Mucin-type *O*-glycosylation controls pluripotency in mouse embryonic stem cells via Wnt receptor endocytosis

Federico Pecori, Yoshihiro Akimoto, Hisatoshi Hanamatsu, Jun-ichi Furukawa, Yasuro Shinohara, Yuzuru Ikehara and Shoko Nishihara
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AUTHORS: Federico Pecori, Yoshihiro Akimoto, Hisatoshi Hanamatsu, Jun-ichi Furukawa, Yasuro Shinohara, and Shoko Nishihara
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The manuscript refers to a novel aspect of mucin-type O-glycosylation claiming their involvement in the regulation of mouse embryonic stem cell (ESC) pluripotency. Authors provide multiple lines of evidences supporting their hypothesis that the C1GalT1 catalysed formation of T antigen on ESCs controls the maintainance of pluripotency and self-renewal ability of mouse ESCs. They further give mechanistic insight into cellular processes affected by aberrant O-glycosylation (block of T antigen formation) by pinpointing the promotion of canonical Wnt signaling in ESCs (blocked endocytosis of the Wnt receptor frizzled-5 carrying T antigen).

The manuscript reports solid work on a highly significant topic with great impact on cell biology and cellular glycobiology. All claims of the paper are supported by experimental evidence and there is no major concern with respect to the content or to formal aspects of data presentation. Before publication the manuscript should however be revised according to the minor points listed below.

Comments for the author

Abstract:
Authors should revise the summary of their work, as this does not contain all relevant information. In particular, the text as provided mentions only that there are specific biological effects exerted by changes in O-glycosylation pattern, but it does not specify the structures involved.

P3, line 101: Although the term “aberrant O-glycosylation” is often used in the context of cancer-associated O-glycosylation changes, it remains unclear.
“Aberrant” structures (generally short precursor glycans) are found also in normal epithelial tissues, only their relative expression rate is shifted towards higher levels. It would be more appropriate to refer to “aberrant glycoprofiles or patterns”.

Fig. 1C,D The methodical descriptions of thisexperimental part are insufficient. Were replicate anaöyses performed? Are the presented data mean values or just showing representative data? Are authors aware of the fact that MALDI-MS does not allow reliable quantification of components in mixtures? The use of an internal standard does not overcome this problem. What refers GN4 to? In Fig. 1C relative amounts of selected structures are presented. Why were these data not combined with those in Fig. 1D?

P7, line 214 f:
Could authors comment on the T-antigen mediated fzd5 endocytosis whether or not it is dependent on galectin-3 or any other galectin?

Reviewer 2

Advance summary and potential significance to field

This manuscript is focused on analyzing the impact of the O-glycosylation Wnt receptor resulted in the maintenance of the pluripotency of mouse embryonic stem cells (ESC). This study brings innovation because there is no study demonstrated the role of O-glycosylation in the maintenance of ESC. The authors have identified C1Galt1 that is inducing the mucin-type O-glycosylation elongation in ESC maintenance. The silencing of C1Galt1 promotes canonical Wnt signaling in ESCs and induce the pluripotency. Furthermore, they identified knockdown of C1Galt1 to decrease the T-antigen on Frizzled-5, which is essential for the pluripotency maintenance in ESC.
Overall, this study demonstrate the importance of O-glycosylation on Wnt signaling and pluripotency of mouse ESC. However, several queries need to be clarified conceptually and methodologically.

Comments for the author

- There are several O-glycosyltransferase are emerging for different steps of glycosylation; however, the authors focused on only ST6GalNAc-I, C1GalT1, or β3GnT6. Is there any study shown the importance of Tn and sTn for ESC maintenance? Rationale is highly needed.
- The transient knockdown of C1Galt1 is showing the decreased T-antigen. However, the accumulation of Tn and sTn may be high in ESC. Do Tn and sTn truncated structure also cause the effect in pluripotency and self-renewal of ESC?
- Mouse and Human have a different set of glycosylation pattern. Do these T-antigen alterations occur in human ESC?
- Most of the results are shown only in one mouse ESC. Transient know down may have an only transient effect on ESC maintenance. How are the results on stable knock down?
- The knockdown of C1Galt1 decreases the internalization of T-antigen; however, the result is indicating in a few cells. The fluorescent quantification in multiple fields is essential to prove the concept.
- C1Galt1 silencing is not decreasing the significant level of Oct3 and Sox2 protein, which are important pluripotent markers in ESC.
- The representation of alkaline phosphatase staining is missing in control and C1Galt1 KD cells.
- The transient knockdown of C1Galt1 is showing the reduction in colony formation and alkaline phosphatase staining how the transient knockdown maintained several days for colony formation assay.
- Mesoderm and ectoderm markers are missing in the lineage differentiation analysis.
- Any defective embryoid body observed in the C1Galt1 KD ESCs compared to control ESC, which may prove the differentiation potential of C1Galt1 KD ESCs.
- It is also essential to prove the reduction of T antigen on Fzd5 and alteration of interaction with ZNRF3/RNF43, which may give a precise mechanism which is a speculated conclusion written in the discussion.

