EMBO reports

Manuscript EMBOR-2012-35736

Kindlin-2 forms a transcriptional complex with -catenin and TCF4 to enhance Wnt signaling

Yu Yu, Junzhou Wu, Ting Zhao, Yunling Wang, Ting Zhao, Bo Ma, Yuqing Liu, Weigang Fang, Wei-Guo Zhu and Hongquan Zhang

Corresponding author: Hongquan Zhang, Peking University Health Science Center

Review timeline:

Submission date:	28 November 2011
Editorial Decision:	05 December 2011
Resubmission date:	11 January 2012
Editorial Decision:	01 February 2012
Revision received:	25 March 2012
Editorial Decision:	04 April 2012
Revision received:	28 April 2012
Accepted:	21 May 2012

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 05 December 2011

Thank you for the submission of your research manuscript to our editorial office. I would like to apologize for the small delay in getting back to you with a decision on your study. I have now had the opportunity to carefully read it and I have also discussed it with the other members of our editorial team and with an additional advisor of relevant expertise in the field.

We acknowledge that you provide evidence that kindlin-2 interacts with and stabilizes the active form of beta-catenin and, in a complex with beta-catenin and TCF4, regulates beta-catenin target gene expression, thereby regulating wnt signaling and cancer cell invasion. Both we and our advisor appreciate the potentially interesting nature of theses findings. However, our advisor also indicated that the manuscript as it stands is not yet sufficiently developed to warrant consideration for publication here. For example, s/he notes that a potential contribution of kindlin-2 localized at focal adhesions or adherens junctions on tumor cell invasion has not been considered. S/he also pointed out that it remains unclear whether kindlin-2 stabilizes beta-catenin only by preventing GSKb binding or whether it also regulates GSK-3b activity through its signaling properties at focal adhesions. From a more technical point of view, the advisor was concerned that some of the findings are not well controlled. For example, only one shRNA has been used and no rescue studies have been performed. In addition, the kindlin-2/beta-catenin interaction has only been analyzed in tumor and not in normal cells.

Based on these considerations we have decided not to proceed with the in-depth review process of your manuscript at this point. Given the interesting topic, we would, however, have no objections to consider a new manuscript on the same topic if at some time in the future you obtained data along the lines described above. However, I have to say, that if you were to send a new manuscript, this would be treated as a new submission and would be evaluated again, also with respect to the literature and the novelty of your findings at the time of resubmission.

I am sorry to have to disappoint you on this occasion, and hope that this will not prevent you from considering EMBO reports for publication of your work in the future.

Yours sincerely,

Editor
EMBO Reports

Resubmission - authors' response 11 January 2012

I greatly appreciated your comments and suggestions for our manuscript entitled “Kindlin-2 forms a transcriptional complex with β-catenin and TCF4 to enhance Wnt signaling”. The advice has improved the quality of our work very much. The suggested experiments have been thoroughly followed and completed. I will list all of them one by one as following:

1. s/he notes that a potential contribution of kindlin-2 localized at focal adhesions or adherens junctions on tumor cell invasion has not been considered.

   We used the following experiments to understand the possible involvement of integrin β1 subunit in the interaction of Kindlin-2 with β-catenin and Kindlin-2 regulated Wnt signaling. For this, we generated an integrinβ1-binding deficient Kindlin-2 mutant, the Kindlin-2 QW mutant. Using this mutant we performed Co-IP assay and found the Kindlin-2 QW mutant also interacted with β-catenin. The experiments are shown in Figure 1C. Besides, we also performed the TOP/FOP assay for examination of the role of integrin β1 in Kindlin-2-regulated Wnt activation. The experiments are shown in Figure 4D.
2. S/he also pointed out that it remains unclear whether kindlin-2 stabilizes beta-catenin only by preventing GSK-3β binding or whether it also regulates GSK-3β activity through its signaling properties at focal adhesions.

   This is a very important question. To clear out if Kindlin-2 also regulates GSK-3β activity, we detected GSK-3β activation by measuring its GSK-3β-Ser9 phosphorylation. We found that Kindlin-2 does inhibit GSK-3β activation by increasing GSK-3β-Ser9 phosphorylation. Therefore, Kindlin-2 regulates Wnt signaling via both preventing β-catenin binding to GSK-3β and inhibiting GSK-3β activation, the two ways. Figure 3G and H described these findings. Thus, it is interesting to distinguish which one is important for Kindlin-2 regulation of Wnt signaling: preventing β-catenin binding to GSK-3β or inhibiting GSK-3β activation? Figure 5E tells a story that Kindlin-2 is required for β-catenin binding onto the Wnt responsive elements. The answering for the myth is that Kindlin-2 binds to β-catenin and stabilizes β-catenin to enhance its occupancy on the Wnt responsive elements is more important than Kindlin-2 inhibition of GSK-3β activity.
3. From a more technical point of view, the advisor was concerned that some of the findings are

not well controlled. For example, only one shRNA has been used and no rescue studies have been performed.

The points for our kind advisor are very critical and needs highly concerned. We applied two siRNAs in our repeating experiments and Kindlin-2 shRNA resistant mutant has been used in the rescue experiment. The new experiments are shown in Figure 3A and 4E. In addition, we added a new experiment to set up a functional link between Kindlin-2-induced tumor cell invasion and the requirements of β-catenin, Axin2 and Snail in a cell invasion assay as shown in Figure 5I.

4. In addition, the kindlin-2/beta-catenin interaction has only been analyzed in tumor and not in normal cells.

In our new experiments we also examined the Kindlin-2/β-catenin interaction in normal cells as shown in Figure 1B and Figure 2D-E.

I hope we have fixed the weak parts in our manuscript appeared in the previous version. On behalf of all the authors of this manuscript, I appreciate your kind consideration of our manuscript can be sent out for reviewing and be eligible for publication in EMBO Reports.

In this manuscript, we identified a direct molecular interaction of Kindlin-2 with unphosphorylated, but not with phosphorylated β-catenin. This finding represents a novel mechanism for maintaining the stability of β-catenin. The interaction allows Kindlin-2 to form a tripartite transcriptional complex with β-catenin and TCF4. The tripartite complex occupies Wnt responsive elements and promotes Wnt target gene Axin2 expression, and in turn functionally regulates tumor cell invasion. Our results unravel a new regulator of Wnt signaling, and may provide a direction for understanding the mechanistic association of Kindlin-2 with tumor invasion. These data are novel and of particular significance because many laboratories are considering these proteins for candidate cancer therapeutic targets.

1st Editorial Decision 01 February 2012

Thank you very much for the submission of your research manuscript to our editorial office. We have now received the full set of reports from the referees that were asked to assess it. As the detailed reports are pasted below I will only repeat the main points here. As you will see, while appreciating the potential interest of the findings, the referees also raise a substantial number of concerns about the conclusiveness and completeness of the data and suggest a number of additional experiments to strengthen the manuscript.

For example, referee 1 feels that the functional significance of the kindlin-2/beta-catenin interaction needs to be strengthened. S/he suggests to improve the reporter assays and to use wnt ligands to activate the pathway. This referee also feels that the functional significance of the kindlin-2/beta-catenin interaction in EMT needs to be strengthened. To clearly establish a positive role for kindlin-2 in the wnt pathway, s/he suggests analyzing its effect on other wnt target genes. Finally, this reviewer makes some technical comments, for example on the co-localization and the interaction experiments (referee 2 also comments on the latter point). One of the main points raised by referee 3 is that it should be shown that endogenous kindlin-2 has the same effects as the overexpressed protein. Along these lines, this referee points out that for cells overexpressing kindlin-2, more than

one independent clone should be analyzed. S/he also states that the effects of kindlin-2 knockdown/overexpression should be better characterized. I would also like to bring up one point that our advisor previously considered crucial and I apologize if I have not been clear enough in my last letter to you: it would be important to use the siRNA-resistant kindlin-2 version in an actual experiment. In other words, in an experiment in which knockdown of kindlin has some effect, it should be shown that the resistant version can rescue these effects. On a more formal notice, it also seems as if figure 3A is not mentioned in the text.

