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Wnt-Induced Deubiquitination FoxM1 Abrogates the Inhibition of ICAT to β-catenin transactivation

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(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 16 September 2015

Thank you for the submission of your manuscript entitled "Wnt-Induced Deubiquitination FoxM1 Abrogates the Inhibition of ICAT to β-catenin transactivation" and for your patience during the review process. We have now received the reports from the two referees that accepted the invitation to review your study, which I copy below.

As you can see from their comments, both referees are rather supportive of your manuscript and recommend publication provided that certain concerns are dealt with. I believe the referee concerns are rather clear and straightforward, so I will not repeat them here, but in any case please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems.

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

REFEREE REPORTS

Referee #1:

In this ms Chen et al analyse the Wnt-dependent stabilisation of FoxM1 and its role in the activation of beta-catenin target genes. The authors build on previous results from their group regarding the role of FoxM1 as a positive regulator of Wnt signalling, and provide evidence that i) FoxM1 is a novel post-transcriptional target of Wnt signalling, ii) FoxM1 is stabilised and deubiquitinated by Wnt signalling and USP5, thereby upregulating beta-catenin target genes, iii) mechanistically,

FoxM1 protects b-catenin from ICAT-dependent inhibition, iv) USP5 contributes to FoxM1-driven proliferation in glioma cells.

Overall this is a solid paper with high quality data that dissects a novel axis in Wnt signalling. The ms is well written and the conclusions supported by the data.

This referee only has few suggestions to improve the ms:
1) The roles of Wnt signalling stabilising other GSK3 targets besides beta-catenin have been explored previously (Kim et al., 2009, Taelman et al., 2010, Acebron et al., 2014, Stolz., et al 2015). FoxM1 seems to be part of this post-transcriptional Wnt signalling (also termed Wnt/STOP). Thus, the authors should consider discussing their data in that context.

2) The authors wrote: "a E3 ubiquitin ligase that regulates FoxM1 has been reported (Laoukili et al, 2008; Park et al, 2008)". It will helpful for the reader to indicate that those papers refer to APC/C-Cdh1.

3) The authors identified the motif containing S474 as the critical GSK3 degron in FoxM1. This motif is independent of the KEN- and D-boxes of the N-term of the protein that are modulated by APC/C-Cdh1. Interestingly, this motif LWEWPS(474)PAPS is close to a consensus degron for FBXW7: ΩxΩΩΩ(S/T)Pxx(S/T/E) [Ω=hydrophobic]; but not for beta-TrCP, which regulates beta-catenin in the Axin complex. The stabilisation of GSK3/FBXW7 targets by Wnt signalling has been reported before (Acebron et al, 2014), as well as their dependency on the Axin/APC complex (e.g. Stolz et al 2015). Identifying the E3 ligase involved in the GSK3-dependent degradation of FoxM1 will strengthen the message of the ms. However, this referee understands that analyzing all possible E3 ligases is clearly beyond the scope of the ms. But, since FBXW7 seems the most likely possibility, the authors could perform an experiment, similar as shown in Figure 3E and 4G, where FoxM1-Ub can be analysed upon GOF or LOF of FBXW7.

4) The authors did not analyse or comment on the type of ubiquitin chains in FoxM1, which are most likely K48-linked ubiquitin chains. If the authors have access to K48-Ub specific antibodies they should re-blot some of their blots and check it; alternatively the authors should discuss this possibility, as some deubiquitinases often favour a particular type of ubiquitin chain.

5) The title is quite long and it may be difficult to understand for the non-specialised reader.

Referee #2:

In this interesting manuscript Chen and colleagues follow up on their Cancer Cell paper from 2011. In this manuscript they show foxm1 is normally degraded in a gsk3/axin dependent mechanism. This can be suppressed by wnt signalling activation. Usp5 can also increase stability by reducing the ubiquitination and subsequent degradation of foxm1. Mechanistically the authors suggest that this increased level of foxm1 then competes ICAT for b-catenin binding which then drives increased expression of wnt target genes. To confirm findings in vivo the authors then show correlation between foxm1, cyclin d1 and then usp5 in human glioma sections (from only 30 patients). Overall there is a lot of potentially interesting findings in this paper but I think more work is needed in the general applicability of their findings and extra details/controls are needed for the experiments done so far.