First revision

Author response to reviewers' comments

To ensure our manuscript complies with the manuscript formatting guidelines, the figure legends were moved to the end of the manuscript, the figures were removed from the manuscript and provided as a separate file for each item, the supplementary figures were reformatted, the supplementary materials and methods and supplementary references were added to the main text, and the funding sources were provided in full and matched with Crossref. Moreover, we collate all the supplementary information, including supplementary figures and related legends, movie captions, and supplementary table into a single separate file. In the revised manuscript and supplementary information, modified or added sentences and words are highlighted in yellow. A formatted PDF copy of this file was also uploaded as Supplementary Information.

Reviewer #1

Abstract:
Authors should revise the summary of their work, as this does not contain all relevant information. In particular, the text as provided mentions only that there are specific biological effects exerted by changes in O-glycosylation pattern, but it does not specify the structures involved.

During the manuscript revision process, we found that mucin-type *O*-glycosylation mediates Frizzled-5 endocytosis via galectin-3 binding to T antigen. Therefore, in accordance with the reviewer's comment and our new findings, we have modified the Abstract as follows: "Here, we

identify the elongation pathway via C1GalT1 that synthesizes T antigen (Galβ1-3GalNAc) as the most prominent among mucin-type *O*-glycosylation modifications in ESCs. Moreover, we show that mucin-type *O*-glycosylation on the Wnt signaling receptor Frizzled-5 regulates its endocytosis via galectin-3 binding to T antigen, and that reduction of T antigen results in the exit of the ESCs from pluripotency via canonical Wnt signaling activation." (page 2, lines 41-47).

P3, line 101:
Although the term "aberrant O-glycosylation" is often used in the context of cancer-associated O-glycosylation changes, it remains unclear. "Aberrant" structures (generally short precursor glycans) are found also in normal epithelial tissues, only their relative expression rate is shifted towards higher levels. It would be more appropriate to refer to "aberrant glycoprofiles or patterns".

Thank you for this suggestion; we have accordingly modified the term "aberrant *O*-glycosylation" to "aberrant glycoprofiles" (page 4, line 105).

Fig. 1C,D
The methodical descriptions of this experimental part are insufficient. Were replicate anaöyses performed? Are the presented data mean values or just showing representative data? Are authors aware of the fact that MALDI-MS does not allow reliable quantification of components in mixtures? The use of an internal standard does not overcome this problem. What refers GN4 to? In Fig. 1C relative amounts of selected structures are presented. Why were these data not combined with those in Fig. 1D?

We thank the reviewer for the thoughtful consideration of our experimental results. The MALDI-MS presented data were obtained from a single technical and biological replicate in two different cell lines to ensure consistency (Fig. 1C, Fig. S1A,B). In accordance with the reviewer's comment, we added the following sentence to the figure legends representing MALDI-MS results: "The data were obtained from a single technical and biological replicate." (page 25, lines 821-822; Supplementary Information page 1, lines 13-14). An explanation of the previously validated methodology used to reliably quantify the amount of *O*-glycans in mixtures (Furukawa et al., 2015a) was included in the manuscript as follows: "Quantitative analysis of *O*-glycans in ESCs was performed by mass spectrometry using optimized microwave-assisted β-elimination in the presence of a pyrazolone (BEP) method for *O*-glycomic analysis that has been validated with a mixture of equal quantities of four glycans (Furukawa et al., 2015a)." (pages 4-5, lines 135-139). The definition of GN4 was included in the Materials and Methods section "Tandem MALDI-TOF MS analysis" as follow: " bis-PMP-labeled N, N′, N″, N‴-tetraacetyl chitotetraose (GN4)." (page 14, line 449). In accordance with the reviewer's suggestion, we combined the data from Fig. 1C and Fig. 1D into a single figure (Fig. 1C).

P7, line 214 f:
Could authors comment on the T-antigen mediated fzd5 endocytosis whether or not it is dependent on galectin-3 or any other galectin?