From the analysis of these comments it becomes clear that significant revision is required before the manuscript may become suitable for publication in EMBO reports. However, given the potential interest of your study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees must be addressed and their suggestions (as detailed above and in their reports) taken on board. 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.

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

1. Do the contents of this manuscript report a single key finding? YES

If NO, could parts of the manuscript be removed to create a more focused study? Would it still be of sufficient quality for publication in EMBO reports?

Yes

2. Is the main message supported by compelling experimental evidence? NO

If NO, please indicate IN ORDER OF PRIORITY which additional experiments are ESSENTIAL (including controls and statistical analyses, and/or those experiments of low technical quality that must be repeated). Could they be conducted within three months? Yes.

Although the interaction of Kindlin-2/β-catenin is established with great care, the function in canonical Wnt/β-catenin signalling is not free of issues.

Major
1. The authors call a 2-fold induction of the TOP/FOP system in 293T cells "strong" (page 7 on my count). Researchers in the Wnt field will object to this conclusion, as these cells are able to induce the reporter (TOPFlash 10 TCF optimal sites) at least two/three orders of magnitude higher. But as the LiCl effect was also very low, the reporter assay cannot be working very efficiently. This casts some doubt to the results. In addition, the authors should activate the pathway with an upstream ligand such as Wnt3a to test their effect in a more relevant mechanism, before they can show that Kindlin-2 is required for Wnt/β-catenin signalling.

2. This reviewer is not convinced about the role of Kindlin-2 as a positive regulator of the Wnt

pathway as Axin2, its main target, is a textbook case of negative feedback in Wnt/β-catenin signalling. This could be the reason why reporter activation is in fact very modest. If Kindlin-2 is required to efficiently activate the negative regulator Axin2 in Wnt/β-catenin signalling, the functional role for Kindlin-2 in this pathway cannot simply be summarized as a positive one. Validating other Wnt target genes would strengthen this main conclusion.

3. The ms does have potential to show a functional role for the Kindlin-2β-catenin interaction in EMT, but fails to do so. Although the effect of Kindlin-2 on Axin2 transcription seems clear, the functional consequences are poorly defined, except for an enhancement in cellular motility. To support the mRNA data, authors should show Axin2 protein levels in the conditions of Fig. 5B and C, and in Kindlin-2 depleted Hs578T cells. Furthermore, the authors focus on a link between Kindlin-2/Axin2 and Snail, referring to Yook et al. 2006. They should be able to validate this link by showing that Axin2 knockdown in MCF7-Flag-Kindlin-2 is able to inhibit the increase in Snail stability. The role of Snail in epithelial-to-mesenchymal transition is well established, although the authors do not assess this process in the manuscript. In order to give a functional relevance to the interactions described and their link to Snail, the authors need to show that Kindlin-2 is required for the Wnt/Axin/Snail-mediated EMT programme, which is believed to underlie the tissue-invasive character of breast cancers. Do authors see induction of EMT marker genes (fibronectin, Twist, Vimentin, loss of E-cadherin, etc) upon modulation of Kindlin-2 function (overexpression or knockdown)? The relationship of the function of Kindlins in focal adhesions should also be addressed within this perspective.
These points can be addressed in 3 months.

3. Have similar findings been reported elsewhere (e.g. on a closely related protein; in another organism or context)? NO

4. Is the main finding of general interest to molecular biologists? YES

This ms shows a new role for Kindlin-2 (FERMT-2 gene product) in binding and stabilizing β-catenin, which plays an important role in canonical Wnt/β-catenin signalling. Specifically, the Kindlin-2/β-catenin complex enhances TCF-4-mediated Axin2 transcription. In addition, the authors implicate this mechanism in a positive role Kindlin-2 may have in tumour cell invasiveness. Because Kindlin-2 has been previously implicated in cancer, and as Kindlin-2 was found to be highly expressed at the tumour invasive front and in metastasis (An 2010 Int. J Cancer), this mechanism could be relevant.

5. After appropriate revision, would a resubmitted manuscript be most suited for publication:

in EMBO reports

6. Please add any further comments you consider relevant:

Moderate
1. The microscopy is not convincing. In Fig. S1, the DAPI stain is all over the place and does not outline the nuclei. In Fig. S2, Kindlin-2 is stained throughout the cell, which means there is no specific co-localisation with β-catenin. Worse, in the lower merged panel a different field is depicted than in the single channels, so that the view avoids one cell in which differential localization is apparent.

2. There have been concerns in literature (Van Noort 2007 Blood, Maher 2009 Biology Direct) about the unfortunate naming and marketing of an antibody (clone 8E4) that is not as specific for unphosphorylated β-catenin as the ABC antibody (clone 8E7) although it is sold as such, by the same company. Moreover, even the 8E7 antibody (to be preferred over 8E4) has been reported to

have specificity issues on westernblot, so the authors should indicate the height of protein markers and/or show a larger portion of the blot, as well as the type of antibody used; Millipore sells both aforementioned antibodies, as well as a number of other alleged dephospho-specific antibodies.

3. Can the authors explain the considerable difference between the GSK3β-Ser9 blots in Figs. 3G and 3H?

4. Reciprocal coIP is not direct evidence for a tripartite complex, as there could also be the 3 combinations of 2 proteins present. An accepted way to show a tripartite complex is a two-step IP. Alternatively, interaction mutants or protein knockdown, as in Fig 4C, could be used to characterize complex formation and functional linkage. The authors must be more careful about their interpretations of 4A and at least show the third protein in each of the individual IP experiments in 4A and 4C.

5. To be sure they are not looking at off-target effects, the authors should confirm key experiments with at least a second RNAi : Fig 3B, E, 5C, D, F.

6. In the Boyden Chamber in vitro cell invasion experiments, why are the controls (the same cell type MCF7) of 5G, 5H and 5I more than different by 3-fold or more (i.e. 0 - 4 - 12) and yet the s.d. is low?
Minor
1. Page numbers would have been nice. Result sections are too long and require more subdivisions (along with the figures for instance).

2. At least once the authors may want to mention the FERMT2 gene name.

3. HEK293T cells are hardly "normal human cells" as they have been manipulated by viral entities twice; perhaps "non cancer-derived cell line" would be more concise.

4. What is the reference for this QW mutant? Authors should either show its functional limitations or refer to published results.

5. The GST-β-catenin fragment 1-159 interacts with Kindlin-2 less efficiently than the 1-781 or 1-89 fragments. Can the authors comment on this surprising and potentially interesting observation?

6. The results in Fig. 5E, if in fact labelled correctly as percentage input, are in stark contrast with Fig 5A. Otherwise, the results might be normalized to the control values? A change of label would be in order. Also, error bars (Fig. 5A and E) would indicate that, at least in this representative one out of three experiments, the duplicates were close to each other.

7. Figures 5H and I are referred to as I and J in the text. Figure 3A is not mentioned in the main text and the cell type in 3C is not indicated.

Referee #2:

In this manuscript, the authors identify a role of kindlin-2 in Wnt signaling. Interaction between kindlin-2 and β-catenin is demonstrated, shown to be regulated by phosphorylation and to control downstream signaling by the Wnt pathway. Overall, the data are very interesting, convincing and novel. The major concern from a kindlin standpoint is whether the interaction between β-catenin and kindlin-2 is direct or indirect. The data supporting this interaction, as well as all the subsequent interaction are by pulldown experiments. An independent approach to demonstrate that kindlin-2 interacts with β-catenin and that this interaction is direct needs to be documented.

