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Nuclear Receptor Binding Protein 1 (NRBP1) regulates intestinal progenitor cell homeostasis and tumor formation

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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 11 August 2011

Thank you very much for submitting your research manuscript on the function of NRBP1 in tumor suppression for consideration to The EMBO Journal editorial office.
I did receive comments from three scientists that vary in their overall assessment with two being in favor of publication (ref#2, #3) and one very much resistant, based on current limited molecular insight. I have thus discussed the paper with colleagues here at the office and on face value we decided to offer the chance for major revisions addressing the critical points in the current version of the study. Specifically, ref#2 raises minor points that should not take too long to be addressed experimentally but would substantiate the findings. Ref#3 emphasizes the need for definitive molecular insight and thus demands convincing support for the molecular function of NRBP1 as component of the elogin/cullin complex. This should include evidence for substrate ubiquitination and, in the best case, in-vitro reconstitution of this relationship. On balance, we do NOT insist on recapitulating the NRBP1/Ras-cooperation in a mouse model.
Though still relatively demanding, I assume that such amendments would be possible during standard revisions. We would however also be able to grant additional time for necessary experimentation upon your request. Please do not hesitate to contact me in case of further questions (preferably via E-mail).

Lastly, I do have to formerly remind you that it is EMBO_J policy to allow a single round of revisions only and that the ultimate decision depends on the content and strength of your adequately modified version.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

The submission from Wilson, Adams, Fraser and colleagues reports on studies of NRBP1 orthologues in various systems, ranging from C. elegans to mouse to human. While much ground is covered by the authors in hopes of clarifying how NRBP1 may be functioning in cell fate, signaling and tumorigenesis, the collection of studies and data presented here has major gaps and weaknesses, and there are many uncertainties about if and how NRBP1 might be connected to the Ras, Notch, and Wnt pathways or specific factors like Sall4. Due to the limitations of the paper, there remain many questions about how NRBP1 inactivation may lead to the tumor phenotypes in NRBP1 cKO mice described in the paper. Another fairly pervasive problem with the paper is that the quality of the images presented in many figures is very poor and, due partly to data quality problems and poor presentation, the authors' claims lack convincing support. A few of the many issues with studies, data and claims are commented on below:

1) The C elegans studies and the cell culture work indicates that NRBP1 interacts in some way with Ras signaling, but the authors' studies and data here do essentially nothing to clarify how NRBP1 is enhancing Ras-dependent phenotypes or what the relevance to the Ras pathway is for the mouse knockout phenotype described in the paper. At present, Figure 1 has essentially no relevance or connection to the rest of the paper.
- Minor issue with Figure 1 - Panel D, Y-axis -> shouldn't the label be "Foci" and not colonies?

2) Figure 2 - The overall quality of the photos and figure preparation is very poor. The fact that the phenotypes of cKO and Lgr5-EGFP-IRESCreERT2 are not really all that similar raises some questions of whether the reported epithelial phenotype seen in cKO mice is due in part to non-cell-autonomous effects of inactivating NRBP1 in various stromal cell types. Some other issues with the studies and data shown are the following:
- Panels E and F - Claims about crypt fission are not convincing. In panel E, all that seems apparent is that the epithelium shows altered, disordered morphology in the limited numbers crypts shown.
- Panel G - No convincing evidence of NRBP1 expression in LI is seen.
- Panel H - There seems to be great variability from crypt to crypt - even in the limited number of crypts shown - for Paneth cell numbers and location. Why is this?
- Panel I - It seems hard to define individual vertical crypts in cKO tissue - how then to quantitate?
- Lack of convincing similarity of Lgr5-targeted and cKO phenotypes is highlighted in panel J. Authors have no convincing argument for why such differences are seen.

3) Figure 3 - The genes reported on in the figure are likely regulated by many pathways besides Wnt, and expression of both Myc and Ccnd1 is linked to cell cycle status. The quality of the staining is very poor and the claims made are not well supported.

4) Figure 4 - The studies suggest some role for NRBP1 in regulating Sall4 levels, but don't really dig deep enough to establish that NRBP1 has a key function in regulating Sall4 protein ubiquitination and half-life. Also, given the uncertainties about how Sall4 may be a target gene of Wnt signaling versus a key factor in Wnt target activation (Bohm et al. BBRC 2006; Shuai et al. Cancer Genet Cytogenet 2009), the authors' studies offer minimal data on the outstanding issues. Studies of endogenous Wnt target genes - Lgr5, Dkk1, Nkd1, Axin2 - would have been preferable, along with some clues about how Sall4 may be acting on Wnt signaling.

5) Figure 5 - The photos in the panel are of poor quality and the claims lack strong support.
- Panel C - not clear that the colorectal tumor was invasive and the photo in panel iii is useless as currently presented.
- Panel D panels - Without comparison to normal tissues, not clear how to interpret the IHC data - is panel vi really the beta-catenin staining?

Referee #2:

In this ambitious and elegant manuscript Wilson and colleagues identify NRBP1 from a genetic screen of ras interactors in C elegans then genetically delete NRBP1 in mice which imposes a crypt progenitor phenotype and acts as a tumour suppressor. Finally they show NRBP1 is downregulated in human cancer where low expression correlates with a poor prognosis. Mechanistically NRBP1 appears to be part of the Cul5 E3 ligase complex. This complex degrades a number of proteins such as SALL4, which has been shown to negatively regulate wnt signalling. Importantly in intestinal extracts SALL4 is upregulated and there is deregulated Wnt signalling. Overall I think this is an important study, given the authors cover so much ground it is always easy to find minor faults throughout such a big study but these are trivial and don't detract from what is an excellent study.

Specific comments.