During the manuscript revision process, we found that Frizzled-5 endocytosis is mediated by galectin-3 binding to T antigen (Fig. 7A-F). Therefore, we modified the Results and Discussion sections as follow: "Galectins (Lgals) belong to a family of carbohydrate-binding proteins that bind to β-galactose-containing glycoproteins (Johannes et al., 2018). Previous studies have reported that Lgals, in particular Lgals3, have a stimulatory effect on endocytosis (Merlin et al., 2011; Gao et al., 2012; Lepur et al., 2012; Lakshminarayan et al., 2014). Moreover, frontal affinity chromatography analysis has demonstrated that T antigen is a ligand for Lgals9, followed by Lgals2, Lgals3, and Lgals4 (Iwaki and Hirabayashi, 2018). Therefore, we hypothesized that Lgals are involved in Fzd5 endocytosis. Immunostaining using an anti-Fzd5 Ab in permeabilized ESCs showed a marked reduction of internalized Fzd5 in ESCs treated with a high concentration of lactose, demonstrating that Lgals regulate Fzd5 endocytosis (Fig. 7A,B). RNA-seq analysis showed that, among the Lgals that can bind T antigen, *Lgals3* was the most highly expressed in ESCs (Fig. 7C). Consistently, addition of Lgals3 resulted in an enhancement of internalized Fzd5, confirming that Lgals3 is involved in Fzd5 endocytosis in ESCs (Fig. 7D,E; Movie 7)." (page 8, lines 254-267), and: "In this study, we demonstrated that T antigen on the Wnt receptor Fzd5 modulates endocytosis of Fzd5 via

Lgals3 (Fig. 7F)" (page 10, lines 331-333). Detailed methodology for galectin dissociation, galectin-3 addition, and RNA-seq was added to the Materials and Methods sections entitled "Cell culture" and "Real-time PCR and RNA-seq analysis" (page 13, lines 408-411; page 13, lines 425-430). RNA-seq data generated for this study has been deposited in the GEO repository under accession number GSE152688. The above-mentioned reference (Iwaki and Hirabayashi, 2018) was added to the References section (page 19, lines 640-642).

Reviewer #2

There are several O-glycosyltransferase are emerging for different steps of glycosylation; however, the authors focused on only ST6GalNAc-I, C1GalT1, or β3GnT6. Is there any study shown the importance of Tn and sTn for ESC maintenance? Rationale is highly needed.

We thank the reviewer for the thoughtful consideration regarding the rationale of our study. Mucin-type *O*-glycosylation is a step-wise process initiated in the Golgi by the attachment of *N*-acetylgalactosamine (GalNAc) to form the Tn antigen; this process involves a large family of up to 19 transferases. Subsequently, Tn antigen is elongated by ST6GalNAc-I, C1GalT1, or β3GnT6 to synthesize sTn antigen, T antigen, or Core 3 structure, respectively. Furthermore, T antigen and Core 3 structure undergo further branching (Fig. 1A). In addition to sTn antigen, T antigen, and Core 3 structure, the Tn antigen can also be extended to form Core 5 to 8 structures. However, Core 5 to 8 structures have an extremely restricted occurrence and the glycosyltransferases involved in their formation remain to be fully characterized (Brockhausen et al., 2009); thus, they are not considered in this study.

Tian et al. reported that mice deficient for *Galnt1*, which was shown to be the most abundantly expressed among the 19 enzymes involved in the formation of Tn antigen during murine embryonic submandibular gland development, showed a reduction in FGF signaling (Tian et al., 2012), a key signaling pathway involved in ESC differentiation (Lanner and Rossant, 2010). Moreover, Carter et al. previously demonstrated that a novel culture medium that includes the primitive growth factor NME7AB, which binds to the extracellular domain of the cleaved form of the mucin-type *O*-glycosylated protein MUC1, maintained human ESCs in an undifferentiated state (Carter et al., 2016), indicating that mucin-type *O*-glycosylation is involved in the pluripotency network. However, the function of mucin-type *O*-glycosylation in mouse ESC pluripotency and its relationship to signaling is unknown. The partial functional redundancy of ppGalNAc-Ts makes analysis of their function highly complex (Bennet et al., 2012); we therefore focused on the catalyzation step following Tn antigen formation that is selectively performed by ST6GalNAc-I, C1GalT1, or β3GnT6. To clarify our rationale, we modified the Introduction section as follows: "*Galnt1* is the most abundantly expressed of the 19 ppGalNAc-Ts during murine embryonic submandibular gland development; mice deficient for *Galnt1* show a reduction in FGF signaling (Tian et al., 2012)." (page 3-4, lines 101-103); "However, the function of *O*-glycosylation in ESC pluripotency network and its relationship to signaling in ESCs remain unknown." (page 4, lines 110-112), and added the following text in the Results section: "In addition to sTn antigen, T antigen, and Core 3 structure, the Tn antigen can also be extended to form Core 5 to 8 structures. However, Core 5 to 8 structures have an extremely restricted occurrence and the glycosyltransferases involved in their formation remain to be fully characterized (Brockhausen et al., 2009); thus, they are not considered in this study." (page 4, lines 129-133). The above-mentioned relevant references were added to the References section (page 18, lines 579-582; page 22, lines 738-740).