Referee #3:

This is a potentially interesting work describing the interaction of FERMT2 with b-catenin and a potential function of FERMT2 in b-catenin mediated gene regulation. The study is however rather

vague in the description of the experimental procedures and controls and is therefore difficult to judge. Most importantly, it is not clear if only cells engineered to express FERMT2 are used or if any of the reported findings also exist in the parental cells when analyzing endogenus proteins. Furthermore, for the experiments where engineered cells have been used, no controls of at least three independent clones are shown to ensure that we are not just looking at an artifact of a subclone of the parental cell line. This needs to be improved. In addition, it is not described what are the general, cellular effects of overexpression or knock down of FERMT2. Do the cells loose substrate adhesion or cell-cell adhesion? Are there alterations in cell death, apoptosis or proliferation? Much of the shown effects may be indirect and controls for other proteins/pathways which would be affected by such changes in cellular phenotype are missing.

Major points:

Fig1: an interaction of endogenous FERMT2 and endogenous b-catenin needs to be shown, not just from cells transfected to express the proteins

Fig 1: Is b-catenin localized to focal contacts in the presence of FERMT2

Fig 1: The N and C terminus of b-catenin are of very different sequence so it appears unlikely that both parts are bound by the same domain within FERMT2. Show that the C-terminus of b-catenin can compete out the N-terminus of b-catenin bound to FERMT2

Fig. 3C: FERMT2 seems not to be localized to the nucleus in striking contrast to b-catenin

Fig. 3E: the conclusion is not justified, GSK3b decreases b-catenin levels in both FERMT2 expressing and knockdown cells. The experiments were not described in sufficient detail. Are these cells engineered to express b-catenin and FERMT2 or are we looking at endogenous proteins?

Fig. 3G+H: what is the link between FERMT2 and GSK3, how does FERMT2 influence phosphorylation of GSK3? This is an important point which should be analyzed in more detail.

Fig.4 repeat with endogenous proteins

Fig.4D+E: as before, is this endogenous protein or cells engineered to express the proteins? Since FERMT2 increases b-catenin levels, the very modest 2x increase in in reporter activity could be due to just that. This is further supported in 4E where increased signaling by GSK3b inhibition via LiCl gives the identical fold change when comparing control siRNA with or without LiCl and FERMT2 siRNA with or without LiCl. The current data don't support the conclusion that FREMT2 affects Wnt signaling. The assay needs to be repeated with constant amounts of b-catenin, e.g. by expressing a non-degradable mutant of b-catenin.

Fig.5: the experiments need to be repeated with cells expressing only the endogenous FERMT2 protein.

Fig.5C+D: the effects of FERMT2 on the axin2 expression are much more pronounced than the reporter assays had suggested. What is the cellular fate upon downregulation of FERMT2. Do the cells loose substrate adhesion, cell-cell adhesion? Are there alterations in cell death, apoptosis or proliferation? Axin2 is known to be regulated by other inputs in addition to b-catenin/TCF, so could effects on other cellular processes explain the changes in axin2 expression levels?

Fig.5E: this effect could be again due to the upregulation/stabilization of b-catenin in response to FERMT2 and does not allow concluding that the triple complex is involved in transcriptional regulation of axin2. This conclusion needs to be removed from the text or shown under conditions which do not induce stabilization of b-catenin.

The link between FERMT2 and invasion is rather complex and cases of enhanced invasion upon loss of FERMT2 have been described (Shi & Wu 2008). This needs to be included in the last paragraph.

Minor points:

The official name for kindlin2, FERMT2, should be used at least once in the text.

For Fig1c, the methods section should provide details which exact mutant of FERMT2 was used.

The paragraph "Similar to Kindlin-2, Smad3 reportedly protects... leaves room for future investigation." contains no data and needs to be moved to the last paragraph.

1st Revision - authors' response 25 March 2012

Referee #1:
Major
1. The authors call a 2-fold induction of the TOP/FOP system in 293T cells "strong" (page 7 on my count). Researchers in the Wnt field will object to this conclusion, as these cells are able to induce the reporter (TOPFlash 10 TCF optimal sites) at least two/three orders of magnitude higher. But as the LiCl effect was also very low, the reporter assay cannot be working very efficiently. This casts some doubt to the results. In addition, the authors should activate the pathway with an upstream ligand such as Wnt3a to test their effect in a more relevant mechanism, before they can show that Kindlin-2 is required for Wnt/β-catenin signalling.
Response:
We appreciated this important comment and suggestion from this reviewer, which enlarged our imagination and extended our understanding on the important role of Kindlin-2 - β-catenin interaction on the activation of Wnt signalling, which we did not think from this angle before. Thank you very much indeed. We completed the following experiments:
We have changed the former TOP/FOP system (with 3 TCF optimal sites) with a new SuperTOP/FOPFlash system (8 TCF optimal sites, Addgene plasmid 12456). We answer this question at three layers. First, we expressed Kindlin-2 alone in 293T cells, and like before we could only see approximately 3-fold induction in SuperTOP/FOPFlash system. Since the 293T cells do not express high amount of endogenous β-catenin and there is only basic activation of Wnt signaling in this cell line. Accordingly, to face this low induction of Kindlin-2 on the Wnt signaling activation, we interpreted the scenario as lack of adequate β-catenin. Therefore, there is no proper Kindlin-2 -β-catenin interaction that forms a tripartite complex with TCF4 to activate Wnt signaling as indicated in Fig.1 and Fig.2. Kindlin-2 alone could not induce higher Wnt signaling activation. Secondly, when we transfected the non-degradable β-catenin (S37Y) alone we observed an induction of approximately 190-fold in the SuperTOP/FOPFlash system. Surprisingly, when β-catenin (S37Y) co-expressed together with Kindlin-2, we saw an approximately 1480-fold induction on top of the background. And an approximately 8-fold induction on top of β-catenin (S37Y) expression alone (Fig 4E), suggesting a very strong synergistic effect for Kindlin-2 interaction with the non-degradable β-catenin on the activation of Wnt signaling. Thirdly, we combined depletion of Kindlin-2 by siRNAs with the addition of upstream ligand Wnt3a and GSK-3β inhibitor LiCl as well as β-catenin (S37Y) expression. Importantly, knockdown of endogenous Kindlin-2 lead to approximately 50% cutting down of Wnt signaling activation even with the presence of Wnt3a or LiCl or β-catenin (S37Y) expression (Fig 4F). These data strongly suggest that Kindlin-2 is required for Wnt/β-catenin signalling. This question also leads us to think further that non-degradable β-catenin mutations in human cancers may associate with increased Kindlin-2 and make the tumors more aggressive.

2. This reviewer is not convinced about the role of Kindlin-2 as a positive regulator of the Wnt pathway as Axin2, its main target, is a textbook case of negative feedback in Wnt/β-catenin signalling. This could be the reason why reporter activation is in fact very modest. If Kindlin-2 is required to efficiently activate the negative regulator Axin2 in Wnt/β-catenin signalling, the functional role for Kindlin-2 in this pathway cannot simply be summarized as a positive one. Validating other Wnt target genes would strengthen this main conclusion.

Response:
We appreciate this important comment and suggestion. To this end, we have examined the role of Kindlin-2 in regulating other Wnt target genes, including CyclinD1, LEF-1, Twist, MMP2, sFRP-1

and Versican in both gain- and loss of function experiments. Ectopic expression of Kindlin-2 in MCF-7 breast cancer cell could upregulate the above Wnt target genes at various levels. Knock down the endogenous expression of Kindlin-2 greatly decreased the gene expression of the above Wnt target genes as determined by qPCR. These results are shown in the supplementary figure (SFig 8). We also described these Wnt target genes in the text (page 8). These results clearly demonstrated that Kindlin-2 do play an important role, as a positive regulator, in Wnt signalling pathway.

