al., 2010; Vinyoles et al., 2014). The possible role of multivesicular endosomes in mitosis and spindle formation should therefore be discussed.
3. Perhaps "Wnt/STOP protein stabilization" could be more informative if used in the title itself.

 

Minor points

1. What this paper needs most is a few still images of how microtubule polymerization changes (e.g., in Fig 1A). We normal readers need to be able to visualize this for at least in one example.
2. It would be good to show a normal and abnormal karyotype in Figure 3. This will enrich the paper.
3. In page 11 line 6 "increased by more than 100%" might cause confusion. Simply, "more than two-fold" instead of "more than 100%", would be more straightforward.
4. In figure 4B, the authors may want to use GSK3 RNAi or other means of GSK3 inhibition (e.g., BIO) in addition to Axin or APC RNAi. However, one imagines that GSK3 is required at many stages of cell cycle progression. Perhaps CycY RNAi could also be useful in future work.

1st Revision - authors' response 04 December 2014

**Responses to comments from Reviewer #1**

> *1. There is a fundamental underlying problem with the experimental system used in this manuscript. Specifically, HCT116 cells constitute a poor model because they express a mutant form of b-catenin that is not degraded and thus not sensitive to Wnt signalling. The authors refer to the Wnt signalling state in these cells as basal, which is clearly not the case. In fact, the lack of changes in b-catenin in response to knocking down APC or Axin confirms this.*

Response:
HCT116 cells harbor a heterozygous mutation in the *CTNNB1* gene. However, several previous studies have demonstrated that the mutation in one *CTNNB1* allele does not confer resistance towards Wnts, which might be expected as pointed out by the reviewer. It is also clear from the literature that stabilization of β-catenin alone represents a poor indicator of Wnt signaling. Instead, activation of β-catenin driven gene transcription represents a more reliable and robust marker for Wnt signaling. In fact, treatment with Wnt3a or loss of *APC* or *AXIN1* greatly induces Wnt signaling and β-catenin dependent gene transcription in HCT116 cells (e.g. Voloshanenko et al. (Boutros lab), Nature Communications 2013; Hadjihannas et al. (Behrens lab), PNAS 2006; Li et al. (Clevers lab), Cell 2012 and see our Figures E1A, E2A, E2E and E5A). This effect might be explained by the presence of the remaining wildtype *CTNNB1* allele as suggested before (Li et al., Cell 2012). Thus, HCT116 cells remain responsive towards incoming Wnts and therefore, we believe that it is reasonable to refer Wnt signaling in the absence of additional Wnts to as "basal".

Nevertheless, there might be the remaining possibility that the mono-allelic *CTNNB1* mutation in HCT116 cells could be involved in triggering an increase in microtubule assembly in response to loss of *LRP5/6* or *DVL2*. To exclude this possibility we repeated our key experiments using non-transformed RPE1 cells (human retina epithelial cells), which harbor no known genetic alteration in the Wnt signaling pathway. Importantly, using these cells we fully confirmed our results. In particular, we show that loss of *LRP5*, *LRP6* or *DVL2*, but not of *APC* or *CTNNB1* results in an increase in microtubule plus end assembly rates in RPE1 cells during mitosis (see our new Figures 1B and E1B). We also demonstrate that abnormal microtubule assembly rates are suppressed upon concomitant loss of proteasome activity (see our new Figure 4B) or upon repression of *AXIN1* in RPE1 cells (see our new Figures 4D and E5B).
Thus, the increase in microtubule assembly rates triggered by loss of *LRP5/6* or *DVL2* do not require the mono-allelic mutation of *CTNNB1* present in HCT116 cells, but rather represents a true outcome of inhibition of Wnt signaling.

> *2. Figure E1 provides the basis for many of the experiments. This figure reveals inconsistencies that make interpreting the data difficult. For instance, when b-catenin is depleted the levels of Axin1 decrease, the double knockdown of LRP5 and 6 results in poor depletion of Lrp6, and in the b-catenin knockdown LRP6 also decreases. All these changes are predicted to contribute to the reported differences*

 

*in microtubule dynamics making it difficult to draw clear conclusions. A minor point is the lack of evidence for an effect of the APC siRNA on APC levels.*

Response:
We regret the inconsistencies on our control western blots showing protein levels in response to various transient siRNA transfections. We again repeated the set of transfections several times and we detected, as expected form transient transfections, slightly varying down-regulations of the respective proteins. However, upon repression of *CTNNB1* we consistently found lowered protein levels for Axin-1, which might indicate that *AXIN1* expression might be positively influenced by β-catenin. To our knowledge, this is not yet reported in the literature. Since siRNA-mediated down-regulation of *AXIN1* or C*TNNB1* does not affect microtubule plus end assembly rates we conclude that a potential functional link between β-catenin and Axin-1 is not of key importance for the mitotic microtubule phenotype. The lowered levels of Axin-1 in response to *CTNNB1* repression appear to be a consistent effect that is (still) presented in the revised Figure E1A.