The transient knockdown of C1Galt1 is showing the decreased T-antigen. However, the accumulation of Tn and sTn may be high in ESC. Do Tn and sTn truncated structure also cause the effect in pluripotency and self-renewal of ESC?

We thank the reviewer for the thoughtful consideration of our experimental results. We showed that reduction of T antigen results in accumulation of Tn antigen, but not sTn antigen by HPA and SNA lectin staining, respectively (Fig. S2A-D). Therefore, a truncated sTn structure is not involved in the observed effect on the pluripotency and self-renewal of ESCs. To investigate whether the accumulation of Tn antigen contributed to the loss of pluripotency in ESCs, we focused on the mechanism regulating Frizzled-5 endocytosis. In this analysis, we observed that galectin-3, a carbohydrate-binding protein that binds to T antigen, is involved in Frizzled-5 endocytosis (Fig. 7A-F) (further details are provided in response to the last comment). Thus, we modified the text as

follow: "*C1GalT1* KD resulted in a significant increase in Tn antigen and a mild decrease in sTn antigen at the cell surface, stained by *helix pomatia* agglutinin lectin (HPA) and *sambucus nigra* lectin (SNA), respectively (Fig. S2A-D)." (page 5, lines 159-162); and: "Knockdown of *C1GalT1* expression results in a decrease of T antigen on the Wnt receptor Fzd5, reducing the level of galectin-3 (Lgals3)-mediated Fzd5 endocytosis. The retention of Fzd5 on the surface, in turn, promotes excessive canonical Wnt signaling activation via β-catenin stabilization, resulting in the exit from pluripotency (Fig. 7F)." (page 9, lines 285-289). Therefore, ESC pluripotency loss upon *C1GalT1* knockdown is mediated by the disrupted Lgals3-T antigen interaction and not by accumulation of Tn antigen. SNA lectin information was added to the Materials and Methods section "Fluorescence-activated cell sorting (FACS) analysis" (page 14, line 456).

Mouse and Human have a different set of glycosylation pattern. Do these T-antigen alterations occur in human ESC?

A previous comprehensive study of the glycomes of various cell lines reported that the T antigen elongation pathway is the most prominent in conventional human ESCs, similarly to mouse ESCs (Fujitani et al., 2013). However, mouse and conventional human ESCs reflect two different pluripotent states, namely the naïve and the primed state, respectively, which rely on different signaling pathways to maintain the pluripotent state and induce differentiation (Weinberger et al., 2016). Thus, the influence of T antigen on the pluripotency network and differentiation potential is likely to differ between mouse and human ESCs. Currently, we are exploring the role of C1GALT1-mediated elongation pathway in the conventional human ESC pluripotency network. We added the above information to the Discussion section (page 9-10, lines 303-312). The above-mentioned relevant references were added to the References section (page 18, lines 595-598; page 23, lines 753-755).

Most of the results are shown only in one mouse ESC. Transient know down may have an only transient effect on ESC maintenance. How are the results on stable knock down?

Thank you for this comment. In order to answer this, we established stable *C1GalT1* KD ESCs and analyzed the effect on the pluripotency. Similarly to the transient *C1GalT1* KD, stable *C1GalT1* KD ESCs exit from the pluripotency even in presence of LIF (Fig. S3A-D). The following sentence was added to the manuscript: "ESC pluripotency and self-renewal loss following *C1GalT1* KD was further confirmed by using stable *C1GalT1* KD ESCs (Fig. S3A-D)." (page 6, lines 174-175). Detailed methodology was added in the Materials and Methods "Cell culture" (pages 11-12, lines 367-400).