3. The ms does have potential to show a functional role for the Kindlin-2-β-catenin interaction in EMT, but fails to do so. Although the effect of Kindlin-2 on Axin2 transcription seems clear, the functional consequences are poorly defined, except for an enhancement in cellular motility. To support the mRNA data, authors should show Axin2 protein levels in the conditions of Fig. 5B and C, and in Kindlin-2 depleted Hs578T cells. Furthermore, the authors focus on a link between Kindlin-2/Axin2 and Snail, referring to Yook et al. 2006. They should be able to validate this link by showing that Axin2 knockdown in MCF7-Flag-Kindlin-2 is able to inhibit the increase in Snail stability. The role of Snail in epithelial-to-mesenchymal transition is well established, although the authors do not assess this process in the manuscript. In order to give a functional relevance to the interactions described and their link to Snail, the authors need to show that Kindlin-2 is required for the Wnt/Axin/Snail-mediated EMT programme, which is believed to underlie the tissue-invasive character of breast cancers. Do authors see induction of EMT marker genes (fibronectin, Twist, Vimentin, loss of E-cadherin, etc) upon modulation of Kindlin-2 function (overexpression or knockdown)? The relationship of the function of Kindlins in focal adhesions should also be addressed within this perspective.

Response:

Indeed, these questions are very important and we have to answer using experiments. We have added the following new experiments:

(1) As suggested, we have detected the Axin2 protein levels in MCF-7–Flag-Kindlin-2 stable cells, Kindlin-2 depleted Hs578T cells, and Kindlin-2 siRNA depleted 293T cells. These results are shown in Fig. 5B.

(2) As suggested, we detected the effect of Axin2 knockdown in MCF7-Flag-Kindlin-2 stable cells on Snail stability, and found that the increase in Snail stability induced by Kindlin-2 can be inhibited by Axin2 knockdown. In addition, we also observed a rescue of the loss of E-cadherin upon the knockdown of Axin2, indicating that both Snail and its target E-cadherin are changed under Axin knockdown. These results are shown in Fig. 5F. We also describe this result in the text correspondingly (page 9).

(3) As suggested, we added the observation of EMT marker genes (E-cadherin, Vimentin, N-cadherin, fibronectin) upon overexpression or knockdown of Kindlin-2, and identified Kindlin-2 is required for EMT programme. These results are shown in Fig. 5G. We also described the relevance of Kindlin-2 and EMT in the text (page 9).

(4) It is quite wise that this reviewer raised the question that if the role of Kindlin-2 regulation of EMT with any relation to its location in the focal adhesions. To answer this question, we use the Kindlin-2 integrin β1-binding deficient mutant, the Kindlin-2 QW mutant, which is known not to localize to the focal adhesions (ref: Shi, et al. JBC, 282:20455-20466, 2007). With this tool, we transfected Flag, Flag-Klindlin-2-WT, or Flag-Kindlin-2-QW mutant into MCF-7 cells, and then detected the expression of representative EMT markers E-cadherin and Vimentin. The experiments are shown in Fig. 5H, in which we found that both Klindlin-2-WT and Kindlin-2-QW mutant could regulate E-cadherin and Vimentin to the same trend, indicating that Kindlin-2 regulation of the EMT programme is independent of it localization to the focal adhesions or binding to the integrinβ1.

(5) The suggested important reference Yook et al. 2006 (NCB) has been cited and was put as reference 13.

Moderate

1. The microscopy is not convincing. In Fig. S1, the DAPI stain is all over the place and does not outline the nuclei. In Fig. S2, Kindlin-2 is stained throughout the cell, which means there is no specific co-localisation with β-catenin. Worse, in the lower merged panel a different field is depicted than in the single channels, so that the view avoids one cell in which differential localization is apparent.

Response:

(1) We thank for pointing out this microscopic unclarity. According to this comment we have deleted the former Fig. S1, and instead, a new picture of co-localization of Kindlin-2 and β-catenin at tumor invasive front was shown in supplementary Fig. 1A.

(2) It is true as this reviewer pointed out that Kindlin-2 is stained throughout the cell without activation of integrins (i.e., replating cells on a particular ECM, such as fibronectin). Therefore, under normal culture condition without activation of integrins, Kindlin-2 will be stained throughout the cells. For β-catenin, without addition of Wnt ligands, it will distribute throughout the cells. Under these regular culture conditions, what we have observed is that both Kindlin-2 and β-catenin are localized throughout the cell.

As for the different field in the merge panel, it is our mistake. We are sorry for that. We have deleted it and changed to a right one, as shown in supplementary Fig. 1B.

2. There have been concerns in literature (Van Noort 2007 Blood, Maher 2009 Biology Direct) about the unfortunate naming and marketing of an antibody (clone 8E4) that is not as specific for unphosphorylated β-catenin as the ABC antibody (clone 8E7) although it is sold as such, by the same company. Moreover, even the 8E7 antibody (to be preferred over 8E4) has been reported to have specificity issues on western blot, so the authors should indicate the height of protein markers and/or show a larger portion of the blot, as well as the type of antibody used; Millipore sells both aforementioned antibodies, as well as a number of other alleged dephospho-specific antibodies.

Response:

Thank a lot for this precaution. We have double-checked the antibody we used in this study. In our work, the clone 8E7 antibody was used. To take this precaution, the protein markers were carefully indicated, and the specific band of active β-catenin is about 92 kDa as shown in Fig. 4B (arrow pointed).

3. Can the authors explain the considerable difference between the GSK-3β-Ser9 blots in Figs. 3G and 3H?

Response:

We appreciate the concern about the difference. In fact, the difference is mainly from the different exposure times. We have replaced the former short-exposure blot of GSK-3β-Ser9 with a longer-exposure blot in Fig.3G.

4. Reciprocal coIP is not direct evidence for a tripartite complex, as there could also be the 3 combinations of 2 proteins present. An accepted way to show a tripartite complex is a two-step IP. Alternatively, interaction mutants or protein knockdown, as in Fig 4C, could be used to characterize complex formation and functional linkage. The authors must be more careful about their interpretations of 4A and at least show the third protein in each of the individual IP experiments in 4A and 4C.

Response:

We greatly respect this critical comment and suggestion. To this end, we have set up a two-step IP and re-confirm the formation of the tripartite complex as indicated in Fig. 4B. The detail description of the method for doing this , please refer to the Methods part.

5. To be sure they are not looking at off-target effects, the authors should confirm key experiments with at least a second RNAi : Fig 3B, E, 5C, D, F.

Response:

Thank you for addressing this important point.. According to this comment we have confirmed the key experiments with a second Kindlin-2 siRNA (Kindlin-2 siRNA-2), including Fig. 3B, Fig. 3E, Fig. 5B (the right panel, the former Fig. 5C), Fig. 5C (the former Fig. 5D), and Fig. 5E (the right panel, the former Fig. 5F).

6. In the Boyden Chamber in vitro cell invasion experiments, why are the controls (the same cell type MCF7) of 5G, 5H and 5I more than different by 3-fold or more (i.e. 0 - 4 - 12) and yet the s.d. is low?

Response:

We appreciate the reviewer's careful observation for avoiding our misleading. We are sorry that our former figure description is misleading. In fact, the control of Fig. 5I (the former Fig. 5G) is MCF-7 cells, the Fig. 5J (the former Fig. 5H) is Hs578T cells, and the Fig. 5K (the former Fig. 5I) is 293T cells. The invasive ability of the three cell lines is quite different. We have added the description of cell lines applied in Fig. 5I, 5J and 5K .