1. I agree with the authors that the most likely reason for the crypt progenitor phenotype is through an increase in Wnt signalling. The overlap with the microarray following Apc loss is very consistent with this. It is important to note that is still unclear if the OCT4 overexpression phenotype the author cite is down to the overexpression of Wnt signalling. It is possible that the overexpression of OCT4 in the crypt would interact with Myc and SOX4 expression at the base of the crypt to provoke an intestinal progenitor phenotype which is characterised by increased Wnt signalling. I think a good way for the authors to tackle the dependence of Wnt signalling would be grow these crypts ex vivo and see if they form similar organoids to those following Apc loss ie they form spheres that do not have differentiation and do not bud (unlike Wild type organoids), they could then remove the spondin and see if they are still dependent on a Wnt ligand.

2. The above would also answer the question posed by the Lgr5CREER experiment where mice don't get cancer. As recombination is throughout the intestine: mesenchymal, epithelial, neuronal and smooth muscle in ROSACREER mice, it could be down to the concerted loss in all cells that mice develop tumours. Thus it will be of interest to see if the crypt progenitor phenotype is remains in culture. I think the most likely explanation for the Lgr5 CREER result is that this is a very poorly penetrant cre recombinase and thus if there some selection against the NRBP1 knockout cells would be lost. The data from the RosaCREER would suggest this as a significant percentage of these mice show reconstitution form non recombined cells. This is quite an important point that the authors should highlight in the manuscript as it basically makes it unlikely that NRBP1 is an initiating event in CRC, instead it's a co-operator.

3. Do the authors see any tumourigenesis/LOH in the NRBP1 +/- mice.

4. Whats the level or recombination in the ROSACREER mice given a single injection of tamoxifen?

5. The human lung data is very impressive as is the fact the NRBP1 loss leads to lung carcinoma in the mouse. Given the authors have staining for SALL4 working it would be good to see if there is any correlation with NRBP1 within the lung tumours. Here this would be a nice paradigm as increased wnt signalling has been shown in a number of tumours to confer a poor prognosis and drive tumour progression. Also it would be interesting to see if TSC22 was upregulated in lung. It's been recently shown by the Peeper in EMBO that upregulation of the long isoform can overcome oncogene induced senescence and in mice probably the best example of OIS is lung cancer (the McMahon groups work on BRAF) outwith melanoma.
All of these extra experiments i think would be beneficial for the manuscript and should not take too long. The results would be interesting either way.

Referee #3:

In the manuscript titled 'Nuclear receptor binding protein 1 (NRBP1) regulates intestinal progenitor cell homeostasis and tumour formation', Wilson et al. identified NRBP1 as a tumour suppressor. They elegantly carried out a C. elegans RNAi screening and validated the target gene using a mouse model. The NRBP1 knock out mouse shows an interesting phenotype in the intestine. The in vivo data are convincing but the biochemical mechanism of action of NRBP1 is not clearly demonstrated.

Following points outline the major concerns in the manuscript.

1. The biochemical characterization of NRBP1 is not very clear. The connection between NRBP1, ElonginBC and CUL5 is not supported by clear data. First of all, the interaction between NRBP1 and Elongin B/C, as observed by IP/Mass spec, should be validated, as has been done for SALL4.

2. CUL5 did interact with NRBP1 only when all other components of the hypothesized complex have been overexpressed. Authors need to show that NRBP1-Elonging B/C-Cul5 complex is forming. Does the interaction between NRBP1 and CUL5 get reduced if ElonginB/C is knocked down? Is it possible to overexpress one of the components and IP the endogenous protein?

3. The stabilization of SALL4 and Tsc22d2 in NRBP1 cKO mice suggests that NRBP1 regulates the abundance of these proteins but this data in no way demonstrates that an ubiquitin ligase activity is involved. It is absolutely necessary to demonstrate the ubiquitination of the substrates with either overexpressing or knocking down the ligase. This experiment can be done very easily in HCT cells. Moreover, in vitro experiments using purified components are required to convincingly demonstrate a substrate-E3 relationship.

5. Additionally the quality of the data in figure 4 needs to be improved. For example, in fig4C, authors claim that in cKO mice Sal4A and B are stabilized but in the figure there is no band for Sal4A.

6. The authors suggest cooperation between NRBP1 and Ras using a focus formation assay in NIH3T3 fibroblasts. However, a relevant effect of NRBP1 was found in the intestine where Ras plays a major role in oncogenesis. Keeping that in mind, an in vivo model of Ras, eg the KRasG12D mouse model, would have been more convincing rather than murine fibroblasts.

Minor points:
1. Specific deletion of NRBP1 in the intestinal stem cells produced similar effect as cKO mice but to a lesser extent. Authors showed data for staining with anti-lysozyme and alcian blue (fig 2J). The difference is not obvious from the figure. It would have been better if authors could provide quantification of the stainings.
2. The manuscript includes a lot of discussion about NRBP1 ubiquitin ligase activity and how this complex might be regulating WNT pathway and intestinal progenitor cell homeostasis. But in absence of conclusive evidence of the ubiquitin ligase activity the whole discussion part is still a speculation and hypothesis.
3. 'Materials and Methods' section states that IP/Mass spec experiment was carried out using tandem affinity purification but the from the description it looks like the method is a single step affinity purification, not tandem.

1st Revision - authors' response 08 February 2012

We would like to thank the Referees for their time and expertise in reviewing our paper '***Nuclear Receptor Binding Protein 1 (NRBP1) regulates intestinal progenitor cell homeostasis and tumor formation'.*** We have significantly revised our manuscript in light of their comments and we hope that it is now ready for publication.