In contrast to the lowered levels of Axin-1 we, unfortunately, realized that the slightly lower levels of LRP6 after *CTNNB1* repression were not reproducible. We believe that the original result was most likely due to a blotting problem (very outer edge of the membrane). To further confirm this we also performed *CTNNB1* targeting siRNA transfections in RPE1 cells and could also not observe an effect on LRP6 protein levels (see our new Figure E1B). Therefore, we are grateful to the reviewer for pointing out this inconsistency, which we could resolve. We apologize for our mistake and we present a new western blot for LRP6 now in our revised Figure E1A.

Minor point: For detecting the down-regulation of APC we tested several commercially available antibodies, but none of them reliably detected APC in HCT116 or in RPE-1 cells. However, since we used APC targeting sequences that are widely used in the literature and that result in increased Axin-2 protein levels on western blots (see our new Figures E1A, E1B and E5A) and an increase in Wnt reporter activity (see our new Figure E2A) we are confident that APC is indeed sufficiently repressed to trigger a significant activation of Wnt signaling.

*3. Throughout the manuscript the authors claim that many of the manipulations they carry out inhibit Wnt signalling; however, they never actually provide data for this.*

Response:
In our studies we detect altered microtubule plus end assembly rates in response to repression of *LRP5/6* or *DLV2* as well as upon treatment with sFRPs or DKK1. All these treatments are well established to cause an inhibition of Wnt signaling. Examples of published studies include e.g. Uematsu et al., Oncogene 2003; Bilic et al., Science 2007; Ettenberg et al., PNAS 2010; Voloshanenko et al., Nature Communications 2013; Liu et al., Neuron 2014 and many more.

Nevertheless, we agree with the reviewer that it is important to formally demonstrate that Wnt signaling is indeed inhibited in our experimental set-ups. Thus, we measured Wnt reporter activity in response to reduced expression of *LRP5/6* and *DVL2* and after treatment with sFRPs or DKK1 (in the absence of additional Wnts). As expected from the literature, these additional experiments demonstrate a significant inhibition of basal Wnt signaling in all cases. These data are now presented in our new Figures E2A, E2C and E2D.

*4. Figure 1 shows that depleting APC, b-catenin, Axin do not affect MT polymerisation prompting the authors to suggest that these proteins do not contribute to the effects they observe. However, then in Fig 4 they show that depleting these proteins does have an effect and rescues Lrp6 depletion. It is possible that there is a lower limit to the microtubule polymerisation rate and that this prevents any measurable decreases in response to these proteins. However, in their previous paper they show that low levels of taxol can lower polymerisation rate in HCT 116 cells below that in the control situation (Fig 1 D in Ertych et al., 2014)*

 

*suggesting that lowering polymerisation rates is indeed possible. This suggests that a contribution of APC, b-catenin and/or Axin should be possible to measure if they do indeed contribute as suggested.*

Response:
We have indeed shown that treatment with low doses of Taxol can suppress microtubule plus end assembly rates. However, this effect is clearly much more pronounced in cells with increased microtubule growth rates (e.g. in SW480 cells) where we can suppress the assembly rates from 22.9 µm/min to 15.8 µm/min by 0.1 nM Taxol treatment. The same treatment on HCT116 cells results in microtubule growth rate suppression from 14.6 µm/min to 13.6 µm/min and this effect is NOT significant. This indicates that lowering microtubule assembly rates from a "normal" state is much more difficult than suppressing high rates of microtubule growth. This result might help to explain why e.g. *APC* or *AXIN1* depletion does not result in significant lowered microtubule growth rates. It might be possible to reveal a slight reducing effect when analyzing knockout cells, which are, most likely, not viable.
In this regard, it is important to note that overexpression of Aurora-A clearly results in an increase in microtubule assembly rates (Ertych et al., Nature Cell Biology 2014) while Aurora-A depletion does not affect normal microtubule assembly. Thus, while an alteration of a given protein can increase microtubule assembly rates the opposite alteration must not necessarily trigger the opposite effect. We believe that this is a wide-spread phenomenon that can be seen in various biological processes.