Comments

1) Throughout the manuscript, a lot of data is presented in line graph form and it's hard to get a feel for the significance of the data. For example in figure 1 there is a mean from 2 independent experiments and then in figure 6 and 7 a mean of 3 experiments (though it's not clear to mean is there are technical or biological replicates).

2) Often a single siRNA is used and only 1 rescue experiments is performed throughout the paper. Have the authors made crispr lines? Extra siRNAs should be used.

3) The authors suggest this is a general phenotype but focus predominantly on glioma. The model proposed suggest icat and foxm1 interactions should be conserved. Given the authors have antibodies they should look in tissues like the intestine when one might expect foxm1 to be at the base of the crypt. Whilst in islets which have been suggested to have high levels of icat should have low levels of foxm1 (low wnt signalling). The authors should cite the paper by Strom et al development 2007, showing that apc loss cannot transform islets due to high icat.

4) Extra validation of the icat antibody would be helpful.

5) In figure 5 the levels of endogenous Icat looks low in these lines it would be good to repeat in another set of cell lines

6) Some note on westerns blots intensities are required. In figure 1 a v 1c basal levels of foxm1 and b-catenin are different

7) Why was the qt-pcr performed at 8 hours (supplemental figure 1) when westerns done at 6 hours?

8) Given the model, could foxm1 interact with b-catenin in the same destruction complex. It's binds axin and gsk3?

9) Extra wnt targets than cyclin D1 should be examined on human tissue. Many pathways control cyclin d1. The authors could use rnascope technology for axin2

1st Revision - authors' response 09 December 2015

General Responses

We thank the reviewers for their thorough evaluation of our original manuscript and constructive comments and suggestions, which would greatly help us to improve our study. Therefore, we have revised our manuscript accordingly, including new experimental results, clarifications and statements. We strongly believe that we have adequately addressed all the points raised by and to the satisfaction of the reviewers. Above all, our important conclusion is supported by robust data of high-quality.

To facilitate the assessment of our revision, we have provided point-by-point responses herein. Also, we have incorporated the responses into the revised manuscript and updated the figure numbering in the revised submission to reflect the changes we made in response to the reviewers' comments.

Responses to Reviewer #1

"In this ms Chen et al analyse the Wnt-dependent stabilisation of FoxM1 and its role in the activation of beta-catenin target genes. The authors build on previous results from their group regarding the role of FoxM1 as a positive regulator of Wnt signalling, and provide evidence that i) FoxM1 is a novel post-transcriptional target of Wnt signalling, ii) FoxM1 is stabilised and deubiquitinated by Wnt signalling and USP5, thereby upregulating beta-catenin target genes, iii) mechanistically, FoxM1 protects b-catenin from ICAT-dependent inhibition, iv) USP5 contributes to FoxM1-driven proliferation in glioma cells.
Overall this is a solid paper with high quality data that dissects a novel axis in Wnt signalling. The ms is well written and the conclusions supported by the data."

Response: We thank the reviewer for acknowledging the importance and strength of our work.

"This referee only has few suggestions to improve the ms:
1) The roles of Wnt signalling stabilising other GSK3 targets besides beta-catenin have been explored previously (Kim et al., 2009, Taelman et al., 2010, Acebron et al., 2014, Stolz., et al 2015). FoxM1 seems to be part of this post-transcriptional Wnt signalling (also termed Wnt/STOP). Thus, the authors should consider discussing their data in that context."

Response: We thank the reviewer for this important suggestion. We have now cited the above papers in the reference. We also added the discussion about the concept of Wnt/STOP in reference of previous publications including Acebron et al., 2014 and Koch et al, 2015. We agree with the reviewer that FoxM1 is part of Wnt/STOP, and then discussed our data in context of Wnt/STOP concept (in page 9 of the revised manuscript).

"2) The authors wrote: "a E3 ubiquitin ligase that regulates FoxM1 has been reported (Laoukili et al, 2008; Park et al, 2008)". It will helpful for the reader to indicate that those papers refer to APC/C-Cdh1."

Response: Following the reviewer's suggestion, we have specifically indicated APC/C-Cdh1 by modifying the above sentence to "an E3 ubiquitin ligase, APC/C-Cdh1, that regulates FoxM1 has been reported (Laoukili et al, 2008; Park et al, 2008)".

