EMBO reports

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Deconstruction of O-glycosylation - GalNAc-T Isoforms Direct Distinct Subsets of the O-Glycoproteome

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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 03 July 2015

Thank you for your patience while your study was peer-reviewed at EMBO reports. we have now received reports from the two referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, both referees are supportive of the study, although they raise several issues that can be addressed with further discussion/clarification, rewriting or inclusion of some data apparently not shown (ref 2 point 8).

Given these positive evaluations, we are happy to invite revision of your study, which should address all referee concerns. If the referee concerns can be adequately addressed, we would be happy to accept your manuscript for publication.

Revised manuscripts must be submitted within three months of a request for revision unless previously discussed with the editor; they will otherwise be treated as new submissions. You study will be published in report format, which means that the Results and Discussion section will need to be merged into one, and the text overall should be shortened to as close to 40,000 characters (including spaces and references) as possible.

Please note that it is a precondition for publication in EMBO reports that authors make all data no thosted within the paper freely available, where possible in an appropriate public database (in this case the transcriptomic and proteomic datasets). The accession code(s) should be specified in the main text in the first instance where the data are mentioned, and be included in the Methods section under a "Data availability" subheading.

In addition, I have noticed that there is no information on the number of independent experiments, what the bars represent (mean, median) and the type of errors calculated (SE, SD,..) in the legends to figures 1A and 4. Please include this in your revised study.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision)
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
- a separate PDF file of any Supplementary information (in its final format)

EMBO reports now accommodates the inclusion of extra figures (up to five) in the online version of the manuscript. These are presented in an expandable format inline in the main text so that readers who are interested can access them directly as they read the article. They are also provided for download in a separate typeset PDF to accompany the Article PDF. These should be those of particular value to specialist readers, but which are not required to follow the main thread of the paper (and not additional controls or reagent optimization). These should be labeled expanded view, and the rest supplementary.

We also encourage the publication of original source data -particularly for electrophoretic gels and blots, but also for graphs- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please find the edited versions below my signature and let me know if you do NOT agree with any of the changes.

Every EMBO reports paper now includes a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version and they are freely accessible to all readers. The synopsis includes a short standfirst text -I have added my proposal for this text below- as well as 2-4 one sentence bullet points that summarize the paper. These should be complementary to the abstract -i.e. not repeat the same text. This is a good place to be more informative and include, as appropriate, key acronyms and quantitative and organism (yeast, mammalian cells, etc) information. Could you supply a 550 pixels wide by 150-400 pixels high simple graphic outlining the main message of the study? Please ensure that any labeling is readable at this size. We would also need you to provide the bullet points to accompany the standfirst? Do let me know if you would like to modify the standfirst blurb:

"This study shows that, contrary to current assumptions, individual GalNAc-Ts modify distinct subsets of substrates. Such specific regulation of the cellular O-glycoprotome likely impacts many pathways and processes.

2-3 bullet points"

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, please contact me if I can be of any assistance.

Edited title and abstract

GalNAc-T isoforms modify distinct subsets of the O-glycoproteome
GalNAc-type O-glycosylation is found on most proteins trafficking through the secretory pathway in metazoan cells. The O-glycoproteome is regulated by up to 20 polypeptide GalNAc-transferase isoforms (GalNAc-Ts) and the contributions and biological functions of individual GalNAc-Ts are poorly understood. Here, we used a zinc-finger nuclease (ZFN)-directed knockout strategy to probe the contributions of the major GalNAc-Ts (GalNAc-T1 and T2) in liver cells, and explore how the GalNAc-T repertoire quantitatively affects the O-glycoproteome. We demonstrate that the majority of the O-glycoproteome is covered by redundancy, whereas distinct subsets of substrates are modified by non-redundant functions of GalNAc-T1 and T2. The non-redundant O-glycoproteome subsets are related to different cellular processes, and they support the predicted role of GalNAc-T2 in lipid metabolism. These results suggest that GalNAc-Ts have non-redundant glycosylation functions and their contributions to the O-glycoproteome may affect distinct cellular processes, as well as providing a comprehensive resource of individual GalNAc-T substrates. Our study provides a new view on the regulation of the O-glycoproteome, suggesting that the plurality of GalNAc-Ts arose to regulate distinct protein functions and cellular processes.