Minor

1. Page numbers would have been nice. Result sections are too long and require more subdivisions (along with the figures for instance).
Response:
Thanks!
(1) We have added the page numbers.
(2) We have subdivided the result section along with the figures.

2. At least once the authors may want to mention the FERMT2 gene name.
Response:
We have added the Fermitin family homolog 2 (FERMT2) gene name in the introduction section on Page 3. Sorry for this ignorance.

3. HEK293T cells are hardly "normal human cells" as they have been manipulated by viral entities twice; perhaps "non cancer-derived cell line" would be more concise.
Response:
According this reviewer's kind reminder, we have changed the HEK293T from "normal human cells" to "noncancer-derived cell line" in the text on Page 3.

4. What is the reference for this QW mutant? Authors should either show its functional limitations or refer to published results.
Response:
The Kindlin-2 QW mutant is the Kindlin-2Q614A/W615A mutant, a mutant that does not bind to integrin β1and not to be recruited to focal adhesions as described in the previous report (Shi, et al. JBC, 282:20455-20466, 2007). We have rewritten this portion in text on Page 4, and cited as reference 9.

5. The GST-β-catenin fragment 1-159 interacts with Kindlin-2 less efficiently than the 1-781 or 1-89 fragments. Can the authors comment on this surprising and potentially interesting observation?
Response:
We thank this reviewer for awareness of this difference, which have led us to repeat this experiment. As the result shown in Fig.1D, we have not observed an obvious difference in binding efficiency of GST-β-catenin fragment 1-89 and 1-159. An acceptable explanation of the former result is that the amount of each GST-β-catenin fragments incubated with the total lysates in the GST pulldown assay is not equal, which may cause the difference.

6. The results in Fig. 5E, if in fact labelled correctly as percentage input, are in stark contrast with Fig 5A. Otherwise, the results might be normalized to the control values? A change of label would be in order. Also, error bars (Fig. 5A and E) would indicate that, at least in this representative one out of three experiments, the duplicates were close to each other.
Response:
Thank you for this suggestion. We have changed the label, and error bars have been added. The improved figures are shown in Fig. 5A and Fig. 5D.

7. Figures 5H and I are referred to as I and J in the text. Figure 3A is not mentioned in the main text and the cell type in 3C is not indicated.
Response:
We are sorry for the carelessness mentioned.
(1) We have changed the mistakes in the text accordingly.
(2) We have moved the former Figure 3A to the supplementary Fig. 3 , and added the description in the text on Page 5, 8 and 9.
(3) The cell type in 3C is Hs578T cells, and this figure has been moved to supplementary Fig. 4. We have added the figure description of cell lines applied.

Referee #2:
In this manuscript, the authors identify a role of kindlin-2 in Wnt signaling. Interaction between kindlin-2 and β-catenin is demonstrated, shown to be regulated by phosphorylation and to control downstream signaling by the Wnt pathway. Overall, the data are very interesting, convincing and novel. The major concern from a kindlin standpoint is whether the interaction between β-catenin and kindlin-2 is direct or indirect. The data supporting this interaction, as well as all the subsequent

interaction are by pulldown experiments. An independent approach to demonstrate that kindlin-2 interacts with β-catenin and that this interaction is direct needs to be documented.

Response:
We are so grateful for this reviewer's critical comments which are very helpful to us and needs to be highly concerned. To this end, we have identified the interaction between Kindlin-2 and β-catenin is a direct interaction, by direct GST pulldown assays using purified proteins. As shown in Fig. 1F, both full length GST-β-catenin and His-MBP-Kindlin-2 fusion protein were expressed and purified *in vitro*. A direct interaction between GST-β-catenin and His-MBP-Kindlin-2 was confirmed by incubating the two fusion proteins. In addition, we mapped the binding regions between Kindlin-2 and β-catenin, which strengthened the existence of the interaction between the two molecules. Furthermore, we added a new experiment to identify a competition between the N- and C-termini of β-catenin for interaction with Kindlin-2 *in vitro*. These results are shown in Fig. 1G. We also described the direct interaction and the competitional relationship in the text on Page 4. Taken together, we applied several independent approaches and demonstrated that Kindlin-2 and β-catenin not only physically interact with each other but also functionally link together to enhance the Wnt signaling.

Referee #3:
This is a potentially interesting work describing the interaction of FERMT2 with b-catenin and a potential function of FERMT2 in b-catenin mediated gene regulation. The study is however rather vague in the description of the experimental procedures and controls and is therefore difficult to judge. Most importantly, it is not clear if only cells engineered to express FERMT2 are used or if any of the reported findings also exist in the parental cells when analyzing endogenus proteins. Furthermore, for the experiments where engineered cells have been used, no controls of at least three independent clones are shown to ensure that we are not just looking at an artifact of a subclone of the parental cell line. This needs to be improved. In addition, it is not described what are the general, cellular effects of overexpression or knock down of FERMT2. Do the cells loose substrate adhesion or cell-cell adhesion? Are there alterations in cell death, apoptosis or proliferation? Much of the shown effects may be indirect and controls for other proteins/pathways which would be affected by such changes in cellular phenotype are missing.

Response to the general comments and concerns from this reviewer:
We appreciate the comments and valuable suggestions from this reviewer. We answer these questions literally and experimentally.
(1) In the revision of our manuscript, we added more detailed description of the experimental procedures and controls. However, due to the word limitation of the text we put more experimental procedures into the on-line supplements. We are sorry for this inability.
(2) The reviewer's comments were quite right that we should use as much as we can for assay of changes using the parental cell lines for measuring the endogenous proteins. To this end, in the revision of our manuscript, we applied and repeated the experiments using endogenous proteins and measured for more than one cell lines.
(3) For engineered cell lines, what we used were mixed clones (from 6 to 10 individual subclones) after G418 selection from the very beginning of establishing the stable clones, for both the control and experimental cell lines. In way we tried to avoid clonal effects and artifacts.
(4) Overexpression or knockdown of FERMT2 (Kindlin-2) do cause cellular effects. Overexpression of FERMT2 (Kindlin-2) enhances cell spreading and adhesion, migration and the resistance to cisplatin-caused cell death as described in our previous reports (An, et al. 2010, International Journal of Cancer, Ref.8 in this manuscript; Gong, et al. 2010, Cancer letters, Ref. 16 in this manuscript); knockdown of FERMT2 (Kindlin-2) leads to decreased cell spreading and adhesion as well as cell migration, and enhances the sensitivity to cisplatin-caused cell death as described in An, et al. 2010 and Gong, et al. 2010. In addition, overexpression of FERMT2 (Kindlin-2) causes loosed cell-cell contact as indicated by the downregulation of E-cadherin and upregulation of Vimentin, signs of epithelial to mesenchymal transition (EMT), whereas when we knocked down the endogenous FERMT2 (Kindlin-2) we saw the opposite. We have detailed these two aspects in the text. The regulatory role of FERMT2 (Kindlin-2) on cell proliferation is described in the supplementary Fig.9 in the revision of our manuscript.
(5) In terms of the role of FERMT2 (Kindlin-2) on the pathways, as what we dealt with in this report the Wnt signaling pathway, we identified a direct role of FERMT2 (Kindlin-2) on the Wnt signaling by identifying formation of a tripartite complex for FERMT2 with β-catenin and TCF4. Meanwhile,

we also uncovered a molecular cascade – an indirect role of FERMT2 on Wnt signaling, that FERMT2 activates atypical PKC (PKCζ), and the latter phosphorylates GSK-3β at Ser 9 to inactivate GSK-3β, thus Wnt signaling got activated. We have detailed this in the text.

Major points:

Fig1: an interaction of endogenous FERMT2 and endogenous β-catenin needs to be shown, not just from cells transfected to express the proteins.

