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Ring finger protein 14 is a novel regulator of TCF/-catenin-mediated transcription and is required for colon cancer cell survival

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

1st Editorial Decision 01 August 2012

Thank you very much for the submission of your research manuscript to our editorial office. First of all I would like to apologize for the unusual amount of time it has taken us to complete the review process. We have just 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 number of substantial concerns about the completeness of the data.

All referees point out a number of technical concerns (for example missing controls and unequal protein loading) that prevent the data from being fully conclusive. In addition, they also feel that the in vivo data on the role of RNF14 in regulating wnt signaling would need to be strengthened and propose potential ways on how to achieve this. Referee 1 states that additional data for a causal link between RNF14 depletion, wnt signaling and the observed growth defects should be provided. Referee 2 feels that stronger support for the idea that RNF14 increases the binding between TCF and beta-catenin should be provided and suggests testing whether RNF14 and TCF are associated also in the absence of wnt signaling. Finally, both referees 1 and 3 feel that further insights into the
mechanism by which RNF14 affects wnt signaling should be provided, but we would not make this a prerequisite for publication here (if you have these data at hand already, we would of course encourage you to add them though). However, since the request for further mechanistic insights alludes to the fact that referee 3 (and referee 2) do not consider that data on the enhanced interaction between TCF and beta-catenin to be convincing, we would require that these data be strengthened.

From the analysis of these comments it becomes clear that significant revision is required before the manuscript becomes 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 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.

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REFEREE REPORTS:

Referee #1:

In this manuscript, the authors showed that RNF14 positively regulated Wnt signaling using mainly mammalian cell lines. They also showed that RNF14 promoted the binding of beta-catenin to the target gene promoter using ChIP assay. In addition, they demonstrated that overexpression of RNF14 enhanced the Wnt signaling reporter gene expression in zebrafish head. Thus, they discovered a novel Wnt signaling regulator. Their findings are potentially interesting. However, they did not show the details of the molecular mechanisms by which RNF14 promotes Wnt signaling.

Their zebrafish data do not provide any significant information. In addition, controls in overexpression experiments are insufficient.

I think that there are several points that need to be addressed.

Comments (Major):
1. The authors stated,"RNF14 overexpression increased binding of β-catenin to TCF4 by over 6 fold (Fig1C), suggesting that RNF14 affects β-catenin and TCF interaction and thereby may play a role in Wnt signaling". However, their data are not sufficient for stating it. The authors did not present the immunoblotting of the immunoprecipitates with anti-TCF4. In addition, the amount of TCF4 in input lane 2 is different from that in input lane 4. Therefore, comparison of lane 2 with lane 4 in Fig 1C is impossible.

2. In zebrafish experiment, the authors stated,"Wnt signaling is only enhanced in specific regions where Wnt activity is normally detected", although they showed the Rnf14-mediated TOPdGFP activation in midbrain. Truly only midbrain? Wnt signaling controls the patterning of various tissues during early embryogenesis. At least, activation of Wnt signaling in early embryogenesis affects the brain patterning. Does TOPdGFP activation in the midbrain of RNAF14-overexpressed zebrafish embryos reflect the direct effect of RNF14? To confirm their hypothesis, the authors should show the expression pattern of midbrain marker genes (pax7, otx2, wnt1, etc..) and Tcf/Lef genes by in
situ hybridization, as negative controls. And the authors should explain the reason why RNF14 overexpression did not affect Wnt signaling in early embryogenesis.

3. Their current in vitro data alone would be insufficient for publishing this manuscript in EMBO report. The authors have attempted to increase the significance of this study using zebrafish. Zebrafish is one of good tools for investigation of gene function "in vivo". But the authors used zebrafish as "a test tube". Although they stated,"we provide both in vivo and in vitro evidence", they did not show the in vivo function of RNF14. The impact and quality of the current author's overexpression experiment using zebrafish is much less than those of cell line experiments. The authors should show zebrafish Rnf14 knockdown phenotype (in vivo function of RNF14), rnf14 expression pattern, and the relationship between the phenotype and Wnt signaling. These experiments can provide in vivo evidence. Alternatively, the authors should show the detailed mechanisms by which RNF14 activates Wnt signaling (as described below), to strengthen the impact of this study.

4. Supp Fig 2 is one of important figures in this study. To show that RNF14 affects Wnt signaling via TCF/LEF, the mutant lacking TCF/LEF-binding activity is essential. In Supp Fig 2B, the authors have tried to identify the region interacting with TCF1 and stated, "the N-terminal deletion almost completely eliminated binding". However, the amount of the inputted N-terminal deleted RNF14 is very low. Therefore, this figure does not support the authors' statement. The author should perform this experiment again. Which region of RNF14 was isolated in yeast two-hybrid screening? This helps to understand which region of RNF14 is required for the interaction with TCF/LEF. In addition, RNF14 deltaN mutant, which may lack the TCF/LEF binding activity, activated Wnt signaling reporter in lane 6 of Supp Fig 2C, and this activation strength was similar to that observed with the RNF14 wild type (lane 3). The amount of RNF14 deltaN in lane 6 was much less than that of RNF14 wild type in lane 3. These observations suggest that TCF/LEF-binding activity is not required for RNF14 function. The authors should interpret this discrepancy.

5. The authors performed almost all experiments (in both mammalian cells and zebrafish embryos) using full-length wild-type RNF14. Sometimes, the effect caused by gene overexpression does not reflect its functional activation. Therefore, the authors should use the loss or/and gain of function RNF14 mutants (the C-terminus deletion mutants) as a negative control in Co-IP assay (TCF vs. RNF14), zebrafish experiment and ChIP assay.

6. In Fig.4, the authors showed that RNF14 siRNA treatment reduced the growth of cancer cell lines. However, they did not show the relationship of this cell growth reduction with Wnt signaling at all, although they discussed that an RNF14-modulated gene LEF1 is involved in cell survival of T-cell lymphoma. Does overexpression of LEF1 or beta-catenin-TCF fusion protein reverse the cell growth reduction in RNF14-depleted cells? This experiment strengthens this study. At minimum, the authors should show or discuss that the direct inhibition of Wnt signaling with dominant negative Tcf, TCF/LEF RNAi, or etc... also reduces colon cancer cell survival.

7. In Fig. 5D, the authors showed that RNF14 overexpression did not affect TCF4-beta-catenin fusion protein and concluded that RNF14 may stabilize the interaction between beta-catenin and TCF. The authors should confirm that RNAF14 interacts with this fusion protein using co-IP assay. Beta-catenin fusion to TCF4 might affect this binding.

Minor comments

1. In the top and forth panels (anti-TCF4 immunoblotting) in Fig 1C, there were two bands and RNF14 preferentially interacted with the lower band. Are the upper and lower bands the phosphorylated and unphosphorylated forms of Tcf4?

2. The name of zebrafish Wnt reporter TOP:GFP is incorrect. TOPdGFP is correct.

3. In terms of the nomenclature of genes, the authors should NOT use “z-” according to ZFIN. All in the text and figures should be amended accordingly: e.g. z-RNF14 (Rnf14 protein and rnf14 gene are correct). See below for details:
4. In the text, the authors stated, "At approximately 16 hours post fertilization, weak endogenous Wnt activity can be detected in the midbrain region". On the other hand, in the Figure legend, the authors stated, "TOP:GFP in green (left column) represents a region with active Wnt signaling, the midbrain/hindbrain boundary (mhb, arrowhead)". Which (midbrain or mhb) is correct??

Referee #2:

Wu et al. describe a novel regulator of Wnt/β-catenin signalling. They find that the ring finger protein 14 (RNF14) interacts with all members of TCF/LEF transcription factor family and is able to facilitate TCF/β-catenin mediated transcription. The ability of RNF14 to interact specifically with TCF/LEF transcription factors without significant binding affinity to β-catenin distinguishes RNF14 by the majority of known TCF/β-catenin co-activators, which preferentially interact with β-catenin or both, TCF and β-catenin. The authors clearly show the physical interaction between RNF14 and TCF/LEF as well as the functional consequences of such interaction, especially in tissue culture models (HEK293T and colorectal cancer cell lines). The in vivo experiments based on zebrafish, using the Wnt/β-catenin transcription reporter, seem to be rather supportive than conclusive, and may serve as a good starting point for further research. In general the findings described by Wu et al. add another interesting piece to the complex mosaic of Wnt/β-catenin orchestrated transcription and to our understanding of the mechanisms that regulate this process. The manuscript is well written, and potentially suitable for publication in EMBO Reports. However, in my opinion, there are some issues that should be addressed by the authors before the final acceptance. I have the feeling that, if properly answered, these comments could make the manuscript more robust and attractive for the research community.