REFEREE REPORTS

Referee #1:

Summary:

In this manuscript, Schjoldager et al. identify >1000 quantifiable, unambiguously assigned O-glycosites. Using a zinc finger nuclease strategy, the group endeavored to determine the contributions that individual GalNAc-Ts play in O-glycosylation. Specifically, they were interested in determining whether GalNAc-T1 and GalNAc-T2, the most highly expressed GalNAc-Ts in the liver, were fully functionally redundant or held non-redundant glycosylation functions. Indeed, the authors determined that T1 appears to be selectively involved in glycosylation of basement membrane/extracellular matrix organization while T2 plays a role in glycosylation of proteins involved in lipid or cholesterol metabolism.

Significance:

Expanding our knowledge of the specificity GalNAc-Ts play in glycosylation will help researchers to dissect the implications of deregulated GalNAc-Ts in human disease. These studies set the foundation for researchers to interrogate and confirm candidate sites followed by validating promising candidates for the role(s) glycosylation plays in associated diseases.

Manuscript Discussion & Experimental Suggestions:

Overall, the manuscript is well thought out and underscores that while GalNAc-Ts are largely thought of as redundant, GalNAc-Ts play roles that are non-redundant and their expression in a subset of cells is likely to have an important impact on the glycosylation patters within that cell.

(1) Generally, the manuscript was well outlined. However, in several cases, there is redundancy in explanation and the reader would be better served to use the text space for new information rather than repetitive commentary. In addition, there are a number of places where the authors need to proofread for errors:
a. P4 - don't introduce Fig 1B in the first paragraph, it really doesn't need to come into play until the second - unless you are going to list it as 1A.
b. TCL and SEC are defined twice.
c. Titles are not uniformly capitalized.
d. P18, figure legend 1: check periods at the end of sentences.
e. Figure 1: delta T3 should be +T3
(2) The authors allude to the act that GalNAc-Ts work in cooperation. By knocking one out, is it possible that some of the changes in glycosylation are due to changes in specificity because the cooperative effect is not available?

(3) T3 is known to be upregulated in some cancers, so despite the fact that on p8 the authors note that there are few genes that are deregulated, they should address whether those genes are important for cancer growth/metastasis.
(4) Figure 2
a. Are the authors surprised that with the 12 simple cells there are so many proteins that do not overlap with the HepG2 cells?
b. It is not clear what portion of the circle the number 3 associates with - and if it is to be quantitative, this is not representing the right "area".
c. In B, do the circles correspond with unambiguous or total values or is it just a representation?
d. C - are there any proteins with the number of sites 6-8? If not, those numbers can be left off. If so, #sites is hard to determine for 4-8.
(5) Figure 4
a. Although the authors state in the text that there are not changes in the RPKM, it appears that there are changes that would be significant for both GALNT1 and GALNT2. However, the authors on p8 are likely trying to say that there is no evidence of upregulation of transcript levels of the other GALNT family members. Furthermore, it is not clear in the text whether other known GlycoTs were examined in the same genetic background (which is implied) or in a different context in Hansen et al 2015.
b. In the legend, the authors state that for GALNT13, transcript levels in the deltaT2 clones changed compared to the WT. However, looking at the graph, it appears that the +T3 sample is the only one that changed. Can this graph can be better interpreted/represented so that the statistically significant change(s) are easy to see?

In summary, the manuscript is a well designed analysis of the combinatorial nature of the O-Glycome. There is much to like about the manuscript with the caveats listed above. I would suggest that the authors slightly amend their discussion to reflect the many assumptions that go into such a global analysis.

Referee #2:

Our current understanding of the contributions and biological functions of individual GalNAc-Ts is limited. In order to gain further insight to this question, the authors performed an extensive quantitative O-Glycoproteome analysis in liver HepG2 cells. They demonstrate that the majority of the O-glycoproteome is covered by redundancy while distinct subsets are covered by non-redundant functions of GalNAc-T1 and T2. The distinct non-redundant O-glycoproteome subsets appear to be part of different cellular processes, and they present data supporting a predicted role of GalNAc-T2 in lipid metabolism. The results suggest that individual GalNAc-Ts serve limited non-redundant glycosylation functions, and that their contributions to the O-glycoproteome may affect distinct cellular processes. The authors conclude that the scenario provides a new view on the regulation of the O-glycoproteome, suggesting that the plurality of GalNAc-Ts arose to regulate distinct protein functions and cellular processes.