Response:

We appreciate for this very important point. To this end, we identified the interaction between endogenous FERMT2 and endogenous β-catenin in cell lines including MDA-MB-231, Hs578T, HEK293T, and HKC. These results are shown in Fig. 1A (middle and right panel) and Fig. 1B.

Fig 1: Is b-catenin localized to focal contacts in the presence of FERMT2.

Response:

Under normal culture condition without activation of integrins, FERMT2 will be localized throughout the cells. For β-catenin, without addition of Wnt ligands, it will distribute throughout the cells as well. Therefore, under these regular culture conditions, what we have observed is that both FERMT2 and β-catenin are localized throughout the cell (as indicated by Supplementary Fig.1B). However, when cells are replated on ECM (e.g., collagen type I) integrins are activated and FERMT2 is recruited to focal adhesions. While without addition of upstream Wnt ligands, say Wnt3a, β-catenin is not specifically localized at nucleus and distributes throughout the cells including focal adhesions. Therefore, although there is an interaction between FERMT2 and β-catenin we cannot distinguish β-catenin localized in focal adhesions recruited by FERMT2 from non-specific distribution at this stage. However, we do see co-existence of FERMT2 and β-catenin in focal adhesions as well as in cell nuclei (Supplementary Fig.2).

Fig 1: The N and C terminus of b-catenin are of very different sequence so it appears unlikely that both parts are bound by the same domain within FERMT2. Show that the C-terminus of b-catenin can compete out the N-terminus of b-catenin bound to FERMT2.

Response:

We thank this reviewer for the thoughtful idea and important question. To clarify if a competition for binding to FERMT2-2 may occur between the N- and C- termini of β-catenin, we expressed and purified the N-terminus of GST-β-catenin and the C-terminus of His-β-catenin, and then competition assays were performed. As shown in Fig. 1G, we did identify a competition between the N- and C-termini of β-catenin for interaction with FERMT2 *in vitro*. This thoughtful idea leaves room from us to extend our future studies.

Fig. 3C: FERMT2 seems not to be localized to the nucleus in striking contrast to b-catenin.

Response:

In a great ratio of human tumor tissue specimens we did found a FERMT2 nuclear localization (An, et al. 2010, International Journal of Cancer, Ref.8 in this manuscript). Interestingly, FERMT2 do contain a nuclear localization signal (NLS). Using confocal microscopy we did see a detectable amount of FERMT2 existence in the nucleus (Supplementary Fig. 2 and 4). However, as the reviewer pointed out we did see more β-catenin localized in the normal culture condition with the presence of serum.

Fig. 3E: the conclusion is not justified, GSK3b decreases b-catenin levels in both FERMT2 expressing and knockdown cells. The experiments were not described in sufficient detail. Are these cells engineered to express b-catenin and FERMT2 or are we looking at endogenous proteins?

Response:

We are sorry for not providing with sufficient detail. In our revision of the manuscript, the detail description about this experiment (Fig. 3D, the former Fig. 3E) is added in the text on Page 5. In addition, we repeated this experiment in control or Kindlin-2 siRNA-2 treated 293T cells, and similar results were observed in Fig. 3E. We have to point out that the cells used in these experiments express endogenous FERMT2 and β-catenin, while ectopically express GSK3β.

Fig.3G+H: what is the link between FERMT2 and GSK3β, how does FERMT2 influence phosphorylation of GSK3β? This is an important point which should be analyzed in more detail.

Response:

This is a very important question concerning a molecular cascade that mediates FERMT2 signal to GSK3β. Under the hint of the works from Allen Hall's lab, we knew there is regulation of atypical protein kinase C (PKCζ) on GSK-3β activity (Etienne-Manneville and Hall, 2001, Cell; Ref. 11 in this manuscript), we decided to test FERMT2 regulation on PKCζ activity. Interestingly, we did identify that PKCζ activity can be regulated by FERMT2 in both gain- and loss of functional experiments (Supplementary Fig.5). Therefore, PKCζ is indicated to be a link between FERMT2 and GSK3β. We described our newly identified link of FERMT2 – PKCζ– GSK3β in the text on page 6.

Fig.4 repeat with endogenous proteins.

Response:

As suggested from this reviewer, using endogenous proteins we repeated the Co-IP assay in MDA-MB-231 cells with anti-Kindlin-2 antibody. The result is given in Supplemental Fig. 6.

Fig.4D+E: as before, is this endogenous protein or cells engineered to express the proteins? Since FERMT2 increases b-catenin levels, the very modest 2x increase in in reporter activity could be due to just that. This is further supported in 4E where increased signaling by GSK3b inhibition via LiCl gives the identical fold change when comparing control siRNA with or without LiCl and FERMT2 siRNA with or without LiCl. The current data don't support the conclusion that FREMT2 affects Wnt signaling. The assay needs to be repeated with constant amounts of b-catenin, e.g. by expressing a non-degradable mutant of b-catenin.

Response:

We appreciated this important comment and suggestions from this reviewer. In particular, we thank this reviewer very much indeed for the suggestion that to use **"constant amounts of b-catenin, e.g. by expressing a non-degradable mutant of b-catenin"**, which completely changed our current understanding on the important role of FERMT2 - β-catenin interaction on the activation of Wnt signaling, which we did not think from this angle before. We completed the following experiments to face the suggestion:

We have changed the former TOP/FOP system (with 3 TCF optimal sites) with a new SuperTOP/FOPFlash system (8 TCF optimal sites, Addgene plasmid 12456). We answer this question at three layers. First, we expressed FERMT2 alone in 293T cells, and like before we could only see approximately 3-fold induction in SuperTOP/FOPFlash system. Since the 293T cells do not express high amount of endogenous β-catenin and there is only basic activation of Wnt signaling in this cell line. Accordingly, to face this low induction of FERMT2 on the Wnt signaling activation, we interpreted the scenario as lack of adequate β-catenin. Therefore, there is no proper FERMT2 - β-catenin interaction that forms a tripartite complex with TCF4 to activate Wnt signaling as indicated in Fig.1 and Fig.2. FERMT2 alone could not induce higher Wnt signaling activation. Secondly, when we transfected the non-degradable β-catenin (S37Y) alone we observed an induction of approximately 190-fold in the SuperTOP/FOPFlash system. Surprisingly, when β-catenin (S37Y) co-expressed together with FERMT2, we saw an approximately 1480-fold induction on top of the background. And an approximately 8-fold induction on top of β-catenin (S37Y) expression alone (Fig 4E), suggesting a very strong synergistic effect for FERMT2 interaction with the non-degradable β-catenin on the activation of Wnt signaling (**Thanks this reviewer again for letting us using the non-degradable β-catenin**) . Thirdly, we combined depletion of FERMT2 by siRNAs with the addition of upstream ligand Wnt3a and GSK-3β inhibitor LiCl as well as β-catenin (S37Y) expression. Importantly, knockdown of endogenous FERMT2 lead to approximately 50% cutting down of Wnt signaling activation even with the presence of Wnt3a or LiCl or β-catenin (S37Y) expression (Fig 4F). These data strongly suggest that FERMT2 is required for Wnt/β-catenin signalling. This question also leads us to think further that non-degradable β-catenin mutations in human cancers may associate with increased FERMT2 and make the tumors more aggressive.

Fig.5: the experiments need to be repeated with cells expressing only the endogenous FERMT2 protein.

Response:

As suggested by the reviewer, we have repeated the ChIP assay in LiCl-treated 293T cells with anti-FERMT2 antibody (sigma) to track down the endogenous FERMT2. As shown in Supplemental Fig. 7, we could see approximately 2-folds binding of endogenous Kindlin-2 to Axin2 promoter, compared to approximately 5-folds binding of Flag-FERMT2 to Axin2 promoter in MCF-7-Flag-FERMT2 stable cells using an anti-Flag tag monoclonal antibody M2 in ChIP assays). What we saw the lower FERMT2 occupancy on the Axin2 promoter compared to the exogenous expression of

FERMT2 could be caused either by the non-ChIP optimized antibody applied or as what we discussed above that the relatively low endogenous FERMT2 in 293T cells could not bind to β-catenin-TCF4 complex in the nucleus efficiently, and therefore FERMT2 could not occupy Axin2 promoter efficiently.