*5. The authors conclude that Wnt/STOP, in other words a change in the degradation of specific proteins is the mechanism invoked in increasing the microtubule polymerisation rate. However, this is contradictory to the result showing that Wnt 3a does not produce an effect. The explanation provided is that hyperactivation of Wnt signalling is to blame. However, contradictory data are provided in a recently published (Acebron et al (2014) Mol Cell 54: 663-674; cited in this manuscript) showing that over-activation of Wnt does increase the Wnt/STOP activity.*

Response:
Acebron et al., indeed demonstrated that Wnt/STOP can be inhibited by e.g. DKK treatment and activated/induced by Wnt3a treatment. This is associated with de-stabilization and stabilization of target proteins, respectively. However, in our experiments we find a physiological effect (i.e. an increase in microtubule assembly rates and aneuploidy induction) only in response to inhibition of Wnt/STOP. This is in line with our previous work, in which we have identified several examples where decreased proteins levels of certain mitotic regulators (e.g. Chk2 or Brca1) cause an increase in microtubule assembly whereas the overexpression of the same proteins do not decrease microtubule assembly rates. Thus, it is conceivable that Wnt/STOP might regulate those proteins.
As pointed out above there are multiple examples from cellular systems where deregulation of a given protein causes a specific phenotype while the opposite alterations must not necessarily cause the opposite effect.

*6. The statement that WntSTOP is required for proper microtubule assembly is overstated. The only direct evidence for a role of protein degradation in the measured effects is provided by the data in Figure 4 where the effect of MG132 is measured. This is not sufficient proof to conclude that the observed effects are due to WntSTOP, it simply shows that protein degradation by the proteasome is important. In addition, microtubules are clearly assembled properly they just have slightly altered dynamics. One thing that was never discussed is the depolymerisation rate and thus ultimately the parameters of dynamic instability, which together govern microtubule dynamics.*

Response:
The current (first) model describes Wnt/STOP as a Wnt and cell cycle regulated pathway that contributes to the stabilization of proteins at G2/M and during mitosis (Acebron et al., 2014). Our work provides the following evidence that Wnt-mediated stabilization of proteins in mitotic cells is important for proper microtubule assembly: (i) microtubule assembly is

 

increased in response to basal Wnt inhibition (loss of LRP5/6, loss of Dvl2, treatment with sFRPs, treatment with DKK1); (ii) This effect is restricted to mitosis and not present in interphase; (iii) This effect is suppressed by proteasome inhibition (MG132 treatment); (iv) This effect is also suppressed by inhibition of the Wnt-regulated destruction complex (APC, Axin-1); (v) This effect does not require β-catenin/TCF4-mediated transcription (Figure 1A, Figure E1E, E1F); (vi) This effect causes chromosome missegregation and aneuploidy (Figure 2C and Figure 3A, 3C).
Thus, our data provide solid support for the statement that Wnt/STOP is required for proper microtubule assembly in mitotic cells and for the maintenance of a stable karyotype.

In our previous work we demonstrated already that an increase in microtubule assembly rates occurs independent of alterations of other microtubule dynamics parameters. In fact, rates of rescue or catastrophe or overall dynamicity are not changed in cells exhibiting an increase in plus end assembly rates (Ertych et al., Nature Cell Biology 2014). Alterations in minus end depolymerization would inevitably affect spindle size, which we do not observe. To make this point more clear we have added now a brief statement on this in the Results and Discussion section (see page 7, line 20pp).

> *7. There is no or poor correlation with the measured chromosome defects and the relative protein levels in different clones. For instance, the relative protein level of LRP6 increases from clone 1 to clone 3 (Fig E2). However, the induction of abnormal metaphase spindles is highest for clone 3 and lowest for clone 1 (Fig 2B). Similarly, the lowest level of Dvl were achieved in clone 3 (Fig E2) but microtubule dynamics are the least affected in this clone (Fig 2A). Similarly, microtubule polymerisation rates and chromosome defects also do not correlate.*

Response:
It is difficult to compare protein levels determined in lysates from cell populations with data from single cells that were used for measurements of microtubule assembly rates. It is conceivable that different individual cells chosen for the live cell analyses might harbor different protein levels for LRP6 or Dvl2. Unfortunately, it is not possible to perform all assays (microtubule assembly rates, mitotic spindle formation, karyotype analyses and protein content) from the very same individual cells. This intrinsic problem might explain data variability in all living systems, in particular when analyzing individual cells. Nevertheless, we are convinced of the fact that our data clearly indicate that (varying) lowered levels of LRP6 or Dvl2 in various cell clones (which have been gone through a selection process for weeks) produce consistently very similar phenotypes and outcomes.