This group has published several papers about O-glycoproteome by taking advantage of their "simple cell" system in which a gene encoding a chaperone of the core 1 enzyme is knocked out. Here they significantly expand their prior studies by identifying many more O-glycosylation sites using quantitative proteomic methods. Their conclusions about the non-redundant functions of individual GalNAc-T1, 2, and 3 in this paper are novel.

Although this manuscript contains an enormous amount of high quality data with significant conclusions, some minor revisions need to be made:

1. The authors performed an interesting transcriptome analysis not mentioned in the abstract. They should revise the abstract to include the finding from the transcriptome analysis.

2. Figure 4: It is not clear why transcript levels of GALNT1 and 2 are not reduced in the deltaT1 and deltaT2 cells, respectively?

3. A paper about a non-redundant role of a GalNAc-T (Tian E, 2015, PLoS ONE 10(1):e0115861) should be cited and commented on.

4. The fourth paragraph in "GalNAc-T differential O-glycoproteomes in HepG2Sc" in Results section contains some discussion that should be moved to the Discussion section.

5. Figure 2A: Not clear to what the "HepG2 (74)" shown at the bottom is referring.

6. In several figures/tables, the HepG2SC+T3 cells are labeled HepG2SCdeltaT3 (Figure 3C, EV1B).

7. Figure 3D: Not clear how the total number of sites for each cell (75, 81 and 131 for T1, T2 and T3, respectively) was obtained from the data shown in 3A, 3B, and 3C.

8. Figure EV4A and B: Only 11 peptides are shown in Table E3, but it appears many more peptides were assayed? Where is the data for the other peptides? Also, the numbers in the legend for number of peptides tested does not match that shown in the figure.

9. Table EV2:
a. Are the numbers here Log M/L? If so, please provide that information.
b. Some numbers have many more significant figures than others?
c. Why are numbers not found in each column for each peptide? Was the data for these peptides only of sufficient quality for quantitation in the cells where numbers are provided?
d. It would help to put headers for each column at the top of each page.
e. Not clear which column some numbers fall into, e.g. T777 from ADAM9?

1st Revision - authors' response 02 September 2015

Point-by-point response to reviewer comments:

Reviewer #1:
Query 1: P4 - don't introduce Fig 1B in the first paragraph, it really doesn't need to come into play until the second - unless you are going to list it as 1A.
Answer 1: Agree.
Action 1: We have postponed the introduction of Figure 1B to the second paragraph on page 4.

Query 2: TCL and SEC are defined twice.
Answer 2: Agree.
Action 2: Removed on page 5.

Query 3: Titles are not uniformly capitalized.
Answer 3: Agree.
Action 3: All capitalized now.

Query 4: P18, figure legend 1: check periods at the end of sentences.
Answer 4: Agree.
Action 4: Periods are added appropriately.

Query 5: Figure 1: delta T3 should be +T3
Answer 5: Agree.
Action 5: DT3 changed to +T3 in Figure 1 text.

Query 6: The authors allude to the act that GalNAc-Ts work in cooperation. By knocking one out, is it possible that some of the changes in glycosylation are due to changes in specificity because the cooperative effect is not available?
Answer 6: There is no solid evidence that GalNAc-Ts work cooperatively by interactions, but rather that prior glycosylation by one isoform affects the ability of another enzyme to glycosylate a particular substrate site because of the presence of a close proximal GalNAc residue. In our study here, we chose to only address the more isolated single-sites of O-glycosylation by selecting 1-Tn glycopeptides for this very reason, and thus believe we prevent introducing any bias from multiple

Tn peptides into the dataset. Moreover, we assure that protein and transcript level of the other GalNAc-Ts present in HepG2 is not subjected to major changes.
Action 6: To clarify further we have reworded a sentence on page 5 to reflect this as follows:

"In particular, interpretation of the contribution of individual GalNAc-Ts and their substrate specificities is ambiguous because GalNAc-Ts often function successively to cooperate in glycosylation of clustered glycosites through lectin-mediated interactions with partially glycosylated substrates or through direct recognition of a substrate site with and adjacent GalNAc glycosite [1]."