"3) The authors identified the motif containing S474 as the critical GSK3 degron in FoxM1. This motif is independent of the KEN- and D-boxes of the N-term of the protein that are modulated by APC/C-Cdh1. Interestingly, this motif LWEWPS(474)PAPS is close to a consensus degron for FBXW7: ΩxΩΩΩ(S/T)Pxx(S/T/E) [Ω=hydrophobic]; but not for beta-TrCP, which regulates beta-catenin in the Axin complex. The stabilisation of GSK3/FBXW7 targets by Wnt signalling has been reported before (Acebron et al, 2014), as well as their dependency on the Axin/APC complex (e.g. Stolz et al 2015). Identifying the E3 ligase involved in the GSK3-dependent degradation of FoxM1 will strengthen the message of the ms. However, this referee understands that analyzing all possible E3 ligases is clearly beyond the scope of the ms. But, since FBXW7 seems the most likely possibility, the authors could perform an experiment, similar as shown in Figure 3E and 4G, where FoxM1-Ub can be analysed upon GOF or LOF of FBXW7."

Response: We thank the reviewer for this important suggestion. Following this suggestion, we have first tested whether FoxM1 interacts with FBXW7. We found that FBXW7 interacts with wild type FoxM1 but not with S474A mutant (Figure 3F), suggesting the motif LWEWPS(474)PAPS is a degron for FBXW7. Moreover, overexpression of FBXW7 (*i.e.* GOF) induced FoxM1 ubiquitination as compared with control (Figure 3G, lane 4 vs lane 2). Next, since S474 is phosphorylated by GSK3, we determined whether FBXW7-mediated FoxM1 ubiquitination is regulated by GSK3 activation, by using GSK3β CA construct. We found that FBXW7 induced FoxM1 ubiquitination was increased by GSK3β CA transfection (Figure 3G), suggesting that GSK3 activity enhances FBXW7 mediated FoxM1 ubiquitination. Furthermore, we found that knockdown of FBXW7 by siRNA (*i.e.* LOF) inhibited FoxM1 ubiquitination as compared with control siRNA (Figure 3H). More importantly, knockdown of FBXW7 reversed the inhibitory effect of Wnt treatment on FoxM1 ubiquitination (Figure 3H). Collectively, these results suggest that FBXW7 mediated FoxM1 ubiquitination is regulated by Wnt signaling, and that Wnt induced FoxM1 stabilization via a Wnt/STOP mechanism.

"4) The authors did not analyse or comment on the type of ubiquitin chains in FoxM1, which are most likely K48-linked ubiquitin chains. If the authors have access to K48-Ub specific antibodies they should re-blot some of their blots and check it; alternatively the authors should discuss this possibility, as some deubiquitinases often favour a particular type of ubiquitin chain."

Response: Following the reviewer's suggestion, we analyzed the type of ubiquitin chains for FoxM1 ubiqutination by using HA-tagged K48-only or K63-only ubiquitin constructs. We found that only poly-ubiquitin-K48 in FoxM1 was detected by HA antibody (Figure 2F), suggesting that FoxM1-ubquitin chains are K48-linked polyubiquitin chains.

"5) The title is quite long and it may be difficult to understand for the non-specialised reader."

Response: Following the request, we have modified the title to: "Wnt-Induced Deubiquitination of FoxM1 ensures nucleus β-catenin transactivation."

Responses to Reviewer #2

"In this interesting manuscript Chen and colleagues follow up on their Cancer Cell paper from 2011. In this manuscript they show foxm1 is normally degraded in a gsk3/axin dependent mechanism. This can be suppressed by wnt signalling activation. Usp5 can also increase stability by reducing the ubiquitination and subsequent degradation of foxm1. Mechanistically the authors suggest that this increased level of foxm1 then competes ICAT for b-catenin binding which then drives increased expression of wnt target genes. To confirm findings in vivo the authors then show correlation between foxm1, cyclin d1 and then usp5 in human glioma sections (from only 30 patients). Overall there is a lot of potentially interesting findings in this paper but I think more work is needed in the general applicability of their findings and extra details/controls are needed for the experiments done so far."