Major comments:

- Is RNF14 ubiquitously expressed? The presented data show its expression in HEK293T cells and some colon cancer cell lines. On the other hand, zebrafish analysis revealed dramatic decrease of expression during development, but no important positional information is given. Can RNF14 be considered as a general co-factor or a tissue/cell type specific one? Does the expression of RNF14 correlate with Wnt-reporter activity (or with any of the TCF proteins) in other tissues, or in other stages during zebrafish development (or any other vertebrate model organism/tissue)?

- An important question is whether RNF14 is pre-associated with TCFs at target promoters already in the Wnt-OFF state or if it is recruited to TCFs only upon Wnt stimulation. The presented data suggest a β-catenin independent binding, but unfortunately they are not conclusive enough to fully address this point. Moreover the majority of key experiments is based on overexpression (potentially less physiologically relevant results could not be excluded). Additionally the cancer cell lines used have an aberrantly activated Wnt pathway, hence do not allow to address such a question (i.e. Wnt-independent preassociation between TCFs and RNF14). A possible experiment which may uncover an answer to this problem could be a co-immunoprecipitation on endogenous proteins (TCFs, RNF14 and possibly β-catenin) in Wnt-stimulated vs. -unstimulated cells. Such an experimental set-up can be even used in combination with siRNA on RNF14 for ChIP experiments to render data on Fig5 more robust. Alternatively, transfection of dominant negative TCFs may be worth to do (expression of target genes should decrease but DN-TCF should be still associated together with RNF14 to target promoters - if the author's suggestions are correct).

- In vivo data based on zebrafish model seem to be rather supportive than convincingly showing the importance of TCFs - RNF14 association in vivo. Why did the authors choose this stage (16 hpf) if at 15 hpf they detected the lowest levels of RNF14? Would it not be better to check the reporter activity at other stages, and to complement the overexpression approach with parallel morpholino experiments?

- In Fig 2D-E the authors check just the intensity of the reporter, but according the data as presented they could not exclude the role of AR-pathway (stimulated upon RNF14 mRNA microinjection)
which theoretically may increase the proliferation/number of the reporter positive cells, potentially resulting in a more bright signal, without increasing the reporter level per individual cell. Microinjections of few control mRNAs (e.g. RNF14 without AR interaction domain, or RNF14 lacking the ability to bind TCFs) would help to address such issues, and support the role of RNF14-TCF in vivo interaction.

- Fig 1C: The authors conclude that RNF14 transfection increases the affinity of the binding between TCF4 and β-catenin. I think that the co-IP shown is too weak to draw such a conclusion, for the following reasons:
  a) the co-IP was done in unstimulated HEK293T cells, and being TCF/β-catenin transcription very low (almost inactive) in this cell line. How do the authors explain immunoprecipitated β-catenin when pulling down TCF4 in the first place?
  b) RNF14 apparently enhances Wnt signalling (Fig 2 and 3): can the increased binding between TCF4 and β-catenin simply be an indirect effect of such induction?
  c) within the most right lane the two bands representing TCF4 seem to be more abundant than in the second lane: this, alone, would be sufficient to explain an increased β-catenin found in the immunoprecipitated reaction (i.e. input control for TCF4 is not exactly the same for both parallels).
  d) the different levels of immunoprecipitated β-catenin can be simply due to a different IP efficiency with TCF4 in the two samples: a control of an IB with anti-TCF4 in the IP:TCF4 reaction is required to exclude this possibility.

Minor points

- Does RNF14 stimulate β-catenin-transcription without Wnt-stimulation? Such data might emerge, but is not currently clear, from Fig2C (the bars for such parallels are to low to do any conclusion).

- Fig3F: The downregulation of LEF1 upon siRNA treatment against RNF14 does not seem to be very dramatic: why the authors precisely choose particularly this target gene to suggest LEF1 as an important molecule within their model (text on page 9)?

- Fig4: If the authors observed the more dramatic effects on survival of DLD1 colonies upon siRNA treatment against RNF14 why did they not make a growth curve (picture of the colonies) using these cells, and did not study here RNF14 function in more details in this cell line?

- ChIP against RFN14 is performed only with the transfected protein. Since RNF14 is expressed in both DLD-1 and HCT116 cells, ChIP could be performed on the endogenous protein (the best control being the siRNA against RNF14 treated cells). Cells with not aberrantly activated Wnt-signalling could be even tested (Wnt-stimulated vs. unstimulated situation), to confirm β-catenin-independent binding of RNF14 on target loci.

- In Fig5B the proper controls are missing as the authors are only detecting here nuclear proteins. Showing the cytosolic and membranous fractions is crucial to exclude any potential cross-contamination among fractions. This is all the more relevant when proteins such β-catenin are involved.

- In the discussion authors suggested the role of RNF14 in ubiquitylation (e.g. histone H2B). It might be worth to test if RNF14 can play such a role in either monoubiquitylating H2B or ubiquitylating TCFs or β-catenin.

- In the supplementary Fig2C-D, how can the author draw any conclusion on the decreased pathway activation by the RNF14DeltaN when such a lower amount of it is expressed after transfection (not comparable to full length protein)?

Referee #3:

We et al
The authors report that RNF14 binds TCF/Lef. The demonstration of the basic biochemical interaction is very convincing, although some of the details are not quite as clean (see specific
They also show quite nicely that RNF14 boosts β-catenin-TCF transcriptional activity, and that this stimulation occurs only in cells activated by Wnt.

This conclusion is drawn based on gain- and loss-of-function experiments (siRNA) in cell lines (293 and colon cancer lines), and by gain-of-function in Zebrafish embryos carrying a GFP reporter. The images from the latter model are impressively clear (I wonder however why knock down using morpholinos was not attempted?).

While these two major conclusions are quite compelling and may potentially be of interest in the field, this study in its present form lacks of a molecular explanation for the effect of RNF14. The authors argue that RNF14 stabilizes the β-catenin-TCF interaction, but this statement is based on a single experiment that is unfortunately not convincing (see details below). Even if this turns out to be true, one expects to see data on the mode of action of RNF14:

a) First of all, can a RNF14-TCF-β-catenin complex form (see specific comments)?

b) Does RNF14 interact with chromatin-bound TCF? This would be somewhat surprising, since it interacts with the HMG box, and one would thus expect overlap with the DNA binding interface.

c) Could RNF14 compete with a TCF-bound repressor?

d) Or could RNF14 act more indirectly? What about its ubiquitin ligase activity? Could it modify TCF and thus increase its binding to β-catenin (or decrease its binding to a repressor)? I would have thought that this would be the first hypothesis to test!

These are all obvious and rather simple experiments that would bring the manuscript to a higher level of impact in the Wnt field. As it stands, these results merely add another name to the too long list of molecules that somehow influence the pathway.

Specific comments:

1. Fig.1C: Increased β-catenin-TCF interaction in the presence of RNF14 is not convincing: There are two bands for TCF. While the top band is about equal in both lanes, and the lowest band is much more intense in the lane (+RNF14). In the middle panel, it seems that the lowest band is actually the one most efficiently IPed with Flag RNF14.

In the lower panel (TCF IP), one seems only one band. In the absence of molecular weight markers, one cannot guess which of the two TCF forms this band corresponds. The quantification indicates a 6.7x increase in the presence of RNF14, which would make sense only if quantification was performed using the highest band in the input. This figure should be redone, including indication of markers and of single band intensities. Average fold increases of at least 3 replicates should be included.

Also please consider note for supplementary Fig.2 for acceptable blot quantification.

2. Page 5 and Suppl fig2: "Interestingly, overexpressed β-catenin did not immunoprecipitate with flag-RNF14 (supp Fig1D). Thus, RNF14 may not directly interact with β-catenin but is able to bind all four TCF members.”

This experiment shows that RNF14 does not bind directly to β-catenin. But what about TCF binding simultaneously RNF14 AND β-catenin. I don't understand why the FlagRNF14 IP of Figs.1B and C was not blotted for β-catenin, this would bring an important piece to the puzzle.

3. Supplementary fig. 2: The numbers given for the quantification are not believable. Quantification was presumably done on classical films, which give a non linear signal (sigmoid) that saturates within a 2-4 fold range. From my experience, a look at the blot shows that the inputs vary over a relatively wide range. deltaN is particularly much weaker than other constructs. There is a weak band for the pulled down fraction of this construct. This band is certainly weak, of the same intensity as the control GST band, but the amount of control GST input is clearly >10 times higher. That both give the same 0.1 ratio makes no sense. The lack of interaction for the deltaN construct is thus not convincingly demonstrated.