Referee #1:

The submission from Wilson, Adams, Fraser and colleagues reports on studies of NRBP1 orthologues in various systems, ranging from C. elegans to mouse to human. While much ground is covered by the authors in hopes of clarifying how NRBP1 may be functioning in cell fate, signaling and tumorigenesis, the collection of studies and data presented here has major gaps and weaknesses, and there are many uncertainties about if and how NRBP1 might be connected to the Ras, Notch, and Wnt pathways or specific factors like Sall4. Due to the limitations of the paper, there remain many questions about how NRBP1 inactivation may lead to the tumor phenotypes in NRBP1 cKO mice described in the paper. Another fairly pervasive problem with the paper is that the quality of the images presented in many figures is very poor and, due partly to data quality problems and poor presentation, the authors' claims lack convincing support. A few of the many issues with studies, data and claims are commented on below:

We understand the limitations of our paper and we have tried to address these in our manuscript which has now been extensively revised. The referee should appreciate that Nrbp1 is a completely uncharacterized gene and that we have made significant and incisive progress towards 1). establishing an important role for Nrbp1 in cell fate and tumorigenesis and 2). providing mechanistic insights into how Nrbp1 functions. We conclude that the role of this gene is complex and is likely to function in several pathways to elicit the phenotypes we describe in our paper. We were surprised by the comment on image quality. All of the images in the paper now exceed the dpi required by the journal. We would be happy to supply high-resolution tiff files if this referee requires them.

1) The C elegans studies and the cell culture work indicates that NRBP1 interacts in some way with Ras signaling, but the authors' studies and data here do essentially nothing to clarify how NRBP1 is enhancing Ras-dependent phenotypes or what the relevance to the Ras pathway is for the mouse knockout phenotype described in the paper. At present, Figure 1 has essentially no relevance or connection to the rest of the paper.
- Minor issue with Figure 1 - Panel D, Y-axis -> shouldn't the label be "Foci" and not colonies?

Remembering that Nrbp1 was almost completely unknown until we retrieved it from the worm screen described in our paper we feel that Figure 1 is important to our story. In our manuscript we describe how the WNT, RAS and NOTCH pathways converge to regulate vulva induction, pathways that we analyse in our mouse model thoughout the rest of the manuscript.

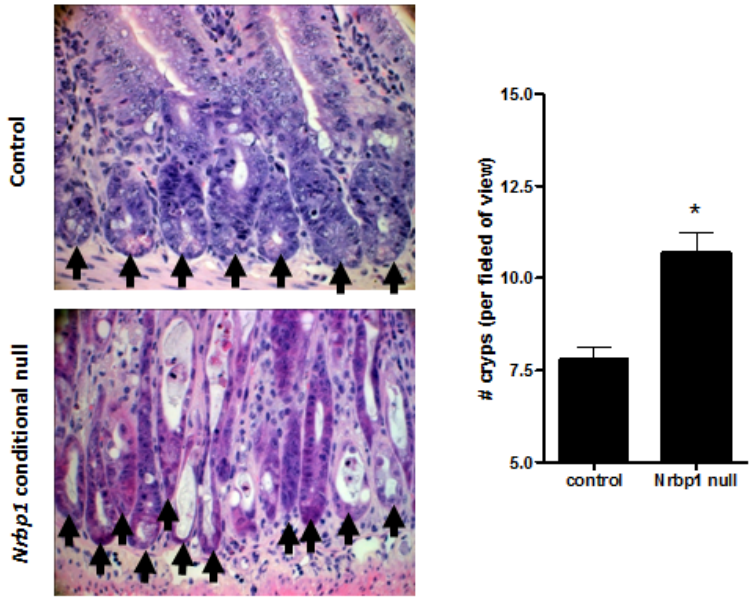
The Y axis has been changed from 'Colonies' to 'Foci'

2) Figure 2 - The overall quality of the photos and figure preparation is very poor. The fact that the phenotypes of cKO and Lgr5-EGFP-IRESCreERT2 are not really all that similar raises some questions of whether the reported epithelial phenotype seen in cKO mice is due in part to non-cell-autonomous effects of inactivating NRBP1 in various stromal cell types. Some other issues with the

studies and data shown are the following:
- Panels E and F - Claims about crypt fission are not convincing. In panel E, all that seems apparent is that the epithelium shows altered, disordered morphology in the limited numbers crypts shown.

As indicated above the images provided in the revised version of the manuscript exceed those required by EMBO and we would be happy to provide the source tiff files if the referee requires them.

Crypt number was calculated as shown below. This phenotype was confirmed by a consultant gastrointestinal pathologist who reviewed slides from the Nrbp1 mice (Mark Arends).