Response: We thank the reviewer for the positive comments and suggestions on our work. We have now increased the human GBM sample number to 50. The new data further confirm the correlations between FoxM1, cyclin D1, Axin2 and USP5 in human GBM tissues. Moreover, we have now performed the experiments suggested by the reviewer. The detailed results have been presented in the revised manuscript. Please see our responses to the reviewer's concerns have been presented in below.

"Comments
1) Throughout the manuscript, a lot of data is presented in line graph form and it's hard to get a feel for the significance of the data. For example in figure 1 there is a mean from 2 independent experiments and then in figure 6 and 7 a mean of 3 experiments (though it's not clear to mean is there are technical or biological replicates)."

Response: We thank the referee for the comments. We apologize for omitting the number of times of an experiment was done in those line graph experiments. We have now specified the number of times and replicates in the figure legend for all the data. We would like to confirm that for the experiments required for quantification and statistical analysis, at least 3 biological replicates per condition are included. For those gel-driven experiments, all experiments have been done in 3 independent biological replicates (3 independent experiments), except Figure 1B. The experiments in Figure 1B were repeated in two independent experiments since we have limited access to radioactive [35S] methionine. This has ensured that the data is reproducible.

"2) Often a single siRNA is used and only 1 rescue experiments is performed throughout the paper. Have the authors made crispr lines? Extra siRNAs should be used."

Response: All of the siRNAs except siFoxM1 used in this study are siRNA products from Santa Cruz Biotechnology, which generally consist of pools of three to five target-specific 19-25 nt siRNAs designed to knockdown gene expression. We apologize for omitting the details and resource of the siRNAs in the original manuscript. We have now added the detail description of these siRNAs in the section of "EXPERIMENTAL PROCEDURES".
The siFoxM1 was the one used in our previous *Cancer Cell* paper and other publications and has been proved to knock down FoxM1 specifically and effectively. Following the reviewer's suggestion, we have used another siRNA against FoxM1 (siFoxM1 #2) to perform key experiments. The results from the experiments showing in Figure 6B and Figure 6H demonstrated that siFoxM1 #2 has similar effect as the siFoxM1 (now designated as siFoxM1 #1 in the revised manuscript).

"3) The authors suggest this is a general phenotype but focus predominantly on glioma. The model proposed suggest icat and foxm1 interactions should be conserved. Given the authors have antibodies they should look in tissues like the intestine when one might expect foxm1 to be at the base of the crypt. Whilst in islets which have been suggested to have high levels of icat should have low levels of foxm1 (low wnt signalling). The authors should cite the paper by Strom et al development 2007, showing that apc loss cannot transform islets due to high icat."

Response: We thank the reviewer for this suggestion.
The expression of FoxM1 and ICAT in intestine have been reported in previous studies. Indeed, FoxM1 is expressed in proliferating epithelium at the base of the crypt (which has high level of Wnt activity), but not in the intestinal villus (Fig. 7 of Ye et al, MCB, 1997, 1626–1641). Moreover, a study of the role of FoxM1 in colon cancer development using conditional FoxM1 knock-out and FoxM1 overexpression transgenic mice has shown that FoxM1 affects β-catenin-TCF-4 signaling in colon cancers (Yoshida et al, GASTROENTEROLOGY, 2007). On the other hand, ICAT expression was detected in normal colorectal tissues, but found to be overexpressed in almost half of colorectal carcinomas (Koyama et al**,** Jpn J Clin Oncol 2002;32(9)358–362). Together, these studies suggested that FoxM1 expression is important β-catenin/TCF activation and colon cancer formation. We have now described these findings from the above studies in the Discussion of our revised manuscript.

We have examined the FoxM1 expression in human normal pancreas tissues and pancreatic cancers and found the level of FoxM1 is lower in normal pancreas tissues (including islets) than in pancreatic cancers (please see the representative IHC below (*figure shown to referees but removed from this review file)*). The results are consistent with the data from The UCSC Genome Browser Database (2008 update), which shows that FoxM1 level is very low in pancreatic islets. However, we have not included these data in the revised manuscript because the conclusions regarding roles of FoxM1 as well as ICAT in transformation of pancreas (including islet) need more comprehensive analyses which are outside of the scope of our current study. Nevertheless, we cited and discussed the findings of the paper by Strom et al., Development 2007 in the revised manuscript.

"4) Extra validation of the icat antibody would be helpful."