Quantification should be done by direct comparison with a dilution series of one sample, blotted on the same membrane. To increase the usable range of signals, I advise switching from classical film exposure to direct imaging of the chemoluminescence with a digital camera.
We thank the referees for their careful reading of our study, and for their suggestions. We have worked hard to address each and every point and below we summarize our results point-by-point. Overall, the new experiments have strengthened our model that RNF14 is a co-factor for LEF/TCFs at Wnt Response Elements, both in human cancer cells and in zebrafish embryos.

Referee #1:

1. The authors stated, "RNF14 overexpression increased binding of β-catenin to TCF4 by over 6 fold (Fig1C), suggesting that RNF14 affects β-catenin and TCF interaction and thereby may play a role in Wnt signaling". However, their data are not sufficient for stating it. The authors did not present the immunoblotting of the immunoprecipitates with anti-TCF4. In addition, the amount of TCF4 in input lane 2 is different from that in input lane 4. Therefore, comparison of lane 2 with lane 4 in Fig 1C is impossible.

We have repeated the immunoprecipitation experiment with TCF4 in HEK293T cells (Figure 1C). We have attempted to make sure that TCF4 is equally expressed in all conditions via overexpression of both TCF4 and FLAG-tagged RNF14. We have also added an RNF14 deletion mutant that is unable to enhance Wnt target gene expression (RNF14ΔC (219-474); Supplementary Figure 2E) and used two different TCF4 antibodies to characterize the immunoprecipitated TCF4. Overall, the data show that TCF4 co-immunoprecipitates with both FLAG-RNF14 and FLAG-RNF14ΔC, i.e. deletion of the C-terminus does not matter (see also supplementary Figure2B for mapping of interaction domains of RNF14). A blot for levels of β-catenin that co-immunoprecipitate with TCF4 shows enhanced interactions when RNF14 or RNF14ΔC are overexpressed (lanes 3,4). Longer exposures of the blots show a smaller TCF4 polypeptide (~65 kDa) present in the Input and enriched in the immunoprecipitates. Using two different TCF4 antibodies, we show that this protein is a truncated TCF4 isoform missing the C-terminus (see Supplementary Figure 1B). The slight increase in the level of truncated TCF4 in Input, and the very noticeable enrichment of this isoform in the immunoprecipitates implies that RNF14 has improved interactions with truncated TCF4. We also note that once again, co-expression of full length RNF14 with TCF4 results in slightly lower levels of full-length TCF4 in both the input and the anti-TCF4 immunoprecipitate. Thus, we think that the unequal loading concern that Reviewer #1 notes may be due in part to a true biological effect of overexpressed RNF14 on TCF4 levels.

2. In zebrafish experiment, the authors stated,"Wnt signaling is only enhanced in specific regions where Wnt activity is normally detected", although they showed the Rnf14-mediated TOPdGFP activation in midbrain. Truly only midbrain? Wnt signaling controls the patterning of various tissues during early embryogenesis. At least, activation of Wnt signaling in early embryogenesis affects the brain patterning. Does TOPdGFP activation in the midbrain of Rナフ14-overexpressed zebrafish embryos reflect the direct effect of RNF14? To confirm their hypothesis, the authors should show the expression pattern of midbrain marker genes (pax7, otx2, wnt1, etc..) and Tcf/Lef genes by in situ hybridization, as negative controls. And the authors should explain the reason why RNF14 overexpression did not affect Wnt signaling in early embryogenesis.

The referee is correct in that Wnt signaling controls many patterning events in early embryogenesis, not just in the midbrain. While we now show multiple lines of evidence that Rnf14 is both necessary and sufficient to alter Wnt signaling levels, we have not observed corresponding phenotypic defects in early zebrafish embryos. The TOPdGFP line used for these experiments only reports relatively high levels of endogenous Wnt activity in living embryos, which is restricted to the mid-hindbrain boundary region in the first 24 hours post-fertilization. Were we to probe TOPdGFP expression with higher levels of sensitivity using in situ hybridization or immunohistochemistry for GFP and confocal microscopy, we would probably detect other areas of reporter gene expression. However, using the midbrain as a marker of endogenous Wnt activity in living embryos enables us to examine larger numbers of embryos for each microinjection regime and check for injection quality by visualizing mCherry simultaneously. Wnt signaling also drives gene expression and patterning in many events subsequent to this early 24hr stage. However microinjected mRNA typically degrades after the first 12-24 hours. To address the referee’s concern, we performed in situ hybridization for otx2 to check for morphological defects in the
midbrain region. We found no changes, confirming that the increased TOPdGFP signal we observe is due to increased Wnt signaling (Figure 2A). We also performed qRT-PCR of endogenous Wnt target genes (sp5, lef1, axin2) and observed similar results (Figure 2C, 2D). We normalized the gene expression data to otx2 levels. Because we observed higher levels of Rnf14 at the earlier stage of development (Supplementary Figure 2F), we did test for the role of Rnf14 on Wnt target expression at early (6 hpf) and late (24 hpf) stages. We demonstrated that Rnf14 has a stronger effect at the earlier stage. We also constructed two deletion mutations of Rnf14 and compared the effects of overexpression on target gene expression. This experiment was performed in part due to this Referee’s comment that more mechanistic details were needed (see our response below to comment #5). Finally, we make the point here, and in the manuscript, that Rnf14 does not ectopically activate Wnt signaling when overexpressed (Fig 2), but rather maximizes the effect of naturally stabilized β-catenin. Thus, unlike other genetic manipulations that overactivate Wnt signaling and cause bona fide axis duplication (i.e. stabilized β-catenin, loss of APC function), overexpressed Rnf14 only reveals the maximal potential of endogenously provided β-catenin.

3. Their current in vitro data alone would be insufficient for publishing this manuscript in EMBO report. The authors have attempted to increase the significance of this study using zebrafish. Zebrafish is one of good tools for investigation of gene function “in vivo”. But the authors used zebrafish as “a test tube”. Although they stated, “we provide both in vivo and in vitro evidence”, they did not show the in vivo function of RNF14. The impact and quality of the current author’s overexpression experiment using zebrafish is much less than those of cell line experiments. The authors should show zebrafish Rnf14 knockdown phenotype (in vivo function of RNF14), rnf14 expression pattern, and the relationship between the phenotype and Wnt signaling. These experiments can provide in vivo evidence. Alternatively, the authors should show the detailed mechanisms by which RNF14 activates Wnt signaling (as described below), to strengthen the impact of this study.

To respond to these comments, we now include loss-of-function experiments with a rnf14-specific morpholino (MO) and show that it reduces Wnt signaling activity. To control for MO efficiency, we constructed an expression construct containing the MO-binding site fused to mCherry and showed that our MO effectively knocks down the reporter (Figure 2E). When we knock down endogenous rnf14 expression at different doses of MO we observe consistent, dose-responsive decreases in mRNA levels of axin2, lef1 and sp5 at 6 hours but less so at 24 hours when we hypothesize that MO levels decline (Figure 2F, 2G). We also show rnf14 expression patterns by qPCR, as included in our original manuscript (Supplementary Figure 2F), and we did whole mount in situ hybridization with an antisense RNA probe to rnf14, but found expression to be ubiquitous (data not shown, but consistent with rnf14 in situ hybridization data published in ZFIN). Thus, RNF14 is likely a ubiquitously expressed factor as others have suggested in mammalian studies.

4. Supp Fig 2 is one of important figures in this study. To show that RNF14 affects Wnt signaling via TCF/LEF, the mutant lacking TCF/LEF-binding activity is essential. In Supp Fig 2B, the authors have tried to identify the region interacting with TCF1 and stated, “the N-terminal deletion almost completely eliminated binding”. However, the amount of the inputted N-terminal deleted RNF14 is very low. Therefore, this figure does not support the authors’ statement. The author should perform this experiment again. Which region of RNF14 was isolated in yeast two-hybrid screening? This helps to understand which region of RNF14 is required for the interaction with TCF/LEF. In addition, RNF14 deltaN mutant, which may lack the TCF/LEF binding activity, activated Wnt signaling reporter in lane 6 of Supp Fig 2C, and this activation strength was similar to that observed with the RNF14 wild type (lane 3). The amount of RNF14 deltaN in lane 6 was much less than that of RNF14 wild type in lane 3. These observations suggest that TCF/LEF-binding activity is not required for RNF14 function. The authors should interpret this discrepancy.