Query 7: T3 is known to be upregulated in some cancers, so despite the fact that on p8 the authors note that there are few genes that are deregulated, they should address whether those genes are important for cancer growth/metastasis.
Answer 7: Agree.
Action 7: Even though a limited number of transcripts are affected (14 up, 53 down) we addressed this question by inserting the following paragraph on page 9:

"Despite a limited number of transcripts being affected by *de novo* introduction of GalNAc-T3 (Fig. EV5 and Table EV6), it may be interesting to note that Matrix Metalloproteinase 14 (MMP14 also known as MT1-MMP) was down-regulated more than 50 fold by expression of GalNAc-T3 ($HepG2^{WT}$+T3). MMP14 promotes cellular migration and invasion *in vivo*, and a number of specific substrates have been reported [41]. Specifically, we identified a non-redundant glycosite (T263) in close proximity to the MMP14 cleavage site in Matrix Metalloproteinase 11 (MMP11 also known as Stromelysin-3 [42]) (^{253}VQHLêYGQPWPTVTSRT^{268}, cleavage site arrow and glycosite underlined). MMP11 is inactivated by the MMP14 processing in this site [42]. MMP11 is upregulated in cancer and suggested to play a role in invasive growth [43-45], and it is possible that the upregulation of GalNAc-T3 (and perhaps its paralog GalNAc-T6 with similar specificity [46] often found in cancer [47, 48] may help block MMP14 mediated inactivation of MMP11 and thus promote invasive growth".

Query 8: Re. Figure 2, are the authors surprised that with the 12 simple cells there are so many proteins that do not overlap with the HepG2 cells?
Answer 8: We are not surprised for the following reasons: i) the 12 cell lines are predominantly of different tissue/organ origin and different protein expression profiles must be expected, and as we reported in Steentoft et al., EMBO 2013 analysis of each of the 12 cell lines added 10-30% new glycopeptides; ii) most of the cell lines express different repertoires of GalNAc-Ts and are thus expected to cover different substrates; and iii) our previous work in Schjoldager et al., PNAS 2012 and in Steentoft et al EMBO 2013 was performed using the older LTQ-Orbitrap XL ETD spectrometer, while the newer more sensitive LTQ-Orbitrap Velos Pro was used this study.
Action 8: We have included a short sentence on p.5 to this effect:

"More than 50% of the O-glycoproteins identified are novel compared to our previous analysis of 12 human SimpleCell lines from different organs (Steentoft et al., 2013) (Fig. 2A), which may partly be attributed to use of a more sensitive mass spectrometer."

Query 9: It is not clear what portion of the circle the number 3 associates with - and if it is to be quantitative, this is not representing the right "area".
Answer 9: Agree.
Action 9: We have moved the numbers outside the area they represent, and added arrows to direct the reader instead.

Query 10: In Figure 2B, do the circles correspond with unambiguous or total values or is it just a representation?
Answer 10: Thank you for pointing this out. We agree that the representation may not be clear enough. We meant to incorporate both, all identified entries (ambiguous/unambiguous), all quantified entries (ambiguous/unambiguous) and all quantified monoglycosylated entries (ambiguous/unambiguous).
Action 10: We have changed Figure 2B legend text to the following:

"(B) Relative numbers of all identified sites, quantified sites and quantified single sites identified in this study".

Query 11: Figure 2C - are there any proteins with the number of sites 6-8? If not, those numbers can be left off. If so, #sites is hard to determine for 4-8.
Answer 11: Yes there are proteins with 6-8 sites identified and we agree that the axis is hard to read.
Action 11: We have formatted Figure 2C and divided the left Y-axis in two segments in order to read the lower numbers for 4-8 sites.