Response: We thank the reviewer for this suggestion. In response to this question and the question #5, we have now used another ICAT antibody (from abcam) and repeated several key experiments. We have now validated this new ICAT antibody by using the knockdown and overexpression ICAT approach. Our new data (Figure 5D and Figure 5G in the revised manuscript) are consistent with the data from using the old ICAT antibody (from Santa Cruz).

"5) In figure 5 the levels of endogenous Icat looks low in these lines it would be good to repeat in another set of cell lines"

Response: We agree with the reviewer for this observation; thus, we have reviewed our experimental procedure. We found that it was caused by short exposure time on these western blots. The levels of endogenous Icat in these cell lines are indeed not low. Therefore, we did not repeat the experiments in another set of cell lines. Instead, we have repeated the experiments using new ICAT antibody with better exposure time in western blotting. These new better quality figures further confirms that the levels of endogenous Icat in these cell lines are not low (Figure 5D and Figure 5G in the revised manuscript).

"6) Some note on westerns blots intensities are required. In figure 1 a v 1c basal levels of foxm1 and b-catenin are different"

Response: Thanks for this suggestion. We have now added a note in the figure legend of Figure 1 "noted that the basal levels of FoxM11 and b-catenin in A and C are similar but look different due to variations on the exposure time of the Western blots."

"7) Why was the qt-pcr performed at 8 hours (supplemental figure 1) when westerns done at 6 hours?"

Response: We have repeated the experiment by performing the qt-PCR at 6 hours. The result has shown that Wnt treatment did not change the FoxM1 mRNA level at 6 hours (supplemental figure 1 in the revised manuscript).

"8) Given the model, could foxm1 interact with b-catenin in the same destruction complex. It's binds axin and gsk3?"

Response: Although Axin and GSK3 form a phosphorylation complex which can bind both FoxM1 and beta-catenin, but the E3 ligase in the beta-catenin destruction complex is beta-TrCP, while the E3 ligase in the FoxM1 destruction complex is FBXW7. Therefore, FoxM1 does not interact with beta-catenin in the same destruction complex.

"9) Extra wnt targets than cyclin D1 should be examined on human tissue. Many pathways control cyclin d1. The authors could use rnascope technology for axin2."

Response: Following the instruction of the reviewer, we have analyzed the RNA expression levels of axin2 by using rnascope technology in 50 human GBM samples (please see Methods for detailed experiment procedure). Our new data from rnascope and IHC analyses (Figure 7I, Figure 7J and Figure S4 in the revised manuscript) showed that the Axin2 mRNA levels in the GBM samples are directly correlates with the protein levels of FoxM1 and USP5. Moreover, statistical analyses of the correlations revealed that they are significant (Figure 7I and Figure 7J in the revised manuscript).

2nd Editorial Decision 13 January 2016

Thank you for the submission of your revised manuscript to The EMBO Journal. As you will see below, your article was sent back to the original referees, who now consider that you have properly dealt with the main concerns originally raised in the review process, and therefore I am writing with an 'accept in principle' decision. This means that I will be happy to formally accept your manuscript for publication once a minor issue has been addressed.

Browsing through the manuscript I have noticed a cosmetic issue that will need to be addressed in the final version of the paper. As you correctly noticed, supplementary information is now called "Appendix". This means that figure names must be changed to "Appendix S1", "Appendix S2", etc. Tables follow a similar pattern ("Appendix table 1", etc.). Please make sure that in-text call-outs to the figures are properly corrected. No changes are needed in the "Appendix" file itself, just the reference to the figures should reflect that they are contained in the Appendix.

Thank you very much for your patience. I am looking forward to seeing the final version of your manuscript. Congratulations in advance for a successful publication.

Referee #1:

My general evaluation is similar as in the first submission: this is a solid paper with high quality data that dissects a novel axis in Wnt signalling. The ms is well written and the conclusions supported by the data. The authors have addressed all the issues rose by this referee and provided important data on the E3 ligase involved in FoXM1 degradation. In this referee's opinion the ms should be accepted in EMBO Journal.

Referee #2:

The authors have responded to all my points. I believe they have dealt with them all adaquately and they have significantly strengthened this submission.

I therefore think this is a very interesting submission that will be of relevance to people in the Wnt signalling and Glioma fields and is now ready for publication,