The referee is referring to a protein interaction experiment shown in Supplementary Figure 2A/B and transient transfection studies in Supplementary Figure 2C-E. In short, the yeast-two-hybrid screen was performed with the 88 amino acid TCF-1 HMG DNA binding domain. Multiple, independent clones of nearly full-length RNF14 were isolated. In the in vitro mapping experiment performed here (with
bacterial recombinant protein), we find that the nature of the interaction with RNF14 is complex. Overall it appears that the N-terminal half of RNF14 is **sufficient** for interaction, but it may not be wholly **necessary**. Since the other referees had comments about the mapping, we repeated the entire experiment with additional deletions and the results are shown in Supplementary Figure 2B. In summary, the N-terminal region is most important and its deletion greatly compromises TCF-1 binding. However, binding is not completely eliminated. Thus, either other regions of RNF14 can compensate, or there are two regions of binding and our deletion eliminates only one at a time. This interpretation is supported by the transient transfection data in Supplementary Figure 2C where overexpression of RNF14ΔN does indeed show a slight increase in Wnt reporter gene expression. We should note that RNF14ΔN is unstable when expressed in mammalian cells and this is shown in the western blot in panel C. We do not agree that these data mean that RNF14-LEF/TCF binding is irrelevant because as the *in vitro* interaction studies show – the interaction is specific, but complex and likely involves multiple points of contact.

5. The authors performed almost all experiments (in both mammalian cells and zebrafish embryos) using full-length wild-type RNF14. Sometimes, the effect caused by gene overexpression does not reflect its functional activation. Therefore, the authors should use the loss or/and gain of function RNF14 mutants (the C-terminal deletion mutants) as a negative control in Co-IP assay (TCF vs. RNF14), zebrafish experiment and ChIP assay.

We have used *rnf14ΔC* deletion mutations in the zebrafish setting (*rnf14ΔAR, rnf14ΔC*; Figure 2), and in the co-immunoprecipitation experiments (Figure 1). The results show that while the Androgen Receptor Binding domain at the extreme C-terminus is not necessary, deletion of additional sequences completely eliminates activity and enhancement of Wnt target gene expression. We note here that RNF14ΔC retains the ability to co-immunoprecipitate with TCFs.

6. In Fig 4, the authors showed that RNF14 siRNA treatment reduced the growth of cancer cell lines. However, they did not show the relationship of this cell growth reduction with Wnt signaling at all, although they discussed that an RNF14-modulated gene LEF1 is involved in cell survival of T-cell lymphoma. Does overexpression of LEF1 or beta-catenin-TCF fusion protein reverse the cell growth reduction in RNF14-depleted cells? This experiment strengthens this study. At minimum, the authors should show or discuss that the direct inhibition of Wnt signaling with dominant negative Tcf, TCF/LEF RNAi, or etc... also reduces colon cancer cell survival.

Many studies from many different groups have reported detailed analyses for how Wnt signaling uses LEF/TCFs to direct cancer cell survival. Dominant negative LEF/TCF expression in colon cancer cells has a strong, negative effect on the cell cycle if the isoform contains the long E-tail (found in TCF-1 and TCF-4). Overexpression of other dnLEF/TCF isoforms does not affect the cell cycle. Thus, while reduction of RNF14 did not have dramatic effects on the growth of colon cancer cells, we observed a significant decrease in clonogenic survival of these cells. The rescue experiment suggested by the referee is a good suggestion – although challenging - since the knockdown requires multiple siRNA treatments and an additional transient transfection to introduce the rescue construct. We nevertheless attempted the experiment and we do indeed observe that a TCF4-β-catenin fusion protein rescues survival in the clonogenic assay (Figure 4F, G). These data suggest that a primary function of RNF14 in colon cancer cells is to mediate Wnt signaling.

7. In Fig 5D, the authors showed that RNF14 overexpression did not affect TCF4-beta-catenin fusion protein and concluded that RNF14 may stabilize the interaction between beta-catenin and TCF. The authors should confirm that RNAF14 interacts with this fusion protein using co-IP assay. Beta-catenin fusion to TCF4 might affect this binding.

This was a good suggestion and we addressed this concern by performing a co-immunoprecipitation experiment. The TCF4-β–catenin fusion protein is very active in Wnt reporter assays and it works very
nicely to rescue clonogenic survival. However, we were unable to detect an interaction in co-immunoprecipitation studies. Either this is due to the fact that this fusion protein is not very abundant and difficult to detect, or as the referee wondered, RNF14 cannot interact with this polypeptide. Since we are not sure which of these two possibilities is correct, we have removed these data. We show that RNF14 enhances b-catenin occupancy of Wnt Response Elements (Figure 5 C, D) and co-immunoprecipitates with TCF4 (Figure 1C). Therefore, our conclusion that RNF14 stabilizes b-catenin association at WREs remains an important part of our model.

Minor comments

1. In the top and forth panels (anti-TCF4 immunoblotting) in Fig 1C, there were two bands and RNF14 preferentially interacted with the lower band. Are the upper and lower bands the phosphorylated and unphosphorylated forms of TCF4?

These two bands are due to cleavage of TCF4 (see answer to comment #1), not due to phosphorylation. That the C-terminal deletion enables greater interaction with RNF14 is extremely interesting, but follow-up studies lie beyond the scope of this report.

2. The name of zebrafish Wnt reporter TOP:GFP is incorrect. TOPdGFP is correct.

Corrected – thank you.

3. In terms of the nomenclature of genes, the authors should NOT use "z-" according to ZFIN. All in the text and figures should be amended accordingly: e.g. z-RNF14 (Rnf14 protein and rnf14 gene are correct). See below for details: https://wiki.zfin.org/display/prot/Conventions+For+Naming+Zebrafish+Genes

All nomenclature is corrected in the text

4. In the text, the authors stated, "At approximately 16 hours post fertilization, weak endogenous Wnt activity can be detected in the midbrain region". On the other hand, in the Figure legend, the authors stated, "TOP:GFP in green (left column) represents a region with active Wnt signaling, the midbrain/hindbrain boundary (mhb, arrowhead)". Which (midbrain or mhb) is correct??

Expression begins at the mid-hindbrain boundary and spreads throughout the midbrain, so we use the generic abbreviation mb to refer to both.

Referee #2:

1. Is RNF14 ubiquitously expressed? The presented data show its expression in HEK293T cells and some colon cancer cell lines. On the other hand, zebrafish analysis revealed dramatic decrease of expression during development, but no important positional information is given. Can RNF14 be considered as a general co-factor or a tissue/cell type specific one? Does the expression of RNF14 correlate with Wnt-reporter activity (or with any of the TCF proteins) in other tissues, or in other stages during zebrafish development (or any other vertebrate model organism/tissue)?

As described above, we performed in situ and rnf14 expression appears to be ubiquitous in early embryos (data not shown). Therefore, our findings in the TOPdGFP zebrafish line suggest that rnf14 is likely to be a ubiquitously available factor for Wnt signaling and its functions are therefore manifest through the prism of cell-type and stage-specific actions of TCF/β-catenin complexes (Figure 2).

2. An important question is whether RNF14 is pre-associated with TCFs at target promoters already in the Wnt-OFF state or if it is recruited to TCFs only upon Wnt stimulation. The presented data suggest a β-catenin independent binding, but unfortunately they are not conclusive enough to fully address this point. Moreover the majority of key experiments is based on overexpression (potentially less physiologically relevant results could not
be excluded). Additionally the cancer cell lines used have an aberrantly activated Wnt pathway, hence do not allow to address such a question (i.e. Wnt-independent preassociation between TCFs and RNF14). A possible experiment which may uncover an answer to this problem could be a co-immunoprecipitation on endogenous TCFs (e.g. TCF1) in Wnt-stimulated vs. -unstimulated cells. Such an experimental set-up can be even used in combination with siRNA on RNF14 for ChIP experiments to render data on Fig5 more robust. Alternatively, transfection of dominant negative TCFs may be worth to do (expression of target genes should decrease but DN-TCF should be still associated together with RNF14 to target promoters - if the author’s suggestions are correct).

The referee makes a very good point and we have addressed it using chromatin immunoprecipitation with cells in which Wnt signaling is in an OFF-state (HEK293T cells). Because our RNF14 antibody is not ChIP compatible, we expressed FLAG-tagged RNF14 and used anti-FLAG antisera in the chromatin immunoprecipitation assay (Figure 5 I, J). We observe that RNF14 is present at bona fide Wnt response elements in the AXIN2 and SP5 promoters in the Wnt-OFF state. We also performed ChIP for β-catenin and observed no occupancy until Wnt signaling was activated by the small molecule BIO (Figure 5F,G). Its dramatic increase at WREs served as positive control for the experiment. BIO treatment did not affect RNF14 binding much – its levels showing no statistically significant change comparing the Wnt-OFF versus Wnt-ON state. We also examined what happens to β-catenin and TCF4 occupancy when endogenous RNF14 is knocked down. In colon cancer cells, knockdown of RNF14 reduces β-catenin occupancy but has no effect on TCF-4 binding (Figure 5C,D,E). The effects on β-catenin occupancy are less striking in 293T cells when BIO is used to strongly overactivate Wnt signaling, but they are still significant. Knockdown of endogenous RNF14 reduces β-catenin occupancy modestly. We did not have time to repeat this experiment and titrate the level of BIO activation to see if further reduction of β-catenin binding is observed when RNF14 expression is eliminated. In summary, we conclude that RNF14 occupies Wnt Response Elements with LEF/TCFs in the absence of Wnt signaling. We thank the referee for this suggested experiment.