> *8. There are no control experiments for the APC or Axin knockdown experiments to show the link between tubulin dynamics and chromosome mis-segregation. Previous data in the literature has shown chromosomes mis-segregation in APC-depleted cells; however, in those cases direct effects of APC on microtubules were implicated. Here the authors make the link between microtubule dynamics and chromosome defects but they only provide measures of this activity selectively.*

Response:
This is an excellent suggestion. Indeed, loss of APC has been associated with impairment of microtubule-kinetochore attachments (e.g. Fodde et al. Nature Cell Biology 2001). In contrast, no such role has been described for Axin-1.
To thoroughly address the point made by the reviewer we determined the generation of lagging chromosomes in response to loss of APC or loss of Axin-1 in the absence or presence of low doses of Taxol (see our revised Figure 2C). The results fully confirm the reviewer´s and our expectation: loss of APC, but not of Axin-1 causes lagging chromosomes and thus, chromosome missegregation. However, in contrast to LRP5, LRP6 or DVL2 repression, this effect was NOT suppressed by Taxol indicating that chromosome missegregation in response to loss of APC occurs independently of an increase in microtubule plus end assembly. This is in full agreement with our data showing that loss of *APC* does not cause any alteration in microtubule plus end assembly rates (Fig. 1A and new Figure 1B). These new data are now described in the Results and Discussion section (see page 10, line 3pp).

 

Moreover, to provide an additional link between Wnt-mediated protein stabilization and chromosome missegregation we also determined lagging chromosomes during anaphase in response to loss of LRP6 or DVL2 and concomitant depletion of Axin-1 (see our new Figure 4E). These new experiments demonstrate that not only the increase in microtubule dynamics, but also chromosome missegregation is suppressed upon concomitant inactivation of the Wnt-regulated destruction complex. We are convinced that this now provides a clear link between mitotic Wnt signaling/Wnt-STOP, altered microtubule plus end assembly and chromosome missegregation. These new experiments are described in the text on page 12, line 16pp.

> *9. There is no evidence provided for a functional relationship between mutations in the investigated proteins and mutations or defects in cancer. In fact, colorectal cancer invariably carries APC mutations, which are associated with elevated Wnt signalling. In that scenario, mutations that lower Wnt signalling (i.e. defective LRP 5/6) should restore normal microtubule polymerisation rates and rescue any associated phenotype. Furthermore, in their previous publication the authors state that "Reconstitution of proper microtubule assembly rates by chemical or genetic means suppresses CIN and thereby, unexpectedly, accelerates tumour growth in vitro and in vivo" (Ertych et al., 2014). Together these considerations question the relevance of the new findings in the context of cancer. They also indicate the likely significant contribution of Wnt-independent functions of APC, Dvl, etc. particularly their direct effect on microtubules and microtubule dynamics in mitosis, which are only mentioned in passing in the discussion.*

Response:
As the reviewer points out, it is apparent that hyper-activity, but not loss of Wnt signaling is strongly associated with human cancer. However, in our study we are not primarily focusing on the cancer-associated role of Wnt signaling, but instead we revealed an important requirement of basal Wnt signaling and, in particular, of Wnt/STOP during a normal unperturbed cell cycle. In fact, we demonstrate that basal Wnt activity is required for proper microtubule dynamics, proper execution of mitosis and for the maintenance of a stable karyotype and euploidy. This reinforces the importance of Wnt signaling during embryonic cell divisions and mitoses.

On the other hand, it is clear that various human diseases are associated with aneuploidy. One prime example is cancer. Here, loss of APC might indeed contribute to chromosome missegregation (e.g. Fodde et al., 2001), but this appears to be independent of de-regulation of microtubule plus end dynamics as we demonstrate in our revised Figure 2C. In addition, direct functions of different Wnt signaling proteins, which we discuss in our Introduction (see page 4, line 16pp) and in our Results and Discussion section (see page 10, line 3pp) might also contribute to aneuploidy in cancer. However, clear evidence for this is still lacking.

Although not central to our study it is interesting to note that the induction of aneuploidy in response to Wnt inhibition might be of relevance for human diseases other than cancer. In fact, it has been recently shown that inhibition of Wnt signaling upon loss of LRP6 or after induction of DKK1 in neurons is associated with the pathology of Alzheimer´s disease (Liu et al., Neuron 2014; Caricasole et al., J. Neuroscience 2004), which is characterized by high-grade aneuploidies in the brain. As an interesting perspective for future studies we have included these new aspects at the end of our Discussion section (see page 14, line 3pp).