Query 12a: Regarding Figure 4, although the authors state in the text that there are not changes in the RPKM, it appears that there are changes that would be significant for both GALNT1 and GALNT2. However, the authors on p8 are likely trying to say that there is no evidence of upregulation of transcript levels of the other GALNT family members.
Answer 12a: We agree that there is variation in the transcript levels of *GALNT1* and *GALNT2* in comparison to WT transcript levels, and as the reviewer correctly point out we state that there is no evidence of *compensatory* changes (i.e. upregulation of one *GALNT* in response to loss of another). We do not see higher transcript levels of *GALNT1* in *GALNT2* KO compared to WT and vice versa.
Action 12a: We have tried to clarify this by inserting the following on p. 8, 3rd paragraph:
"The transcriptomes of two independent clones of each HepG2^{WT}DT1/DT2/+T3 KO's were analysed by RNA-sequencing (RNAseq, data is available at www.ebi.ac.uk/arrayexpress with accession number E-MTAB-3844), and although we saw minor differences in *GALNT1* and *GALNT2* transcript levels, we found no evidence of compensatory changes in transcript levels in any members of the large *GALNT* family (Fig. 4)".
Question 12b: Furthermore, it is not clear in the text whether other known GlycoTs were examined in the same genetic background (which is implied) or in a different context in Hansen et al 2015.
Answer 12b: We merely refer to Hansen et al., in order to point to an updated list of glycosyltransferase genes. When we refer to transcript levels in the manuscript we only refer to the RNAseq dataset obtained in this study and thus the same genetic background (HepG2).
Action 12b: To clarify this point we added the following on p.9, 1st paragraph:

"…or other known glycosyltransferase genes (For an updated list of glycosyltransferase genes see Hansen et al, 2015).

Query 13: In the legend, the authors state that for GALNT13, transcript levels in the deltaT2 clones changed compared to the WT. However, looking at the graph, it appears that the +T3 sample is the only one that changed. Can this graph can be better interpreted/represented so that the statistically significant change(s) are easy to see
Answer 13: Agree
Action 13: DT2 corrected to +T3.

Reviewer #2:
Query 1: The authors performed an interesting transcriptome analysis not mentioned in the abstract. They should revise the abstract to include the finding from the transcriptome analysis.
Answer 1: We agree and thank the reviewer for acknowledging this analysis.
Action 1: Abstract revised to reflect this.

Query 2: Figure 4: It is not clear why transcript levels of GALNT1 and 2 are not reduced in the deltaT1 and deltaT2 cells, respectively?
Answer 2: Gene inactivation by introduction of minor insertions or deletions (indels) at the targeted site may or may not affect transcript levels, and only the predicted protein products are important to consider.
Action 2: None.

Query 3: A paper about a non-redundant role of a GalNAc-T (Tian E, 2015, PLoS ONE 10(1):e0115861) should be cited and commented on.
Answer 3: Agree and we regret to have missed this citation.
Action 3: We now cite this on p.6, 2nd paragraph):

"… which is in agreement with and confirms the proposed association of murine *Galnt1* and BM/ECM formation (Tian et al, 2012, Tian et al, 2015)."

Query 4: The fourth paragraph in "GalNAc-T differential O-glycoproteomes in HepG2Sc" in Results section contains some discussion that should be moved to the Discussion section.
Answer 4: Agree.
Action 4: Results and Discussion have been merged.

Query 5: Figure 2A: Not clear to what the "HepG2 (74)" shown at the bottom is referring.
Answer 5: Agree.
Action 5: We have changed Figure 2 legend to:

"(A) Overall identified O-glycoproteins in all HepG2 cell lines (631 glycoproteins) with comparison to the HepG2 O-glycoproteome presented in Schjoldager et al., 2012 (74 glycoproteins) and the human O-glycoproteome identified in 12 SCs presented in Steentoft et al., 2013 (662 glycoproteins)."

Query 6: In several figures/tables, the HepG2SC+T3 cells are labeled HepG2SCdeltaT3 (Figure 3C, EV1B).
Answer 6: Agree.
Action 6: DT3 corrected to +T3

Query 7: Figure 3D: Not clear how the total number of sites for each cell (75, 81 and 131 for T1, T2 and T3, respectively) was obtained from the data shown in 3A, 3B, and 3C.
Answer 7: The data presented in figure 3A,B,C are the total number of peptides quantified in both TCL+SEC not taking any possible overlap in sites identified between TCL and SEC into account. In Fig.3D venn diagram any overlap between isoform-specific sites found in both TCL and SEC are missing giving rise to a slightly lower number than what is seen in the bar-diagram. In panels E and F comparing for instance T1 TCL (44) + T1 SEC (36) = 80 with the red colored bars in panel A 28 (singlets) + 52 (<-1) = 80 adds up.
Action 7: In order to make this clearer we added the following to the legend of Figure 3:

"(D) Venn diagram showing the distribution of candidates for isoform-specific sites among $HepG2^{SC}$DT1, T2 and +T3 applying a $\log_{10}$ (+/-1) cut-off (excluding sites identified in both TCL and SEC for each isoform)".