3. In vivo data based on zebrafish model seem to be rather supportive than convincingly showing the importance of TCFs - RNF14 association in vivo. Why did the authors choose this stage (16 hpf) if at 15 hpf they detected the lowest levels of RNF14? Would it not be better to check the reporter activity at other stages, and to complement the overexpression approach with parallel morpholino experiments?

This particular TOPdGFP reporter line best reports Wnt signaling in the midbrain region at 16 hpf which is why we chose this time point (Figure 2A, B). Nevertheless, to address the Referee’s comment we examined earlier (6 hour) and later (24 hour) time points in our Rnf14 overexpression studies (Figure 2C, D). We observed that overexpression of either wildtype rnf14 or rnf14ΔAR (missing the Androgen Receptor binding domain) increased axin2, lef1 and sp5 mRNA levels (but did not change otx2 levels – not shown). The effect was most obvious at 6 hours. Interestingly, expression of rnf14ΔC – which is inactive in our transient transfection assays (Supplementary Figure 2E), acts like a dominant negative and lowers mRNA levels of these targets (but again – does not affect otx2 mRNA levels). We also performed morpholino knockdown as described above in response to Referee #1 (see comment #3). Briefly, knockdown of endogenous rnf14 had a dose-response effect on endogenous Wnt target gene expression at 6 hours, though this effect was weaker at the 24 hour timepoint.

4. In Fig 2D-E the authors check just the intensity of the reporter, but according the data as presented they could not exclude the role of AR-pathway (stimulated upon RNF14 mRNA microinjection) which theoretically may increase the proliferation/number of the reporter positive cells, potentially resulting in a more bright signal, without increasing the reporter level per individual cell. Microinjections of few control mRNAs (e.g RNF14 without AR interaction domain, or RNF14 lacking the ability to bind TCFs) would help to address such issues, and support the role of RNF14-TCF in vivo interaction.
As described above, we addressed this question by microinjecting wildtype and \textit{rnf\_AR} mRNA. The latter being a deletion mutant that eliminates Androgen Receptor binding. For reasons that stem from the complicated domain mapping studies (see response to Referee \#1, comment \#4), we were unable to define a mutant completely lacking TCF interaction. We used \textit{otx2 in situ} hybridization to show that the midbrain region is not enlarged with RNF14 overexpression. Nevertheless, we observe that both overexpression of either wildtype or \textit{rnf14\_AR} increases Wnt target gene expression (Figure 2C). We normalized the data to \textit{OTX2} mRNA levels to ensure that transcript levels were not due simply to an increase of the midbrain region.

5. Fig 1C: The authors conclude that RNF14 transfection increases the affinity of the binding between TCF4 and \(\beta\)-catenin. I think that the co-IP shown is too weak to draw such a conclusion, for the following reasons:

a) the co-IP was done in unstimulated HEK293T cells, and being TCF/\(\beta\)-catenin transcription very low (almost inactive) in this cell line. How do the authors explain immunoprecipitated \(\beta\)-catenin when pulling down TCF4 in the first place?

b) RNF14 apparently enhances Wnt signalling (Fig 2 and 3): can the increased binding between TCF4 and \(\beta\)-catenin simply be an indirect effect of such induction?

c) within the most right lane the two bands representing TCF4 seem to be more abundant than in the second lane: this, alone, would be sufficient to explain an increased \(\beta\)-catenin found in the immunoprecipitated reaction (i.e. input control for TCF4 is not exactly the same for both parallels).

d) the different levels of immunoprecipitated \(\beta\)-catenin can be simply due to a different IP efficiency with TCF4 in the two samples: a control of an IB with anti-TCF4 in the IP:TCF4 reaction is required to exclude this possibility.

We have repeated this co-immunoprecipitation multiple times, and show an example in Figure 1C. An extensive description of this experiment is provided in our response to Referee \#1 (comment \#1), but briefly here – we find that either expression of wildtype RNF14 or RNF14\_C, enhances the amount of \(\beta\)-catenin that co-immunoprecipitates with TCF-4. At this point in the study, we moderate our interpretation of the results to say that the data suggest “...that RNF14 affects \(\beta\)-catenin and TCF interaction”. Later in the manuscript when we observe by chromatin immunoprecipitation that RNF14 knockdown reduces \(\beta\)-catenin occupancy at target genes, we make a stronger conclusion that RNF14 action increases the association or stability of \(\beta\)-catenin occupancy with LEF/TCF complexes at Wnt Response Elements.

**Minor points**

1. Does RNF14 stimulate \(\beta\)-catenin-transcription without Wnt-stimulation? Such data might emerge, but is not currently clear, from Fig2C (the bars for such parallels are too low to do any conclusion).

Overexpression of RNF14 does not activate transcription in the absence of \(\beta\)-catenin (Figure 1G). This is evident with a Wnt reporter plasmid (TOPflash), as well as co-transfection controls.

2. Fig3F: The downregulation of LEF1 upon siRNA treatment against RNF14 does not seem to be very dramatic: why the authors precisely choose particularly this target gene to suggest LEF1 as an important molecule within their model (text on page 9)?

LEF1 is a very well characterized Wnt target gene and has been shown as such in many different cell types, tissues, developmental stages and diseases. We also show that other “classic” Wnt target genes such as AXIN2, MYC, and SP5 are equally responsive in their down regulation (Figure 3G, H).

3. Fig4: If the authors observed the more dramatic effects on survival of DLD1 colonies upon siRNA treatment against RNF14 why did they not make a growth curve (picture of the colonies) using these cells, and did not study here RNF14 function in more details in this cell line?

In this revision we provide our data on DLD-1 growth and RNF14 knockdown (Figure 4A). The affect of...
RNF14 loss is most dramatic in DLD1 cells grown as colonies due to the fact that this more robust cell line could withstand continuous treatment with fresh siRNA over the course of the experiment (Figure 4C). However, like the HCT116 cells, loss of RNF14 in DLD1 cells does not affect proliferation very much (Figure 4A). In our hands, colon cancer cell growth in vitro culture is very robust in regular media (even when dominant negative TCFs are expressed!). Wnt-directed phenotypes, including “survival” is more evident in the stringent colony-growth assay. We did not perform the growth experiment in limiting serum or nutrients to reveal growth phenotypes because control experiments show that the cells do not survive siRNA treatments in these conditions. In the future, it will be interesting to explore how RNF14 activities influence some or all of the Wnt-directed functions in cancer in other experimental paradigms.

4. ChIP against RFN14 is performed only with the transfected protein. Since RNF14 is expressed in both DLD-1 and HCT116 cells, ChIP could be performed on the endogenous protein (the best control being the siRNA against RNF14 treated cells). Cells with not aberrantly activated Wnt-signalling could be even tested (Wnt-stimulated vs. unstimulated situation), to confirm β-catenin-independent binding of RNF14 on target loci.

Unfortunately, all RNF14 antibodies tested to date do not work for chromatin immunoprecipitation. This forced us to use epitope-tagged versions of RNF14, which then of course, forced us to use an overexpression setting. We have nevertheless answered the question of occupancy and find that RNF14 is present at Wnt response elements in the absence of Wnt signaling and/or β-catenin.

5. In Fig5B the proper controls are missing as the authors are only detecting here nuclear proteins. Showing the cytosolic and membranous fractions is crucial to exclude any potential cross-contamination among fractions. This is all the more relevant when proteins such β-catenin are involved.

This control is now shown in Figure 5B. We have clear separation between nuclear and cytoplasmic fractions.

6. In the discussion authors suggested the role of RNF14 in ubiquitylation (e.g. histone H2B). It might be worth to test if RNF14 can play such a role in either monoubiquitylating H2B or ubiquitylating TCFs or β-catenin.