> *10. Another consideration is that the microtubule polymerisation rates differ by 25-30% in different controls making the 25-30 % difference in the selected clones relatively minor changes.*

 

Response:
It is important to note that the different clones have gone through different selection procedures. Therefore, it is important to generate and to measure the appropriate control clones (e.g. with control shRNAs etc.). When comparing these proper controls to the different experimental conditions we obtained significant results for each experiment. In addition, microtubule assembly rate measurements always show some variability. Even in untreated cells we usually find assembly rates ranging from 11 to 21 µm/min in an individual cell (see e.g. untreated HCT116 cells shown in Figure 1F). This might reflect the variability of microtubule assembly rates in living cells.

**Responses to comments from Reviewer #2**

> *The reviewed manuscripts describes a previously unrecognized role of Wnt signaling in regulating microtubule assembly rates and thereby affecting mitotic spindle formation and proper chromosome segregation. The authors provide solid, although preliminary evidence that the recently described Wnt-dependent stabilization of proteins (Wnt/STOP) during mitosis likely affects the regulation of microtubule assembly and spindle formation during mitosis in human cells.*
> *This is a nice and relevant study that brings to our attention the previously unappreciated, β-catenin independent functions of Wnt signaling during mitosis. Given the frequent chromosomal instability and mitotic errors in cancer cells and the fact that Wnt-signaling is often affected in cancer, it is of a broad biological significance and interest of scientific community to explore the possible links between Wnt signaling and mitosis. The experiments are carefully done, with appropriate controls, and they are logically linked together. The authors do a nice work in testing the possible involvement of the novel Wnt/STOP pathway, thereby mapping new directions for the future research. The article is appropriate for publishing in EMBO Reports and will be of interest to broad scientific community. Before publishing, several minor issues should be addressed:*
>
> *1. The Wnt signaling pathway is arguably rather complex. Therefore, the authors should pay more attention to the description of the Wnt pathway in the introduction. A little bit more explanations and clarifications of the pathway and the individual players would be very useful.*

Response:
We now included a more detailed introduction on Wnt signaling. However, due to strict space limitations we were not able to do this in a comprehensive manner.

> *2. The language of the manuscript should be extensively edited. The sentences are often very long, complex and rather difficult to navigate. Some expressions are unnecessary complicated and rather confusing, e.g. ". . .increased rates of microtubule assembly rates . . ." (page 5) or ". . . abnormal metaphase spindle structures . . ." (page 9) - why not abnormal metaphase spindles?*

Response:
We now edited the text throughout and omitted in particular long and difficult to understand sentences. We hope that the text is now easier to read and to understand.

> *3. Some details about the experiment with MG132 should be presented already in the text. MG132 has strong and multifaceted effects on cells and therefore specifying that the cells were arrested in mitosis and treated only for one hour would improve the understanding for the reader (everything is correctly explained in Material and Methods, which are by the way very clearly and neatly written).*

Response:

 

As suggested by the reviewer, we now included details for the MG132 experiments in the results section (see page 12, line 8).

*4. Page 11, the authors show the percentage of cells deviating from modal number, but do not state whether the modal number changes during the culturing. This would be useful information and the modal number should be added to the figure or to the text. Of course, the data is available in the supplementary data (Figure E2), but just a simple statement that the modal number remained 45 in all cases would make the reading easier.*

Response:
As suggested, we now included the modal number into the Figures 3A and 3C and mention this also in the text (page 11, line 10)

*5. Finally, it would be useful to provide a simple schematic model of the findings.*

Response:
As suggested, we now include a summarizing model of our findings in our new Figure E6.

**Responses to comments from Reviewer #3**

*Here, Stolz et al. analyzed the potential effects of mitotic Wnt signaling (referred to as Wnt/STOP pathway) on mitosis progression. In a HCT116 colon cancer cell line, the authors investigated microtubule assembly rate upon perturbing Wnt pathway components. Their main finding is that endogenous Wnt signaling during mitosis is required to maintain the proper microtubule assembly rate, and that LRP6 and Dvl are essential components while β-Catenin, Axin and APC are dispensable for this process. An accelerated microtubule assembly rate upon loss of LRP6 or Dvl leads to abnormal mitotic spindle formation and chromosome missegregation that ultimately results in aneuploidy, a hallmark of human cancer. Those are interesting and novel findings. A very novel point is the authors' proposal that Wnt/STOP pathway, a branch of the Wnt pathway conferring protein stabilization during mitosis, is a pathway responsible for the +end microtubule dynamics required for proper microtubule assembly, microtubule-kinetochore (MT-KT) attachment and proper segregation of daughter chromosomes during mitosis.*
*This manuscript reports a novel involvement of the Wnt/STOP pathway in the process of mitosis and will be of general interest to the cancer biology field since chromosome instability is a hallmark of cancer and the Wnt pathway is one of the main signaling pathways involved in cancer development. Publication is recommended after revisions that probably will not require new experiments but rather a more detailed discussion according to the points listed below.*