Query 8a: Figure EV4A and B: Only 11 peptides are shown in Table E3, but it appears many more peptides were assayed? Where is the data for the other peptides?
Answer 8a: Yes many more peptides were assayed. There are multiple sheets in Table EV3. We are concerned that a merged PDF might have hidden these extra sheets.
Action 8a: We have now ensured that all information is readily accessible in the PDF.

Query 8b: Also, the numbers in the legend for number of peptides tested does not match that shown in the figure.
Answer 8b: Agree.
Action 8b: We have corrected the error and stated that we tested 14 peptides in total for T1 and 27 for T2.

Query 9a: Table EV2: Are the numbers here Log M/L? If so, please provide that information.
Answer 9a: Yes
Action 9a: We have added "Log M/L "accordingly.

Query 9b: Some numbers have many more significant figures than others?
Answer 9b: Agree.
Action 9b: The error is now corrected so that all values are shown with two significant figures.

Query 9c: Why are numbers not found in each column for each peptide? Was the data for these peptides only of sufficient quality for quantitation in the cells where numbers are provided?
Answer 9c: Not all glycopeptides are identified in all samples. "All glycopeptides" can be separated into three groups: 1) glycopeptides that were identified, quantified and where glycosites were assigned unambiguously 2) glycopeptides that were identified and where glycosites were assigned but not quantified and 3) glycopeptides that were identified and quantified but where glycosites were not determined (ambiguous site assignment). For data mining we have used glycopeptides with

one glycosite per peptide, unambigously assigned and quantified, therefore not all glycpeptides in the Table have numbers for each sample. The same glycopeptides from different samples may belong to different identification group.
Action 9c: In order to make this more clear we have added the following sentence on p.5:

"All identified and quantitated monoglycosylated peptides identified in the TCL and SEC from HepG2^{SC}DT1/DT2/+T3 samples are summarized in Table EV2, first sheet".

Query 9d: It would help to put headers for each column at the top of each page.
Answer 9d: We are concerned that PDF conversions of our supplemental excel spreadsheets creates an unorganized representation of our data.
Action 9d: The supplemental files are now available as excel spreadsheets to avoid this conversion.

Query 9e: Not clear which column some numbers fall into, e.g. T777 from ADAM9?
Answer 9e: We are concerned that the PDF conversion may have changed the layout of this table.
Action 9e: The supplemental files are now available as excel spreadsheets to avoid this conversion.

2nd Editorial Decision 24 September 2015

Thank you for the submission of your revised manuscript to our offices. We have now received the enclosed reports from the referees that were asked to assess it. As you will see, both referees are very positive about the study.

From the editorial side, there are a few things that we need before we can proceed with the official acceptance of your study.

- As a standard procedure, we edit the title and abstract of a manuscript, both were a bit too long (limit: 100 characters including spaces for the title and 175 words for the abstract). Please find my suggested changes in the attached document.

- I noticed that you have only 4 main figures and 6 Expanded View figures. The scientific report format allows up to 5 figures. If you wish to highlight one of the EV figures in the main manuscript, you could move it there, e.g., EV2, EV4 or EV5, but I leave this up to you.

- Regarding data quantification, can you please specify the size of the scale bar in the legend for Figure EV1?

- Please include the legends for the Expanded View figures and tables in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section.

- Word tables are printed in grayscale. Could you please modify table EV1 and replace the insertions that you labeled with red letters with another highlight, e.g., bold and underlined letters?

Thank you very much and we look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:
Problems have been addressed.

Referee #2:
The authors have responded well to all of the concerns raised in the initial review. This is a very nice contribution!

2nd Revision - authors' response 05 October 2015

Authors made the necessary editorial changes.

3rd Editorial Decision 16 October 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