Fig.5C+D: the effects of FERMT2 on the axin2 expression are much more pronounced than the reporter assays had suggested. What is the cellular fate upon downregulation of FERMT2. Do the cells loose substrate adhesion, cell-cell adhesion? Are there alterations in cell death, apoptosis or proliferation? Axin2 is known to be regulated by other inputs in addition to b-catenin/TCF, so could effects on other cellular processes explain the changes in axin2 expression levels?

Response:

Overexpression or knockdown of FERMT2 do cause cellular effects. Overexpression of FERMT2 enhances cell spreading and adhesion, migration and the resistance to cisplatin-caused cell death as described in our previous reports (An, et al. 2010, International Journal of Cancer, Ref.8 in this manuscript; Gong, et al. 2010, Cancer letters, Ref. 16 in this manuscript); knockdown of FERMT2 leads to decreased cell spreading and adhesion as well as cell migration, and enhances the sensitivity to cisplatin-caused cell death as described in An, et al. 2010 and Gong, et al. 2010. In addition, overexpression of FERMT2 causes loosed cell-cell contact as indicated by the downregulation of E-cadherin and upregulation of Vimentin, signs of epithelial to mesenchymal transition (EMT), whereas when we knocked down the endogenous FERMT2 we saw the opposite. We have detailed these two aspects in the text. The regulatory role of FERMT2 on cell proliferation is described in the supplementary Fig.9 in the revision of our current manuscript.

Although we set up a paradigm in this investigation that FERMT2 - β-catenin - TCF tripartite complex regulates the Wnt target gene Axin2 expression, we agree with the reviewer that other inputs in addition to FERMT2 - β-catenin - TCF tripartite complex may regulate Axin2 expression as well. However, if the other inputs what we refer to here are regulated indirectly by FERMT2 remain unknown. Future works derived by this reviewer's suggestion will be highly desirable for elucidation of the other inputs besides FERMT2 - β-catenin - TCF tripartite on the regulation of Axin2 expression.

Fig.5E: this effect could be again due to the upregulation/stabilization of b-catenin in response to FERMT2 and does not allow concluding that the triple complex is involved in transcriptional regulation of axin2. This conclusion needs to be removed from the text or shown under conditions which do not induce stabilization of b-catenin.

Response:

As suggested, we have removed the conclusion.

The link between FERMT2 and invasion is rather complex and cases of enhanced invasion upon loss of FERMT2 have been described (Shi & Wu 2008). This needs to be included in the last paragraph.

Response:

As suggested, we have added this discussion in the text on Page 9.

Minor points:

The official name for kindlin2, FERMT2, should be used at least once in the text.

Response:

As suggested, we have added the Fermitin family homolog 2 (FERMT2) gene name in the introduction section on Page 3. Sorry for this ignorance.

For Fig1c, the methods section should provide details which exact mutant of FERMT2 was used.

Response:

The Kindlin-2 QW mutant is the Kindlin-2Q614A/W615A mutant. We have rewritten this portion in text on Page 4, and added the reference 9.

The paragraph "Similar to Kindlin-2, Smad3 reportedly protects... leaves room for future investigation." contains no data and needs to be moved to the last paragraph.

Response:

As suggested, we have moved this part to the end of the text before the conclusion section.

2nd Editorial Decision 04 April 2012

Many thanks for submitting the revised version of your manuscript to our editorial office. It was sent back to two of the original referees and we have now received their feedback on it.

As you will see, while both referees appreciate the improvements made during the first round of revision and in principle recommend publication of the study, they still have some concerns that would need to be addressed before acceptance. The concern still raised by referee 1 could be addressed if you have the data at hand, but the reviewer does not make this a prerequisite for acceptance. The concerns of referee 3, however, will need to be addressed, but since the report is pasted below I will not repeat it here.

Based on the overall positive evaluation of the reviewers I would like to give you the exceptional opportunity to revise your study again according to the referee suggestions.

On a more formal note, I would kindly ask you to please identify one of the currently six main figures that can be moved to the supplementary section, as we can only have up to five figures in the main body of the manuscript. As I would suggest leaving the schematic figure (currently Fig 6) as part of the main manuscript, maybe you could re-arrange the remaining figures so that their number is reduced to 4. I could imagine that in some cases, confirmatory results in different cell lines could be moved to the supplementary section.

Also please note that the final version might be sent back to one of the referees again before acceptance, so I would suggest including a letter discussing the changes made in response to the referee comments when you submit your revised version. As per journal policy, manuscripts have to be accepted within 6 months of the first decision, which in your case would be August 1. After this period we are, of course, still interested in publishing your study, but we might need to assess its novelty again at this point.

Please do not hesitate to contact me if you have any further questions.

I look forward to seeing the final version of your manuscript when it is ready.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

With the exception of one point, the comments have been addressed adequately. Major point 1 asked for the use of Wnt3a-conditioned medium to test the role of Kindlin-2 in promoting beta-catenin-mediated gene transcription (TOP/FOP). In response, the authors suggest that, in unstimulated 293T cells, there would be only a low level of beta-catenin that is available to be stabilised and to induce transcription with. This would explain the modest 2~3-fold induction upon Kindlin-2 overexpression. However, treatment with Wnt3a or LiCl can induce a stronger activation also by inhibiting the natural turnover of beta-catenin.
The authors choose to evade the question whether Kindlin-2 is a real Wnt-activator by itself (they removed the original claim from the revised ms) by showing LOF-data for the requirement of this protein in the Wnt/beta-catenin pathway (including the use of Wnt3a-CM, as requested). To address the original point the authors could have performed Kindlin-2 over-expression in combination with Wnt3a and LiCl treatment respectively; a synergistic effect would be expected. I am still interested to see these experiments, but it is no longer a major issue inhibiting acceptance.

Referee #3:

Overall, the manuscript has clearly been improved. There are however still some important aspects which need to be changed/included to support the authors conclusions. In particular, the presence of the endogenous triple complex of FERMT2, TCF4 and beta-catenin is still missing. Moreover, the added data on the signaling mechanism are rather preliminary and correlative and need to be substantiated by functional assays.

Major points:

Fig. S6: This co-IP is not convincing at all. It is an essential point to show the endogenous triple complex of b-catenin, TCF4 and Kindlin2. Please repeat (maybe by precipitating TCF4 instead of Kindlin2).

Fig3G: The effect on GSK3-S9 is not obvious and only correlative. Please show quantified values for the Kindlin2 overexpression experiment and include expression of a GSK3-S9A mutant to prove that the mechanism is indeed via regulation of GSK3 activity and not by sequestering b-catenin to another complex where it just didn't get into contact with GSK3.

Fig. S5: The effect on PKCzeta is not very prominent. Again, functional assessment is missing but essential to prove the mechanism. Please include a PKCzeta knock down to demonstrate that the kindlin2 induced increase of b-catenin requires PKCzeta.

Fig. S1/2: The co-localization is not convincing in either S1 or S2. Both proteins are rather diffusely distributed in the cytoplasm, they differ in their membrane and focal adhesion localization and only show overlap in the nucleus which however was not confirmed by functional, biochemical studies. Therefore please replace "Kindlin-2 was found colocalized with β-catenin in the tumor invasive front and in tumor cells (Supplementary Figure, i.e., SFig 1A-B)." to "Kindlin-2 was found coexpressed with β-catenin...".