**Major points:**

*1. There is a previous paper by Kikuchi et al. (reference #13 of the current manuscript), indicating that endogenous Wnt signaling through Wnt receptors (both Fz and LRP6) and Dvl accounts only for spindle orientation but not for MT-KT attachment, while Dvl is required for MT-KT attachment in a Wnt-independent way. This discrepancy, probably due to cell lines used, merits discussion in the current manuscript.*

Response:
It is indeed interesting that Kikuchi et al. implicated loss of Dvl2 in spindle misorientation. We have previously shown that increased microtubule assembly rates also cause spindle misorientation. Thus, it might be plausible that loss of Dvl2 causes increased microtubule

 

dynamics (as we show in our current work) and this results in spindle misorientation. This function appears to be separate form the role of APC and Dvl2 in mediating microtubule-kinetochore attachments. As suggested, we now discuss this point (see page 10, line 7pp).

> *2. The mechanistic basis of Wnt/STOP pathway proposed by Acebron et al. (reference #10 of current manuscript) is an earlier finding that Wnt induces GSK3 sequestration inside multivesicular bodies (Taelman et al., 2010, Reference #8). Endosomal sequestration of Axin and GSK3 is not only regulating β-Catenin stability but also causes global protein stabilization (Taelman et al., 2010; Vinyoles et al., 2014). The possible role of multivesicular endosomes in mitosis and spindle formation should therefore be discussed.*

Response:
As suggested, we included the requirement of multivesicular endosomes for the inactivation of GSK3 in response to Wnt signaling into our introduction (see 3, line 15pp) and in the Results and Discussion section (see page 9, line 1pp).

> *3. Perhaps "Wnt/STOP protein stabilization" could be more informative if used in the title itself.*

Response:
As suggested, we changed the title to " Wnt/STOP protein stabilization ensures proper microtubule assembly and chromosome segregation".

**Minor points:**

> *1. What this paper needs most is a few still images of how microtubule polymerization changes (e.g., in Fig 1A). We normal readers need to be able to visualize this for at least in one example.*

Response:
Since microtubule assembly rates were determined in living cells we reasoned to provide a model that combines a scheme and a still image. This model is now provided in our new Figure E1D and visualizes the principle of the key assay used in our work.

> *2. It would be good to show a normal and abnormal karyotype in Figure3. This will enrich the paper.*

Response:
As suggested, we now included examples of chromosome spreads showing a normal and an aneuploid karyotype as part of the revised Figure 3A.

> *3. In page 11 line 6 "increased by more than 100%" might cause confusion. Simply, "more than two-fold" instead of "more than 100%", would be more straightforward.*

Response:
As suggested, we changed the sentence accordingly (see page 11, line 9)

> *4. In figure 4B, the authors may want to use GSK3 RNAi or other means of GSK3 inhibition (e.g., BIO) in addition to Axin or APC RNAi. However, one imagines that GSK3 is required at many stages of cell cycle progression. Perhaps CycY RNAi could also be useful in future work.*

Response:
As the reviewer realizes GSK3 has multiple functions during mitosis and in interphase. Previous work has described some severe defects in mitosis after GSK3 inhibition (Tighe et al., BMC Cell Biology 2007). Nevertheless, we used several commercially available GSK3 inhibitors for additional experiments. However, in all cases we either observed cell

 

cycle arrest before mitosis, which excluded any further analyses of mitotic events or we observed severe spindle formation defects (as seen by Tighe et al.), which prevented detailed measurements of microtubule dynamics. In addition, it should be noted that GSK3 inhibition results in rapid onset of apoptosis.
We agree that targeting Cyclin Y is of great interest for future studies, in which we would like to address the role of LRP6 phosphorylation for the regulation of microtubule dynamics. However, we feel that these experiments would be beyond the scope of our current work, but we now mention CDK14-cyclin Y in our Results and Discussion section (see page 8, line 19pp).

2nd Editorial Decision 09 January 2015

Thank you for your patience while we have reviewed your revised manuscript, which, unfortunately, was delayed due to the holiday season. As you will see from the reports below, both referees that were asked to assess the revised version are now positive about its publication in EMBO reports. Referee 1 still feels that the conclusions should be toned down in some instances and that some further clarifications should be added before publication. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once these few minor textual changes have been incorporated.