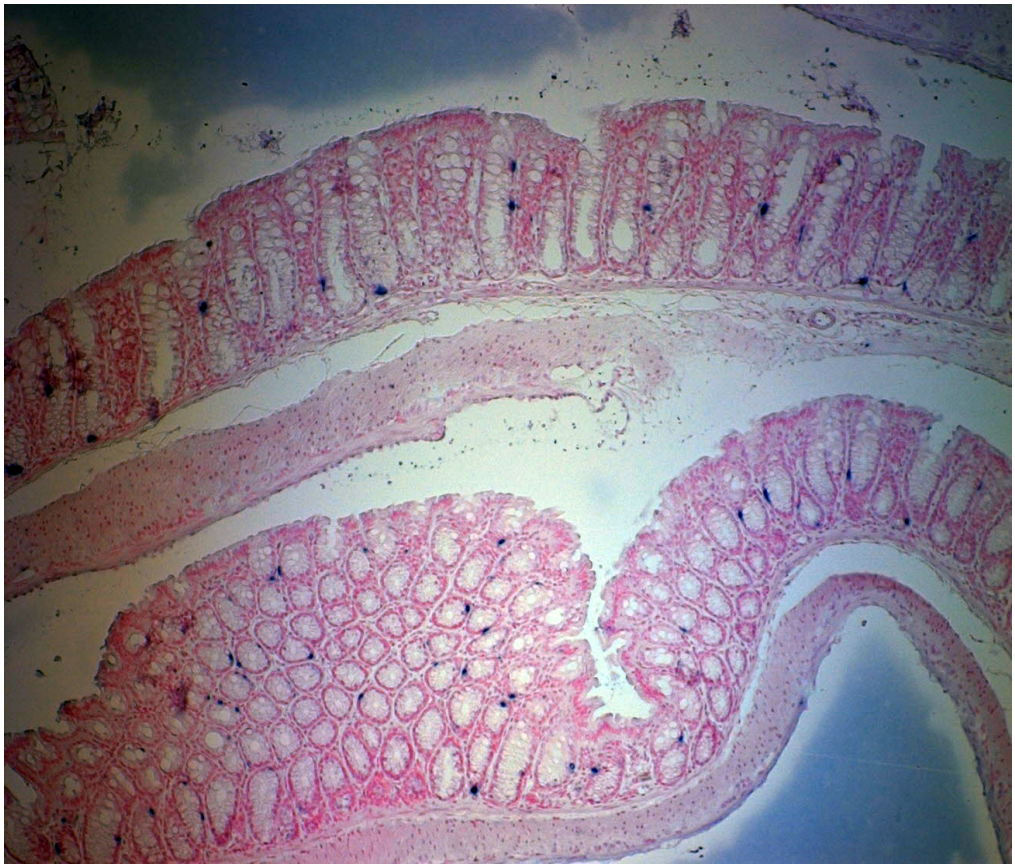


Crypt fission is a prominent feature of Nrbp1 null intestines.

Four lengths of intestinal epithelium were photographed and used to estimate crypt numbers of *Nrbp1*$^{flox/flox}$ *Rosa*$^{CreERT2/+}$, *Nrbp1*$^{flox/flox}$ *Rosa*$^{CreERT2/+}$ and *Nrbp1*$^{flox/+}$ *Rosa*$^{CreERT2/+}$ mice (4 of each genotype). All comparisons were made using the Mann-Whitney U test.

- Panel G - No convincing evidence of NRBP1 expression in LI is seen.

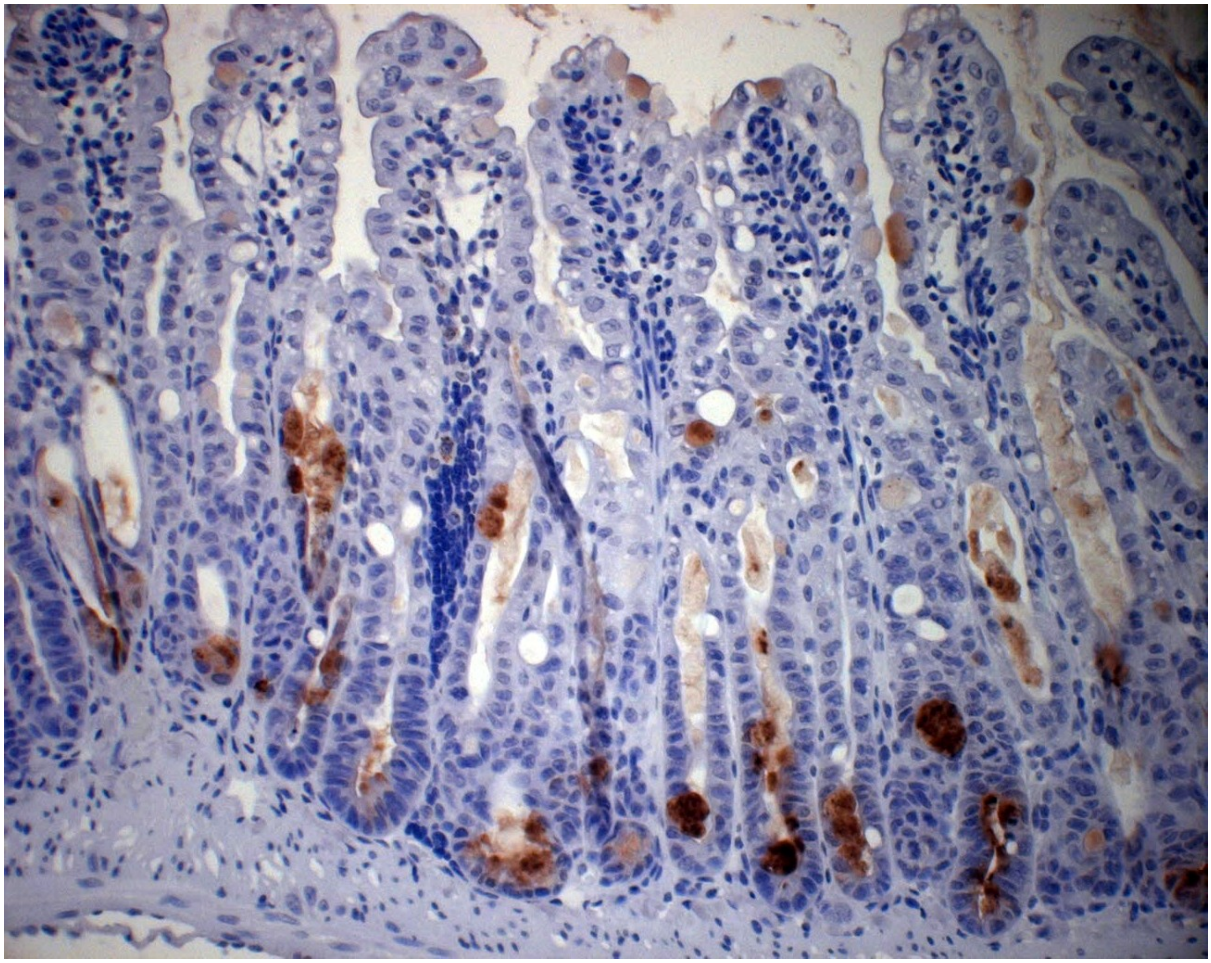
The Nrbp1 ISH staining is representative of the staining we observed. We have provided an image for the referee that clearly shows expression of Nrbp1 in the large intestine in the enteroendocrine cells. This image unequivocally shows large intestine expression of Nrbp1.