We have completed this test and find that histone H2B is most prevalent in the downstream, transcribed body of two different Wnt target genes, AXIN2 and SP5. The level of H2B ubiquitination is very low at the actual Wnt Response Element in the AXIN2 and SP5 promoters. Knockdown of RNF14 does not change the level of ubiquitination in the gene (if anything it is increased for AXIN2). We conclude that H2B ubiquitination is not an outcome of RNF14 activity.

7. In the supplementary Fig2C-D, how can the author draw any conclusion on the decreased pathway activation by the RNF14DeltaN when such a lower amount of it is expressed after transfection (not comparable to full length protein)?

We agree that it is very difficult. Deletion of the N-terminus definitely compromises the stability of the protein. This has made the domain mapping of the LEF/TCF interaction region more complicated, and until we can solve the issue structurally, this aspect of RNF14 action will remain less well understood. What we can say is that the N-terminal region of RNF14 is important for LEF/TCF binding. We cannot yet say whether it is wholly necessary, since RNF14ΔN is unstable compared to other deletion mutants.

Referee #3:

While these two major conclusions are quite compelling and may potentially be of interest in the field, this study in its present form lacks of a molecular explanation for the effect of RNF14. The authors argue that RNF14
stabilizes the β-catenin-TCF interaction, but this statement is based on a single experiment that is unfortunately not convincing (see details below). Even if this turns out to be true, one expects to see data on the mode of action of RNF14:

a) First of all, can a RNF14-TCF-β-catenin complex form (see specific comments)?

See our answer below.

b) Does RNF14 interact with chromatin-bound TCF? This would be somewhat surprising, since it interacts with the HMG box, and one would thus expect overlap with the DNA binding interface.

While an in-depth molecular study of structure, function and DNA binding lies beyond the scope of this report, we can say with strong certainty that RNF14 occupies Wnt Response Elements with TCF (judged by chromatin-immunoprecipitation). RNF14 is not the first factor shown to interact with the HMG DNA binding domain of LEF/TCFs. The High Mobility Group (HMG) domain interacts with DNA in a well-characterized binding/bending fashion that exposes many residues and structural features to solution space. Meaning, there is plenty of opportunity for simultaneous protein/DNA interactions between LEF/TCFs and other factors.

c) Could RNF14 compete with a TCF-bound repressor?

This is definitely possible. LEF/TCFs interact with TLE/Groucho proteins and other repressors such as CtBP and HDACs. We do note however, that CtBP is not a universal LEF/TCF interacting factor and TLE/Groucho proteins are not detected in HCT116 colon cancer cells (Arce et al. BMC Cancer 9:159) – a cell line where RNF14 has significant enhancing effects on TCF/β-catenin complexes.

d) Or could RNF14 act more indirectly? What about its ubiquitin ligase activity? Could it modify TCF and thus increase its binding to β-catenin (or decrease its binding to a repressor)? I would have thought that this would be the first hypothesis to test!

We looked long and hard for any evidence of LEF/TCF ubiquitination and we do not observe any. We nevertheless consider the question an open one for several reasons. One, ubiquitination might be location specific (only on WREs), two, the RING domain is important for RNF14 activity because its deletion compromises activity and it is usually the RING domain that is important for E3 ligase activity. Three, we note that co-expression of RNF14 with TCF-4 consistently and reproducibly lowers the level of protein (see lane 3, Input panel, Figure 1B), and four, it is intriguing that RNF14 appears to interact best with a truncated form of TCF-4. Unfortunately, the short format of EMBO Reports precludes any discussion of this sort in the text. A major goal for future studies will be a search for the putative targets of RNF14 (TCFs, β-catenin, repressors, transcription machinery).

These are all obvious and rather simple experiments that would bring the manuscript to a higher level of impact in the Wnt field. As it stands, these results merely add another name to the too long list of molecules that somehow influence the pathway.

While we agree that these hypotheses point to obvious experiments, we have tried several of them already and we disagree that they are simple in execution. Identification of bona fide targets of E3 ligases, particularly in a transcription regulatory setting where large multi-subunit co-activating and co-repressing complexes are battling it out for occupancy of TCF at Wnt Response Elements is very complex indeed! As for the long list of proteins that influence the Wnt signaling pathway: Most important nodes of regulation (such as transcription activation of Wnt targets) is comprised of many components (witness the multitude of factors in the signalosome at activated Frizzled Receptor complexes, or the
RNF14 definitely joins the crowd. The short format of EMBO Reports makes it difficult to discuss the aspects of RNF14 that make it noteworthy. Here we provide a starter list of these features: RNF14 is one of the few enzymatic components assembled at Wnt target genes. Enzymes present more possibilities for small molecule targeting than DNA binding proteins for example. Recent discoveries of a switch in/switch out behavior of co-repressor complexes for co-activator complexes at WREs are important in both cancer cells and embryonic stem cells. RNF14 action may be an important link in this activity since it stabilizes β-catenin binding. Finally, RNF14 appears to be a shared co-regulator with both the Wnt pathway and Androgen Receptor signaling. There is a history of hints that Wnt and AR signaling are linked. RNF14 may be the possible connection.

Specific comments:

1. Fig.1C: Increased β-catenin-TCF interaction in the presence of RNF14 is not convincing: There are two bands for TCF. While the top band is about equal in both lanes, and the lowest band is much more intense in the lane (+RNF14). In the middle panel, it seems that the lowest band is actually the one most efficiently IPed with Flag RNF14.

The reviewer is correct, the lower band – a truncated form of TCF-4 seems to interact better (see response to comment #1 of Referee #1).

In the lower panel (TCF IP), one seems only one band. In the absence of molecular weight markers, one cannot guess which of the two TCF forms this band corresponds. The quantification indicates a 6.7x increase in the presence of RNF14, which would make sense only if quantification was performed using the highest band in the input. This figure should be redone, including indication of markers and of single band intensities. Average fold increases of at least 3 replicates should be included.
Also please consider note for supplementary Fig.2 for acceptable blot quantification.

We have repeated the domain mapping and interaction study multiple times and have quantitated the degree of pull-down. We use digital detection of proteins on western blots using an ECL system, and FujiFilm platform for detection. In supplementary Figure 2, we present the quantification of single band intensity at the bottom of the panel. In the figure legend we indicate the range of the results from at least three replicate determinations for each deletion mutant.

2. Page 5 and Suppl fig2: "Interestingly, overexpressed β-catenin did not immunoprecipitate with flag-RNF14 (supp Fig1D). Thus, RNF14 may not directly interact with β-catenin but is able to bind all four TCF members."

This experiment shows that RNF14 does not bind directly to β-catenin. But what about TCF binding simultaneously RNF14 AND β-catenin. I don’t understand why the FlagRNF14 IP of Figs.1B and C was not blotted for β-catenin, this would bring an important piece to the puzzle.

As the referee has suggested, we present a more extensive co-immunoprecipitation analysis of RNF14, TCF-4 and β-catenin, both in an overexpression setting (Figure 1C) and with endogenous proteins (Figure 1D). The data show that immunoprecipitation of endogenous β-catenin brings down RNF14. Since we do not observe direct interactions of β-catenin with RNF14, we conclude that our data are suggestive of a tri-partite interaction between RNF14, TCFs and β-catenin. More structure function studies are necessary to define these interactions – but such studies lie beyond the scope of the report here.

3. Supplementary fig. 2: The numbers given for the quantification are not believable. Quantification was presumably done on classical films, which give a non linear signal (sigmoid) that saturates within a 2-4 fold
range. From my experience, a look at the blot shows that the inputs vary over a relatively wide range. deltaN is particularly much weaker than other constructs. There is a weak band for the pulled down fraction of this construct. This band is certainly weak, of the same intensity as the control GST band, but the amount of control GST input is clearly >10 times higher. That both give the same 0.1 ratio makes no sense. The lack of interaction for the deltaN construct is thus not convincingly demonstrated. Quantification should be done by direct comparison with a dilution series of one sample, blotted on the same membrane. To increase the usable range of signals, I advise switching from classical film exposure to direct imaging of the chemoluminescence with a digital camera.

As requested, we have repeated the domain mapping experiment with new preparations of recombinant proteins. The data are consistent, and shown in Supplementary Figure 2B. The western blot was quantitated via digital exposure and analysis. The overall findings are that the C-terminus, the RING domain, and the Androgen Receptor binding domain are not necessary for binding to the TCF-1 HMG DNA binding domain. Deletion of the N-terminus to amino acid 128 is deleterious to TCF-1 binding, but does not completely eliminate the interaction. Thus, the structural basis for RNF14 interaction with TCF-1 is complex and may involve multiple points of contact.
Thank you very much for the submission of your revised manuscript to our office. We have now received the enclosed reports from the referees that were asked to assess it. As you will see, all three reviewers appreciate that the revised version is now much improved and they all recommend publication in EMBO reports. Referees 1 and 2 still raise some issues, but they can mostly be addressed in writing by the addition of further discussions and clarifications (this would be particularly important with regard to the fact that in some instances, knockdown of RNF14 resulted in enhanced, rather than reduced, expression of wnt target genes). Only point 1a of referee 1 requires the addition of a negative control for the morpholino experiment.