Thank you for your contribution to EMBO reports. I look forward to seeing it in print, as I think it is a very intriguing finding.

REFEREE REPORTS:

Referee #1:

The authors addressed almost all the points raised by the reviewers and added a large number of additional points and experiments.

There remain a few issues that should be dealt with:

• The double knock out for Lpr5 and 6 consistently results in poor depletion of Lpr6. This was not addressed in the response.

• The authors should tone down their conclusion that Wnt/STOP is responsible for the effects they observe. They clearly show that canonical WNt signalling is NOT involved and that the effects demonstrated are specific to mitosis. In the absence of a more complete understanding of the Wnt/STOP effect and the possibility that other pathways/mechanisms may exist in addition to Wnt/STOP. In the absence of any direct evidence that the half-life or turnover of relevant proteins is affected the interpretation should be more cautious.

• In response to the comment that Wnt signalling in HCT116 cells is not basal due to the presence of a mutant allele of beta-catenin the authors point out that Wnt signalling in these cells can still be raised and can thus be considered basal. However, they should acknowledge that it is not basal relative to other systems, but is indeed elevated. So I would like to suggest changing their language to call it baseline versus elevated or to define what they mean by basal in this case. Otherwise it seems a bit misleading.

• In their rebuttal the authors state that : "Even in untreated cells we usually find assembly rates ranging from 11 to 21µm/min in an individual cell (see e.g. untreated HCT116 cells shown in Figure

 

1F). This might reflect the variability of microtubule assembly rates in living cells." This makes the point raised in the initial review particularly relevant that the relatively small differences between different cells make interpretation of consequent effects on cell function with difficult. Such high variability between the growth rate of individual microtubules means that the selection of the 20 microtubules chosen in each cell used to create the averages shown can affect the data significantly. Is such variability also found in mitotic cells? If so, does this not mean it is particularly difficult to compare values that only differ by such small values? The authors need to provide some details about how they ensured that their selection was truly random.

Referee #3:

The revised manuscript has now addressed all the previous concerns satisfactorily. This paper has a single key message of the novel involvement of Wnt/STOP pathway in mitosis. The results are very convincing and discussed properly. Addition of a few still images now improved the manuscript greatly, making it more comprehensible to the broad audience of EMBO Reports. Therefore, I would recommend its publication in EMBO Reports without further revision.

2nd Revision - authors' response 12 January 2015

Thank you very much for accepting "in principle" our manuscript now entitled " Wnt mediated protein stabilization ensures proper mitotic microtubule assembly and chromosome segregation".

Since referee #1 asked for some minor text changes, which needed to be addressed before final acceptance, we now submit a revised version of our manuscript with the following changes:

> *1. Comment form reviewer #1: The double knock out for Lpr5 and 6 consistently results in poor depletion of Lpr6. This was not addressed in the response.*

Transient and stable repression of LRP5 and LRP6 in HCT116 cells resulted in 30-50% reduction in protein levels (Fig. E1A, E1G and E3). Transient transfections with siRNAs in RPE1 cells, however, resulted in a significant higher reduction in LRP5/6 protein levels (Fig. E1B). In all cases we consistently detected significant changes in microtubule assembly rates indicating that partial repression of LRP5/6 is sufficient to induce the phenotypes observed. This is now clearly mentioned in the text (page 8, line 13ff): " Interestingly, we found that **partial** repression of *LRP5*, *LRP6* or *DVL2*, which …."

> *2. Comment form reviewer #1: The authors should tone down their conclusion that Wnt/STOP is responsible for the effects they observe. They clearly show that canonical Wnt signalling is NOT involved and that the effects demonstrated are specific to mitosis. In the absence of a more complete understanding of the Wnt/STOP effect and the possibility that other pathways/mechanisms may exist in addition to Wnt/STOP. In the absence of any direct evidence that the half-life or turnover of relevant proteins is affected the interpretation should be more cautious.*

 

Wnt mediated stabilization of proteins (Wnt/STOP) is a novel function of Wnt signaling, which was just very recently identified. It is clear that so far very little is known about this novel and non-canonical role of Wnt signaling, which acts apparently at G2/M. As the reviewer points out, our work demonstrates a non-canonical role of Wnt signaling for proper microtubule assembly during mitosis that involves protein stabilization. By definition (published by Niehrs and collegues), we would call this role "Wnt/STOP". Nevertheless, we now changed the text at several passages in order to tone down our conclusion. In particular, we make clear that (i) little is known about Wnt/STOP and (ii) that we just provide some first evidence for Wnt/STOP being involved in mitotic regulation. In addition, we changed the title in order to avoid the term "Wnt/STOP": " Wnt mediated protein stabilization ensures proper mitotic microtubule assembly and chromosome segregation".