A section of large intestine showing Nrbp1 staining in enteroendocrine cells.

- Panel H - There seems to be great variability from crypt to crypt - even in the limited number of crypts shown - for Paneth cell numbers and location. Why is this?

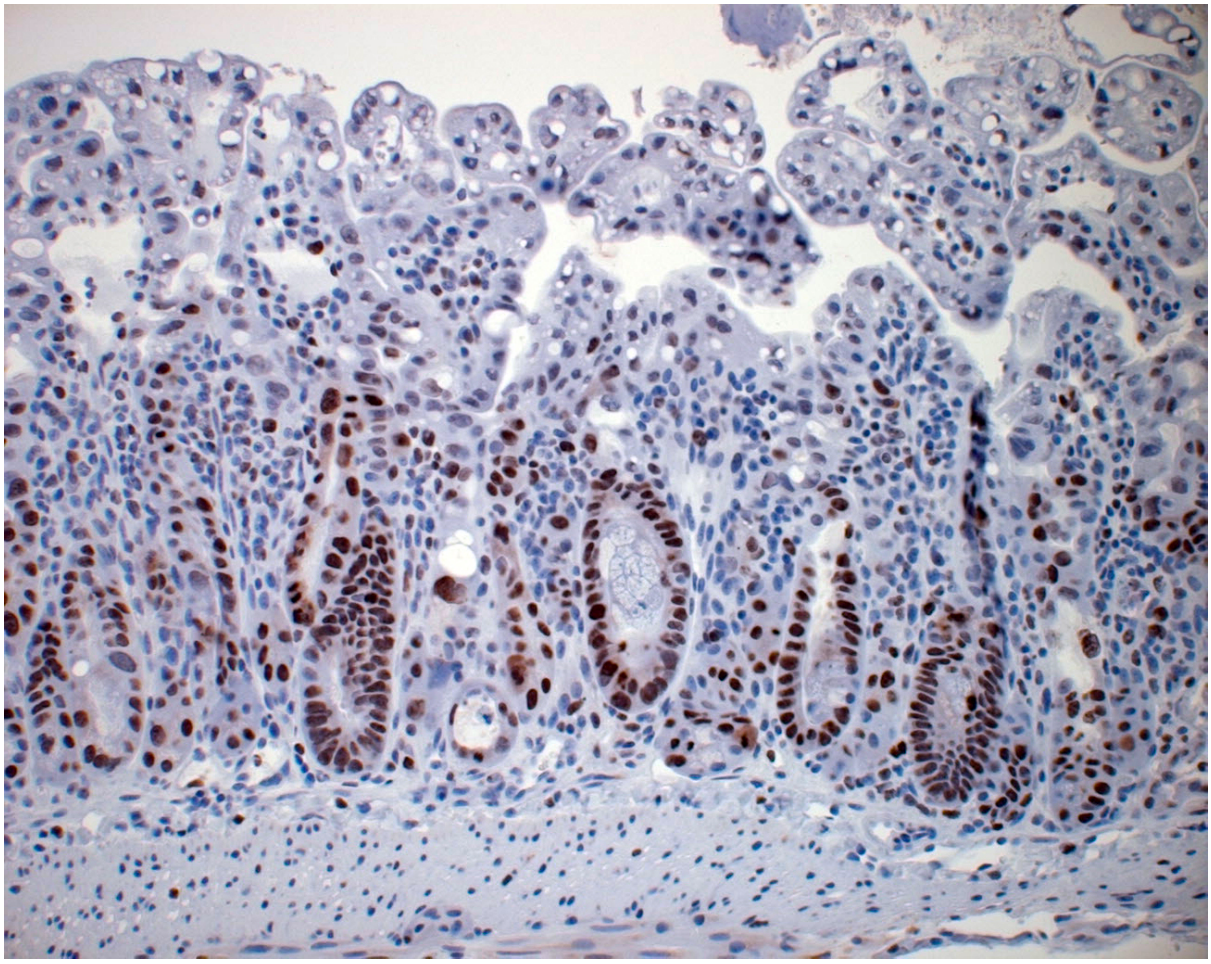
Paneth cells are normally localised in the crypts as they migrate towards high wnt signal after they have been formed in the transit amplifying region of the crypt. If there is abnormal wnt signalling Paneth cells will migrate out of the crypt and become mis-localised, as is seen in the images. In addition, we hypothesise that loss of *Nrbp1* is probably influencing the differentiation of new Patheth cells, therefore Paneth cell number is reduced, but they are relatively long lived cells within the intestine (20 to 40 days) so within the timeframe of analysis (5 days) we were only able to see a modest reduction in the total number of these cells.



A section of small intestine showing Lysosome straining, showing mis-localisation and modest decreased number of Paneth cells.

- Panel I - It seems hard to define individual vertical crypts in cKO tissue - how then to quantitate?

The image provided is representative of the phenotype observed along the entire small intestine. Due to cross cutting within the intestine quantification of the cells that constitute each crypt is only performed when we can view the complete crypt architecture.



A section of small intestine showing Ki67 straining, showing increased staining of proliferative cells.

In all cases, a total of 30 crypts/villi were counted directly from the slides containing small intestine of four *Nrbp1*$^{flox/flox}$ *Rosa*$^{CreERT2/+}$ and controls.