Before we proceed with the acceptance of the manuscript I would kindly ask you to modify the manuscript according to the referee reports and to submit the final version through our website again.

On a more formal note, and as you also mentioned, the current text is slightly too long and I would like to ask you to please go through the text and shorten it slightly. The current character count is 35,000 (including spaces and references) and in the end, it should ideally be cut down to about 31,000 characters. I still think that the message of the manuscript is conceptually short so that EMBO reports is a better fit than The EMBO Journal. I also do not think that shortening of the text should be too problematic, but do let me know if you need help in shortening it. Please note that the materials & methods section should not be shortened any further.

We have also started to encourage authors to submit the raw data of biochemical and/or microscopical images to our editorial office. These data will be published online as part of the supplementary information. This is voluntary at the moment, but if you agree that this would be useful for readers I would like to invite you to supply these files when submitting the revised version of your study.

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

REFEREE REPORTS:

Referee #1:

The authors have done an outstanding job responding to my concerns. I fully support publication of the revised manuscript if the authors improve the following points.

1. Fig. 2F and 2G.
   (a) 20 ng of MO is too much. Higher amounts of MOs may cause non-specific effects. To show that 20 ng of MO does not cause the non-specific effect, at least, the authors should examine whether injection of 20 ng of negative control MO has no effect on Wnt target gene expression.

2. In page 8, the authors stated that "Overexpression of full-length Rnf14 increased levels of Wnt target genes at 6 and 24 hpf, but this increase was less apparent at 24 hpf". However, the effect of full-length Rnf14 on Wnt target gene expression at 6 hpf (Fig. 2C) is almost equal to that at 24 hpf.

3. The authors used two Rnf14 deletion mutants in zebrafish experiments. But they didn't show the detail (structure) of these mutants in this manuscript. They should present more detail information about these mutants in the main text or method section.
Referee #2:

In the revised version of the manuscript Wu et al. present new additional data which clarify and strengthen their statements. This new data together with the authors’ explanations in the rebuttal letter satisfactorily answer my previous concerns. The mechanism of how RNF14 may affect Wnt/β-catenin transcription is now well described and supported by experimental observations, at least in various cell lines. In my opinion, this work now meets the standards of EMBO Reports and might be acceptable for the publication. Nevertheless, some changes/improvements within the text are suggested here to clarify a few minor points.

1) In their rebuttal letter (see previous minor comment n{degree sign}1), the authors say that: „Overexpression of RNF14 does not activate transcription in the absence of β-catenin (Figure 1G). This is evident with a Wnt reporter plasmid (TOPflash), as well as co-transfection controls” I can find here an apparent discrepancy:
If RNF14 addition/overexpression leads to increased binding between endogenous β-catenin and TCF4 in HEK293 cells (Fig 1C 6th panel 3rd lane), why does not it stimulate the Wnt reporter (in the same cellular context) even when no β-catenin is co-transfected (Fig1G)? Intuitively, the increased levels of β-catenin which is bound to TCF4 after RNF14 transfection should potentiate TCF/β-catenin-mediated transcription. Or is it just an experimental artefact (due to the usage of whole cell lysates for co-IP) not reflecting the situation in luciferase reporter assays? Could the authors explain/discuss this?

2) While describing the in vivo experiment performed with zebrafish (Fig2), it is worthy, in my opinion, to clearly emphasize the concept that RNF14 most likely works only as a modulator of Wnt dependent transcription, due to the absence of any morphological/developmental effect upon overexpression or morpholino treatment.

3) In Fig1C second panel: it is a bit foggy which TCF4 band is produced by the transfected plasmid (in the non transfected, as expected, there is none of the two bands, even though TCF4 is endogenously expressed in 293 cells). How could two variants possibly emerge from a transfected TCF4? It most likely could not be a natural splice variant since the authors use a cDNA for transfection. Is it a degradation product or an artefact of transcription? Or do the authors think that RNF14 could stimulate the transcription of an endogenously produced shorter TCF4 variant? Please discuss this point.

4) In Supplementary Fig2H the authors show new interesting data indicating that RNF14 is not involved in Wnt-dependent H2B ubiquitination. My suggestion is to clearly indicate in the main text that the ChIP has been performed in DLD1 cells (i.e. in a „Wnt-ON” situation). The H2B ubiquitination status might be different in the „Wnt-ON” and „Wnt-OFF” situations, although it could be completely RNF14 independent, as suggested by authors.

Typos:

-Page11 last line: FigF,G > Fig5F,G
-Page 17 commenting Fig1F: „overexpressed” is redundantly repeated.

Referee #3:

The authors have satisfactorily answered concerns from all the three reviewers. The revised version is quite fine.
Please find below our responses to referee questions and suggestions. We thank the reviewers once again for a careful read and for their positive assessment of our study. We have corrected typos, addressed questions and modified text in the manuscript to emphasize major points such as our finding that RNF14 acts as a signal amplifier of the Wnt pathway.

Referee #1:
The authors have done an outstanding job responding to my concerns. I fully support publication of the revised manuscript if the authors improve the following points.

1. Fig. 2F and 2G.
(a) 20 ng of MO is too much. Higher amounts of MOs may cause non-specific effects. To show that 20 ng of MO does not cause the non-specific effect, at least, the authors should examine whether injection of 20 ng of negative control MO has no effect on Wnt target gene expression.

   We have removed the 20 ng morpholino data because, as the reviewer points out, this is a very high level of MO to inject. In fact, most zebrafish studies do not report results using this amount. The 20ng dosage was included in the experiment as a top level for a complete dose-response curve, but it is not necessary to include. The revised graphs are shown in Figure 2F and 2G. To recap the results here:

   We observe decreases in Lef1 and Sp5 expression with MO knockdown of RNF14, but no significant changes in Axin2 mRNA. These are more modest changes when we compare them to target gene expression trends with RNF14 overexpression, or when we compare them to Wnt target gene changes in human cells. We note in the results section that fish embryos might be less sensitive to RNF14 knockdown since maternally-derived Rnf14 mRNA/protein could be interfering (see Supp. 2F to see that Rnf14 mRNA is highest at 1.5 hpf), and also because there is another RNF14-related protein that could be acting redundantly (~65% similar protein encoded by a related gene on chromosome 15). Knockdown effects of RNF14 in human cells are much more striking and clear, possibly because only one RNF14 gene is in the human genome.

(b) 5 ng of rnf14 MO slightly enhanced expression of Wnt target genes at 6 and 24 hpf. In addition, 20 ng of rnf14 MO also promoted the expression of axin2 and lef1 at 24 hpf. These results suggest that endogenous Rnf14 might negatively regulate Wnt signaling. The authors should discuss this discrepancy. Although the authors normalized mRNA levels of Wnt target genes to otx2 levels, Wnt signaling also affects otx2 expression at early stage. Knockdown of rnf14 might also affect otx2 expression at 6 hpf.

With the exception of Sp5 (5ng, 6 hpf), all of the small increases in target gene expression at any dose or time are not statistically significant. The small changes are noticeable in part because the 0ng MO data points are set to 1.0 (and thus, no error bars). They may also be noticeable because as the reviewer posits, Otx2 expression might be slightly decreased at 6hpf. Below we show Otx2 expression data for all MO dosages and time points, data that is normalized to the housekeeping EF1A transcript. There are no changes that are statistically significant, including the slight decrease at the 6 hpf, 5 ng MO dosage (p=0.0549). Otx2 levels are steady at 10ng MO, 6hpf (p=0.68) and not significantly changed at any of the MO dosages at 24 hpf.

Other data that show RNF14 is a co-activator include the overexpression studies in zebrafish (Fig. 2C, D). The results clearly show that RNF14 expression enhances target gene expression. A mutant RNF14 that has lost activity (rnf14ΔC; Figure 2C,D) does not enhance expression but in fact strongly lowers expression, a negative effect that is likely due to its competition with endogenous, wildtype RNF14. If RNF14 were a negative regulator, these experiments
would have generated a different outcome.

Finally, as we mention above, RNF14 knockdown experiments in human cells, where there are no other RNF14-related genes, show that knockdown decreases target gene expression, an outcome that clearly demonstrates that RNF14 provides an activating function, not a repressing one.