In the text we made the following changes:

page 2, line 14: Wnt/STOP rather than Wnt-mediated transcriptional control **might be** required for this novel function of Wnt signaling in human somatic cells.

page 5, line 13: **However, this novel role of Wnt signaling is yet poorly understood** and a specific role for the entry into or for the progression of mitosis has not been identified so far.

page 7, line 5: In our work presented here, we reveal a requirement for **Wnt signaling during mitosis that is independent of canonical Wnt signaling** for proper mitotic microtubule plus end assembly and for faithful chromosome segregation in human somatic cells.
page 13, line 9: **Wnt mediated protein stabilization is required for proper microtubule assembly during mitosis**

page 14, line 10: Together, these results **might point into the direction** that Wnt-mediated stabilization of proteins other than b-catenin is required for the maintenance of proper microtubule assembly and for faithful chromosome segregation during a normal mitosis (see Model in Fig E6).

page 14, line 13: Thus, our work **might provide** the first physiological role of Wnt/STOP during an unperturbed cell cycle.

page 14, line 19: This will provide important new insights **for a possible role** of Wnt/STOP as a regulatory pathway for a normal cell cycle and, in particular, for the regulation of a normal mitosis and chromosome segregation.

> *3. Comment form reviewer #1: In response to the comment that Wnt signalling in HCT116 cells is not basal due to the presence of a mutant allele of beta-catenin the authors point out that Wnt signalling in these cells can still be raised and can thus be considered basal. However, they should acknowledge that it is not basal relative to other systems, but is indeed elevated. So I would like to suggest changing their language to call it baseline versus elevated or to define what they mean by basal in*

 

*this case. Otherwise it seems a bit misleading.*

We now included a definition for "basal or baseline Wnt signaling" on page 8, line 7/8: "Therefore, we investigated a potential involvement of **non-induced (= basal or baseline)** Wnt signaling in this process." In addition, according to the suggestion of the reviewer we now clearly state that the measurements were performed in the absence of additional Wnts (= uninduced = baseline), page 8, line 13ff: " Interestingly, we found that partial repression of *LRP5*, *LRP6* or *DVL2*, which led to inhibition of **baseline Wnt signaling in the absence of additional Wnt treatment** (Fig. E2A) triggered an increase in microtubule plus end assembly rates in mitotic, but not in interphase cells (Fig 1A, 1B, E2B)

*4. Comment form reviewer #1: In their rebuttal the authors state that : "Even in untreated cells we usually find assembly rates ranging from 11 to 21µm/min in an individual cell (see e.g. untreated HCT116 cells shown in Figure 1F). This might reflect the variability of microtubule assembly rates in living cells." This makes the point raised in the initial review particularly relevant that the relatively small differences between different cells make interpretation of consequent effects on cell function with difficult. Such high variability between the growth rate of individual microtubules means that the selection of the 20 microtubules chosen in each cell used to create the averages shown can affect the data significantly. Is such variability also found in mitotic cells? If so, does this not mean it is particularly difficult to compare values that only differ by such small values? The authors need to provide some details about how they ensured that their selection was truly random.*

Variability of microtubule assembly rates is a common and known phenomenon. Therefore, we usually measure a total of 400 (!) individual microtubules in order to determine reliable average growth rates. These individual microtubules are always selected randomly. In order to minimize the risk to measure particular classes of microtubules (e.g. astral microtubule versus interpolar or kinetochore microtubules) we used a mitotic synchronization step that induces monopolar mitotic spindles. In those monopolar spindles we cannot discriminate between different classes of microtubules. Thus, microtubules cannot be pre-selected according to their function in bipolar spindles and are therefore randomly selected. Importantly, we have previously shown that the measurements of microtubules in monopolar spindles are comparable with microtubules in bipolar prometaphase spindles (Ertych et al., Nature Cell Biology 2014). This is now clearly described in the Expanded View Method section (page 5, line 10ff): " This synchronization step was useful to ensure measurements of individual microtubules in the same mitotic phase and does not affect the plus end growth rates *per se* [3]. In addition, monopolar spindles do not allow a discrimination between astral and interpolar microtubules and thus, individual microtubules were randomly selected for measurements."

 

Since we have now addressed the requested minor text changes as outlined above, we hope that you will now be able to accept our manuscript for publication in *EMBO Reports*.

Thank you very much for your interest in our work, which will be certainly an important addition to one of the upcoming issues of *EMBO Reports*.

3rd Editorial Decision 13 January 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

 