- Lack of convincing similarity of Lgr5-targeted and cKO phenotypes is highlighted in panel J. Authors have no convincing argument for why such differences are seen.

Lgr5 Cre^{ER} is a relatively inefficient cre driver which recombines less than 10% of intestinal stem cells therefore we obtained exactly the result we expected which was a less profound intestinal phenotype than observed using RosaCre^{Ert2} which is an extremely efficient Cre driver. The fact that we see altered Paneth cell localisation using Lgr5 Cre^{ER} supports our data indicating that Nrbp1 altered Wnt signalling within the crypt. We have clarified the difference between the Lgr5 Cre^{ER} and RosaCre^{Ert2} experiment in the revised text.

3) FIgure 3 - The genes reported on in the figure are likely regulated by many pathways besides Wnt, and expression of both Myc and Ccnd1 is linked to cell cycle status. The quality of the staining is very poor and the claims made are not well supported.

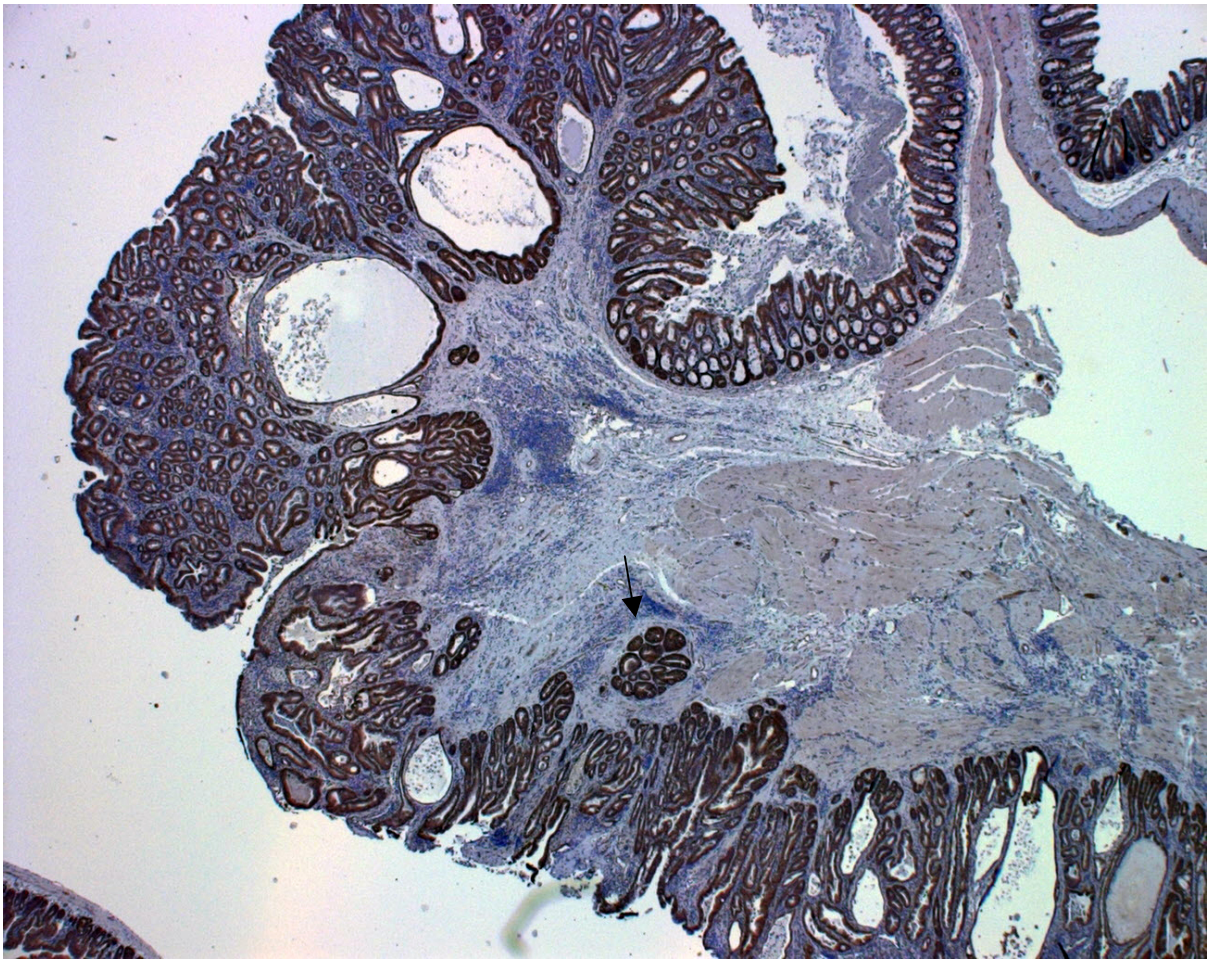
As we discuss in our revised manuscript the fact that Nrbp1 is associated with several key pathways only highlights is importance. The similarity between the expression signatures observed with APC or Nrbp1 lead us to focus on the WNT pathway. We do, however, describe how Nrbp1 affects the Notch pathway as well.

4) Figure 4 - The studies suggest some role for NRBP1 in regulating Sall4 levels, but don't really dig deep enough to establish that NRBP1 has a key function in regulating Sall4 protein ubiquitination and half-life. Also, given the uncertainties about how Sall4 may be a target gene of Wnt signaling versus a key factor in Wnt target activation (Bohm et al. BBRC 2006; Shuai et al. Cancer Genet Cytogenet 2009), the authors' studies offer minimal data on the outstanding issues. Studies of endogenous Wnt target genes - Lgr5, Dkk1, Nkd1, Axin2 - would have been preferable, along with some clues about how Sall4 may be acting on Wnt signaling.