2. In page 8, the authors stated that "Overexpression of full-length Rnf14 increased levels of Wnt target genes at 6 hpf, but this increase was less apparent at 24 hpf". However, the effect of full-length Rnf14 on Wnt target gene expression at 6 hpf (Fig. 2C) is almost equal to that at 24 hpf.

Our statement was meant to refer to the fact that the increases in target gene expression at 24 hpf, are not statistically significant (no asterisk in Fig. 2D). We apologize for the confusion and have changed the sentence to read:

"Overexpression of full-length Rnf14 increased levels of Wnt target genes at 6 hpf, but this increase was less significant at 24 hpf, possibly due to degradation of injected RNA."

3. The authors used two Rnf14 deletion mutants in zebrafish experiments. But they didn't show the detail (structure) of these mutants in this manuscript. They should present more detail information about these mutants in the main text or method section.

The deletions were created based on the domain mapping with the human RNF14 homolog (Supp. 2A, B). To clarify the specific residues that are removed in the zebrafish version, we have modified our sentence in the results section to read:

"A Rnf14 deletion mutant lacking the putative AR domain (zebrafish Rnf14 Δ443-459, supp Fig1A) enhanced Wnt target gene expression while a larger deletion of the C-terminus (Rnf14 Δ219-459) lost the ability to upregulate target genes."

4. In page 12, the authors stated that "knockdown of RNF14 did not alter H2B ubiquitin levels". Which Figure shows this result? I can not find it. Possibly, the authors mean that knockdown of RNF14 did not alter the level of ubiquitinated H2B within the 3' untranslated region of SP5 (supp Fig 2H).

The reviewer is correct, we were referring to the level of H2B ubiquitination within the indicated ORF and 3’ untranslated regions, not the overall level of H2B ubiquitination in cells. We thank the reviewer for catching this. The sentence is corrected to read:

"Furthermore, knockdown of RNF14 did not alter the level of ubiquitinated H2B at the ORF and 3’UTR regions (supp Fig2H) …"

Referee #2:

In the revised version of the manuscript Wu et al. present new additional data which clarify and strengthen their statements. This new data together with the authors’ explanations in the rebuttal letter satisfactorily answer my previous concerns. The mechanism of how RNF14 may affect Wnt/β-catenin transcription is now well described and supported by experimental observations, at least in various cell lines. I my opinion, this work now meets the standards of EMBO Reports and might be acceptable for the publication. Nevertheless, some changes/improvements within the text are suggested here to clarify a few minor points.

1) In their rebuttal letter (see previous minor comment n(degree sign)1), the authors say that: "Overexpression of RNF14 does not activate transcription in the absence of β-catenin (Figure 1G). This is evident with a Wnt reporter plasmid (TOPflash), as well as co-transfection controls" I can find here an apparent discrepancy:
If RNF14 addition/overexpression leads to increased binding between endogenous β-catenin and TCF4 in HEK293 cells (Fig 1C 6th panel 3rd lane), why does not it stimulate the Wnt reporter (in the same cellular context) even when no β-catenin is co-transfected (Fig1G)? Intuitively, the increased levels of β-catenin which is bound to TCF4 after RNF14 transfection should potentiate TCF/β-catenin-mediated transcription. Or is it just an experimental artefact (due to the usage of whole cell lysates for co-IP) not reflecting the situation in luciferase reporter assays? Could the authors explain/discuss this?

We thank the reviewer for thinking deeply about RNF14 and our findings. He/she raises an important point that we have strived to emphasize in the manuscript which is: **RNF14 does not activate Wnt signaling on its own, but instead relies on bona fide Wnt signaling or nuclear-localized β-catenin for its action.** In the co-immunoprecipitation studies that we performed in HEK293T cells (Figure 1B-D), RNF14 and TCFs were overexpressed, and detergent-containing extracts released β-catenin from the plasma membrane (HEK293T cells have a lot of β-catenin at the membrane). This means that co-immunoprecipitations with whole cell extracts will survey interactions between RNF14 and TCF that occur on endogenous target genes in the nucleus, but also interactions that occur between TCF and β-catenin in the complex milieu of the post-harvest extract. In other words, the interactions are bona fide, but the absence of subcellular compartments, such as a nucleus or intact plasma membrane negates morphological or structural levels of regulation that govern how RNF14 influences Wnt signaling.

Intact, living HEK293T cells do not exhibit any active Wnt signaling. Therefore, mere overexpression of RNF14 does not activate Wnt reporters because there is no nuclear localized β-catenin for it to stabilize at Wnt response elements. In contrast, overexpression of β-catenin via transient transfection leads to increases of the protein in the nucleus (Fig.1G). In colon cancer cells (Fig. 1E,F) β-catenin is aberrantly stable and already present in the nucleus. Because TCFs are bound to target genes all the time in both cell types, RNF14 can work with these factors to stabilize β-catenin occupancy and enhance reporter gene or endogenous target gene expression (Fig. 1E,F, Fig.3H). Finally, the in vivo zebrafish experiments very clearly and dramatically demonstrate that RNF14 does not ectopically activate Wnt signaling, but rather enhances bona fide Wnt signals where they occur naturally.

2) While describing the in vivo experiment performed with zebrafish (Fig2), it is worthy, in my opinion, to clearly emphasize the concept that RNF14 most likely works only as a modulator of Wnt dependent transcription, due to the absence of any morphological/developmental effect upon overexpression or morpholino treatment.

We wholeheartedly agree. A thorough discussion of this finding could emphasize this point but because we have been asked to reduce our text by 10%, in-depth discussion of this intriguing activity is not possible. Nevertheless, we have modified our text describing the zebrafish experiments to say:

“Importantly, while injected rnf14:mCherry was present throughout the embryo (Fig2A), Wnt signaling was only enhanced in specific regions such as the midbrain where Wnt activity is normally detected. There is no apparent, aberrant upregulation of Wnt signaling by overexpressed Rnf14 in other regions. These data demonstrate that Rnf14 is a signal amplifier that relies on endogenous Wnt signals for its action, meaning that overexpression itself cannot force ectopic activation.”

3) In Fig1C second panel: it is a bit foggy which TCF4 band is produced by the transfected plasmid (in the non transfected, as expected, there is none of the two bands, even though TCF4 is endogenously expressed in 293 cells). How could two variants possibly emerge from a transfected TCF4? It most likely could not be a natural splice variant since the authors use a cDNA for transfection. Is it a degradation product or an artefact of transcription? Or do the authors think that RNF14 could stimulate the transcription of an endogenously produced shorter TCF4 variant? Please discuss this point.

As the reviewer suggests, the appearance of two polypeptides in extracts with overexpressed TCF4 is striking. However, alternative splicing is not a possible explanation as we use a cDNA plasmid to
overexpress TCF4. An alternative, internal start codon for translation or an artifact of transcription initiation are formal possibilities, but our western blot analysis in Supplementary Figure 1B suggests that both of these explanations are unlikely. Using two different antibodies we show that the difference between the two polypeptides is the presence/absence of the extreme C-terminus. We therefore believe the smaller TCF4 polypeptide is a degradation or protein cleavage-type product. Although we have not directly linked RNF14 to this observation, it is intriguing that RNF14 is an E3 ligase, a class of enzymes with known actions in protein processing.

4) In Supplementary Fig2H the authors show new interesting data indicating that RNF14 is not involved in Wnt-dependent H2B ubiquitination. My suggestion is to clearly indicate in the main text that the ChIP has been performed in DLD1 cells (i.e. in a „Wnt-ON“ situation). The H2B ubiquitination status might be different in the „Wnt-ON“ and „Wnt-OFF“ situations, although it could be completely RNF14 independent, as suggested by authors.

The reviewer highlights an important distinction which is that colon cancer cells that have constitutive, high levels of Wnt signaling might mask the actions of an E3 ligase and its resultant ubiquitin marks that are evident in normal cells with activated Wnt signals. We have therefore revised the text to use the reviewer’s nomenclature for describing the “Wnt-On” DLD1 cells. However, since we are restricted by a character/word limit, we have not described this result and its implications further than that.

Typos:

-Page 11 last line: FigF,G > Fig5F,G
-Page 17 commenting Fig1F: „overexpressed“ is redundantly repeated.

We have corrected these typos, and thank the reviewer for his/her careful reading.

Referee #3:

The authors have satisfactorily answered concerns from all the three reviewers. The revised version is quite fine.

Thank you!
Thank you very much for submitting the final version of your study to EMBO reports. I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. I thought you might be happy to know that Konrad Basler is writing a short highlight (a News&Views-type of article) on your paper to be published in the same issue. I really liked your story and thought it would be nice to give it more of an exposure this way.

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