2nd Revision - authors' response 28 April 2012

Referee #1:

With the exception of one point, the comments have been addressed adequately. Major point 1 asked for the use of Wnt3a-conditioned medium to test the role of Kindlin-2 in promoting beta-catenin-mediated gene transcription (TOP/FOP). In response, the authors suggest that, in unstimulated 293T cells, there would be only a low level of beta-catenin that is available to be stabilised and to induce transcription with. This would explain the modest 2~3-fold induction upon Kindlin-2 overexpression. However, treatment with Wnt3a or LiCl can induce a stronger activation also by inhibiting the natural turnover of beta-catenin.

The authors choose to evade the question whether Kindlin-2 is a real Wnt-activator by itself (they removed the original claim from the revised ms) by showing LOF-data for the requirement of this protein in the Wnt/beta-catenin pathway (including the use of Wnt3a-CM, as requested). To address the original point the authors could have performed Kindlin-2 over-expression in combination with Wnt3a and LiCl treatment respectively; a synergistic effect would be expected. I am still interested to see these experiments, but it is no longer a major issue inhibiting acceptance.

Response:

Thanks this reviewer for helping us to clarify the remaining concerns. As suggested from this reviewer, we have performed Kindlin-2 overexpression in combination with Wnt3a and LiCl treatment respectively. Without too much surprising as the reviewer has predicted, a synergistic effect of Kindlin-2 expression together with addition of either Wnt3a or LiCl was observed that has been shown in the new figure SFig 10.

Referee #3:

Overall, the manuscript has clearly been improved. There are however still some important aspects which need to be changed/included to support the authors conclusions. In particular, the presence of the endogenous triple complex of FERMT2, TCF4 and beta-catenin is still missing. Moreover, the added data on the signaling mechanism are rather preliminary and correlative and need to be substantiated by functional assays.

Major points:

Fig. S6: This co-IP is not convincing at all. It is an essential point to show the endogenous triple complex of β-catenin, TCF4 and Kindlin2. Please repeat (maybe by precipitating TCF4 instead of Kindlin2).

Response:
As the reviewer advised, we have repeated and replaced the previous figure that used endogenous Kindlin-2 for Co-IP with the new figure SFig 9 that employed TCF4 to co-immunoprecipitate β-catenin and Kindlin-2 with the presence of LiCl.

Fig3G: The effect on GSK3-S9 is not obvious and only correlative. Please show quantified values for the Kindlin2 overexpression experiment and include expression of a GSK3-S9A mutant to prove that the mechanism is indeed via regulation of GSK3 activity and not by sequestering β-catenin to another complex where it just didn't get into contact with GSK3.

Response:
We appreciate the constructive idea from this reviewer about the effect of Kindlin-2 on GSK-3β-Ser9. To this end, we first showed a shortly exposed blot from Fig 2G in this new version of manuscript (the former one is Fig 3G) and placed it in SFig 7A in order to see the clear difference of GSK-3β-Ser 9 phosphorylation with or without Kindlin-2 expression; and then we quantified the values of GSK-3β-Ser9 from three independent experiments and placed it in the new SFig 7B.

As suggested, we overexpressed GSK-3β-S9A in both control and Kindlin-2 depletion cells, and the results showed that the presence of endogenous Kindlin-2 prevents the effect of GSK-3β-S9A on β-catenin degradation even with the presence of this constitutive active GSK-3β that suppose to drive β-catenin to be phosphorylated and trigger its degradation (please refer to the newly added figure:

SFig 7C). This finding strongly suggests that the presence of Kindlin-2 and its binding with β-catenin is protective for maintaining β-catenin stability and reduces the effect of GSK-3β phosphorylation on β-catenin. Combining with data indicated in Fig 2F and Fig 2G-H, our findings demonstrated that Kindlin-2 plays a dual role in stabilizing β-catenin by preventing GSK-3β binding to β-catenin on one side, and inhibiting GSK-3β activation via Kindlin-2 – PKCzeta – GSK-3β inhibitory cascade on the other side. Therefore, it is interesting to distinguish which one is important for Kindlin-2 regulation of Wnt signaling: preventing GSK-3β binding to β-catenin or inhibiting GSK-3β activation? To this end, Fig 4C and Fig 4D indicated that Wnt target gene Axin2 level was significantly inhibited by Kindlin-2 knockdown even in the presence of GSK-3β inhibitor LiCl that inactivates GSK-3β kinase activity and also inhibits β-catenin phosphorylation and its degradation, indicating that it is Kindlin-2 that is required for maintaining the Wnt target gene expression in this experiment, but not the inhibition of GSK-3β activation. Under the presence of Kindin-2 and with certain amount of β-catenin, Kindlin-2 – β-catenin – TCF4 complex could bind onto the Wnt responsive elements in the Wnt target genes. Therefore, Kindlin-2 binding to and stabilizing β-catenin enhances the occupancy of Kindlin-2 – β-catenin – TCF4 complex on the Wnt responsive elements, a process that is more critical than Kindlin-2 inhibition of GSK-3β activation via a Kindlin-2 – PKCzeta – GSK-3β inhibitory cascade that prevents the degradation of a fraction of β-catenin that is not bound with Kindlin-2. Taken together, our finding suggests that Kindlin-2 binding to β-catenin rather than inhibition of GSK-3β activation is important in the regulation of Wnt target gene Axin2 expression.

Fig. S5: The effect on PKCzeta is not very prominent. Again, functional assessment is missing but essential to prove the mechanism. Please include a PKCzeta knock down to demonstrate that the kindlin2 induced increase of β-catenin requires PKCzeta.

Response:

Thanks for this reviewer for the interesting experiment that let us to include a PKCzeta knockdown to show that the Kindlin-2 induced increase of β-catenin requires PKCzeta. To scrutinize the regulatory effect of Kindlin-2 on PKCzeta activation, we quantified the changes of PKCzeta phosphorylation with or without addition of Kindlin-2 as indicated in the newly added figure SFig8B.

As suggested, we also did the experiment to knock down PKCzeta using siRNA and then to examine if the Kindlin-2 induced increase of β-catenin requires PKCzeta. The result is just as the reviewer predicted that Kindlin-2 induced increase of β-catenin does require PKCzeta as shown in the newly added figure SFig8C. Furthermore, we also proved that the Kindlin-2 induced increase of β-catenin requires PKCzeta kinase activity as well by using the PKCzeta inhibitor Gö6983 and the PKCzeta pseudosubstrate (please refer to the newly added figure: SFig8C). Taken together, on one side Kindlin-2 – PKCzeta – GSK-3β cascade inactivates GSK-3β to prevent β-catenin degradation; on the other side Kindlin-2 binding to β-catenin and prevents further GSK-3β binding and phosphorylation of β-catenin. As a result, this allows a Kindlin-2 – β-catenin – TCF4 complex to

occupy Wnt responsive elements and consequently to activate Wnt target genes, like Axin2, for example.

Fig. S1/2: The co-localization is not convincing in either S1 or S2. Both proteins are rather diffusely distributed in the cytoplasm, they differ in their membrane and focal adhesion localization and only show overlap in the nucleus which however was not confirmed by functional, biochemical studies. Therefore please replace "Kindlin-2 was found colocalized with β-catenin in the tumor invasive front and in tumor cells (Supplementary Figure, i.e., SFig 1A-B)." to "Kindlin-2 was found coexpressed with β-catenin..."

Response:

As suggested from this reviewer, we have replaced the "Kindlin-2 was found colocalized with β-catenin in the tumor invasive front and in tumor cells (Supplementary Figure, i.e., SFig 1A-B)." to "Kindlin-2 was found coexpressed with β-catenin…" in page 3 in the text.

3rd Editorial Decision 21 May 2012

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.

Yours sincerely,

Editor
EMBO Reports