The referee is completely right that we don't know how Sall4 and Wnt are linked to Nrbp1 beyond the extensive mass spec and expression analysis we performed. Some people have suggested that Wnt regulates expression from the Sall4 promoter (PMID 16899215) while other studies have suggested Sall4 overexpression activates Wnt (19781444). Thus the picture is extremely complex and we would argue that given that we cover so much ground in our paper beyond the scope of this manuscript.

5) Figure 5 - The photos in the panel are of poor quality and the claims lack strong support.
- Panel C - not clear that the colorectal tumor was invasive and the photo in panel iii is useless as currently presented.

The photos in Figure 5C illustrate an overview of the tumours that were found in the Nrbp mice. All the tumours were analysed in detail by a consultant pathologist. We have added an arrow pointing to the invasive part of this carcinoma. Below we provide a picture of one of the invasive carcinomas for this referee. Stained with beta catenin shows the epithelial layer invading the underlying muscle layer (arrow).



Invasive carcinoma stained with b-catenin. Arrow indicates tumour cells that have invaded through into the muscle layer.

- Panel D panels - Without comparison to normal tissues, not clear how to interpret the IHC data - is panel vi really the beta-catenin staining?

We have added images for the normal control samples to the figure so the reader can make the comparison suggested by the referee (all samples were processed for IHC in parallel). We have also corrected the figure legend.

Referee #2:

In this ambitious and elegant manuscript Wilson and colleagues identify NRBP1 from a genetic screen of ras interactors in C elegans then genetically delete NRBP1 in mice which imposes a crypt progenitor phenotype and acts as a tumour suppressor. Finally they show NRBP1 is downregulated in human cancer where low expression correlates with a poor prognosis. Mechanistically NRBP1 appears to be part of the Cul5 E3 ligase complex. This complex degrades a number of proteins such as SALL4, which has been shown to negatively regulate wnt signalling. Importantly in intestinal extracts SALL4 is upregulated and there is deregulated Wnt signalling. Overall I think this is an important study, given the authors cover so much ground it is always easy to find minor faults throughout such a big study but these are trivial and don't detract from what is an excellent study. Specific comments.

1. I agree with the authors that the most likely reason for the crypt progenitor phenotype is through an increase in Wnt signalling. The overlap with the microarray following Apc loss is very consistent with this. It is important to note that is still unclear if the OCT4 overexpression phenotype the author cite is down to the overexpression of Wnt signalling. It is possible that the overexpression of OCT4 in the crypt would interact with Myc and SOX4 expression at the base of the crypt to provoke an intestinal progenitor phenotype which is characterised by increased Wnt signalling. I think a good way for the authors to tackle the dependence of Wnt signalling would be grow these crypts ex vivo and see if they form similar organoids to those following Apc loss ie they form spheres that do not have differentiation and do not bud (unlike Wild type organoids), they could then remove the spondin and see if they are still dependent on a Wnt ligand.

We have performed this experiment as suggested and the results are presented in Supplemental figure 7. We find that organoids from Nrbp1 mice remain responsive to Wnt as expected.

2. The above would also answer the question posed by the Lgr5CREER experiment where mice don't get cancer. As recombination is throughout the intestine :mesenchymal, epithelial, neuronal and smooth muscle in ROSACREER mice, it could be down to the concerted loss in all cells that mice develop tumours. Thus it will be of interest to see if the crypt progenitor phenotype is remains in culture. I think the most likely explanation for the Lgr5 CREER result is that this is a very poorly penetrant cre recombinase and thus if there some selection against the NRBP1 knockout cells would be lost. The data from the RosaCREER would suggest this as a significant percentage of these mice show reconstitution form non recombined cells. This is quite an important point that the authors should highlight in the manuscript as it basically makes it unlikely that NRBP1 is an initiating event in CRC, instead it's a co-operator.

These points have been discussed in the revised manuscript. Our data agrees with the referees' suggestion that the lack of tumours in Lgr5 CREER/Nrbp1 mice is likely to be due to poorly penetrance Cre recombination. We did, however, see key features of the intestinal phenotype observed with ROSACREER such as Paneth cell mis-localisation.

3. Do the authors see any tumourigenesis/LOH in the NRBP1 +/- mice.

A number of $Nrbp^{+/-}$ and $Nrbp^{ko/c}$ mice which were not treated with tamoxifen have been left on the shelf thought-out the time of this experiment and we have never seen any decreased survival, however a large cohort has not been tested experimentally.

4. Whats the level or recombination in the ROSACREER mice given a single injection of tamoxifen?

Analysis of the ROSA26-CreERT2 mouse we used has been performed previously revealing near complete recombination of LoxP alleles in most tissues with a single dose of tamoxifen.

Hameyer D, Loonstra A, Eshkind L, Schmitt S, Antunes C, Groen A, Bindels E, **Jonkers** J, Krimpenfort P, Meuwissen R, Rijswijk L, Bex A, **Berns** A, Bockamp E. Toxicity of ligand-dependent Cre

recombinases and generation of a conditional Cre deleter mouse allowing mosaic recombination in peripheral tissues. Physiol Genomics. **2007** Sep 19;31(1):32-41.

5. The human lung data is very impressive as is the fact the NRBP1 loss leads to lung carcinoma in the mouse. Given the authors have staining for SALL4 working it would be good to see if there is any correlation with NRBP1 within the lung tumours. Here this would be a nice paradigm as increased wnt signalling has been shown in a number of tumours to confer a poor prognosis and drive tumour progression.

Unfortunately, staining for Sall4 in the few remaining sections of the lung tumours failed to show Sall4 positivity.

Also it would be interesting to see if TSC22 was upregulated in lung. It's been recently shown by the Peeper in EMBO that upregulation of the long isoform can overcome oncogene induced senescence and in mice probably the best example of OIS is lung cancer (the McMahon groups work on BRAF) outwith melanoma.

We have tried IHC with the Tsc22D2 and Tsc22D4, but unfortunately not been successful getting it to work, perhaps due to low specificity of antibodies and the high conservation between the isoforms. Thank you for pointing out this interesting paper, we have added this point into the discussion of the paper, and hope to follow this avenue of investigation further.

All of these extra experiments i think would be beneficial for the manuscript and should not take too long. The results would be interesting either way.

Referee #3:

In the manuscript titled 'Nuclear receptor binding protein 1 (NRBP1) regulates intestinal progenitor cell homeostasis and tumour formation', Wilson et al. identified NRBP1 as a tumour suppressor. They elegantly carried out a C. elegans RNAi screening and validated the target gene using a mouse model. The NRBP1 knock out mouse shows an interesting phenotype in the intestine. The in vivo data are convincing but the biochemical mechanism of action of NRBP1 is not clearly demonstrated. Following points outline the major concerns in the manuscript.

1. The biochemical characterization of NRBP1 is not very clear. The connection between NRBP1, ElonginBC and CUL5 is not supported by clear data. First of all, the interaction between NRBP1 and Elongin B/C, as observed by IP/Mass spec, should be validated, as has been done for SALL4.

This experiment is now shown in figure 4B

2. CUL5 did interact with NRBP1 only when all other components of the hypothesized complex have been overexpressed. Authors need to show that NRBP1-Elonging B/C-Cul5 complex is forming. Does

the interaction between NRBP1 and CUL5 get reduced if ElonginB/C is knocked down? Is it possible to overexpress one of the components and IP the endogenous protein?

Figure 4B has been updated to include this data

3. The stabilization of SALL4 and Tsc22d2 in NRBP1 cKO mice suggests that NRBP1 regulates the abundance of these proteins but this data in no way demonstrates that an ubiquitin ligase activity is involved. It is absolutely necessary to demonstrate the ubiquitination of the substrates with either overexpressing or knocking down the ligase. This experiment can be done very easily in HCT cells. Moreover, in vitro experiments using purified components are required to convincingly demonstrate a substrate-E3 relationship.

Using 293T cells we have over expressed SALL4 and TSC22D4, together with either, NRBP1 or NRBP1 shRNAs (+MG132), after IP for either SALL4 or TSC22D4, analysis of ubiquitin levels in these samples provided no evidence of ubiquitination of the potential substrates. Given more time and analysis we would hope to be able to investigate more fully the regulation of these proteins by NRBP1. We have included into the text that this relationship has yet to be confirmed.

5. Additionally the quality of the data in figure 4 needs to be improved. For example, in fig4C, authors claim that in cKO mice Sal4A and B are stabilized but in the figure there is no band for Sal4A.

We have altered figure 4B and have updated the text to be more specific to which Sall4 isoform is stabilised. Importantly although we show binding of Sall4A and Sall4B to Nrbp1 in the intestine we could only detect Sall4B.

Minor points:
1. Specific deletion of NRBP1 in the intestinal stem cells produced similar effect as cKO mice but to a lesser extent. Authors showed data for staining with anti-lysozyme and alcian blue (fig 2J). The difference is not obvious from the figure. It would have been better if authors could provide quantification of the stainings.

The Lgr5 CREER line is a very poorly penetrant cre driver, therefore it is impossible, without crossing the other strains to a Cre reporter strain (to make a quadruple mutant mouse line) to accurately map where recombination has occurred.

2. The manuscript includes a lot of discussion about NRBP1 ubiquitin ligase activity and how this complex might be regulating WNT pathway and intestinal progenitor cell homeostasis. But in absence of conclusive evidence of the ubiquitin ligase activity the whole discussion part is still a speculation and hypothesis.

We have reiterated in the discussion that the ubiquitin ligase activity of Nrpb1 is a hypothesis.

3. 'Materials and Methods' section states that IP/Mass spec experiment was carried out using

tandem affinity purification but the from the description it looks like the method is a single step affinity purification, not tandem.

We performed both tandem and single step purification; we have made this clearer within the results and methods sections.

Additional Correspondence 28 February 2012

Apologies for the delay in getting back to you with a final decision! Given the rather significant revisions, I decided to involve one of the original referees that needed some time before returning comments.

As you will see, this scientist appreciates major improvements of the study but questions the validity of the actual molecular mechanism proposed. Despite physical interaction with E3-ligase complex components, no definitive evidence for ubiquitination of any of the proposed substrates has been revealed. I would thus feel more comfortable if this would be expressed in the paper. Given the huge amount of truly novel and original results however, I am happy to move forward with the study.

Please provide me with an appropriately modified text file to your earliest convenience to enable efficient proceedings.

Please also notice, that The EMBO Journal encourages the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. This is a voluntary policy to present un-cropped/unprocessed scans for the key data of published work. If you agree to this initiative, we would be grateful for one file per figure that combines this information. These will be linked online as supplementary "Source Data" files.

Please let me know if you have any questions about this AND check the URL below for a recent example:
http://www.nature.com/emboj/journal/v30/n20/suppinfo/emboj2011298as1.html

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORT

This is a nice and elegant study. The authors have done a significant amount of work and the revised ms is clearly improved in many ways.

However, there is one big problem I have with this paper:

the authors suggest that NRBP1 function by forming a E3 ubiquitin ligase, thereby controlling the stability of substrates such as Sall4. There is really no evidence whatsoever that corroborates this notion. Figure 4 is by far the weakest Figure of this ms. On the contrary, the authors state in the rebuttal letter that they have been unable to show any ubiquitylation activity associated with NRBP1.

I am uncertain whether it is necessary for the authors to show in this paper exactly how NRBP1 functions, but I would suggest to be very cautious in the interpretation.
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