The knockdown of C1Galt1 decreases the internalization of T-antigen; however, the result is indicating in a few cells. The fluorescent quantification in multiple fields is essential to prove the concept.

In accordance with the reviewer's comment, Fig. 2E was replaced with a representative image of a maximum intensity projection of internal molecules in *C1GalT1* KD cells after immunostaining using PNA-biotin, and fluorescent quantification in multiple fields was added to confirm the reduction of internal T antigen (Fig. 2E,F) (page 25, lines 830-834).

C1Galt1 silencing is not decreasing the significant level of Oct3 and Sox2 protein, which are important pluripotent markers in ESC.

To clearly show a significant reduction at the protein level of the pluripotency markers Oct3/4 and Sox2, we increased the biological replicates from 3 to 5 and replaced the representative western blot image (Fig. 3C,D) (page 26, line 851).

The representation of alkaline phosphatase staining is missing in control and C1Galt1 KD cells.

Thank you for pointing this out; we have added an image showing alkaline phosphatase staining (Fig. 3F).

The transient knockdown of C1Galt1 is showing the reduction in colony formation and alkaline phosphatase staining how the transient knockdown maintained several days for colony formation assay.

C1GalT1 transient KD ESCs were selected for 4 days before plating for an additional 4 days post transfection for the clonogenicity assay. Therefore, the transient knockdown results demonstrate that *C1GalT1* KD ESCs selected for 4 days irreversibly lose the ability to self-renew. To clarify this point, the Results section was modified as follow: "To assess the ability of *C1GalT1* KD cells to self-renew we performed a clonogenicity assay 4 days post transfection" (page 6, line 170-171).
To further confirm this result, we performed a clonogenicity assay using stable *C1GalT1* KD ESCs. Similarly to the result obtained in *C1GalT1* transient KD ESCs, the number of ALP positive colonies was reduced, demonstrating that the self-renewing ability of *C1GalT1* KD ESCs was compromised (Fig. S3D). The following sentence was added to the manuscript: "ESC pluripotency and self-renewal loss following *C1GalT1* KD was further confirmed by using stable *C1GalT1* KD ESCs (Fig. S3A-D)." (page 6, lines 174-175). Detailed methodology was added in the Materials and Methods "Cell culture" (pages 11-12, lines 367-400).

Mesoderm and ectoderm markers are missing in the lineage differentiation analysis.

In accordance with the reviewer's comment, a complete analysis of the expression of differentiation markers was performed and the text modified as follows: "An analysis of the expression of differentiation markers showed that *C1GalT1* KD resulted in an up-regulation of the trophoblast markers *Cdx2* and *Gata3*, suggesting that *C1GalT1* KD induces ESC transdifferentiation toward the trophectoderm (Fig. 3G)." (page 6, lines 175-178). Details on the Real-Time PCR primers used for this analysis were added to Table S1.

Any defective embryoid body observed in the C1Galt1 KD ESCs compared to control ESC, which may prove the differentiation potential of C1Galt1 KD ESCs.

To answer this question, we performed an EB assay from *C1GalT1* KD ESCs and analyzed the early differentiation markers. In this analysis, we found that *C1GalT1* KD ESCs showed an enhanced differentiation potential compared to the control (Fig. S3E,F). The following sentence was added to the manuscript: "Consistently, an EB assay showed an enhanced differentiation potential in *C1GalT1* KD ESCs (Fig. S3E,F)." (page 6, lines 178-180). Details of the methodology were added to the Materials and Methods "Cell culture" (page 11, lines 365-366).

It is also essential to prove the reduction of T antigen on Fzd5 and alteration of interaction with ZNRF3/RNF43, which may give a precise mechanism, which is a speculated conclusion written in the discussion.

In accordance with the reviewer's comment, we investigated the molecular mechanism involved in Frizzled-5 altered endocytosis following reduction of T antigen. In the original manuscript, we put forward two possible mechanisms for the regulation of Frizzled-5 endocytosis by the T antigen: Frizzled-5 interaction with galectins, and/or with Znrf3/Rnf43. Thus, we investigated whether Frizzled-5 endocytosis was altered by galectins dissociation using a high concentration of lactose, or by addition of galectin-3, which we showed by RNA-seq to be the most highly expressed in ESCs (Fig. 7C). We also performed a transient knockdown of *Znrf3*, which we demonstrated by RNA-seq analysis to be highly expressed in ESCs compared to its homolog *Rnf43* (Fig. S7A), and analyzed the changes in Frizzled-5 internalization. In these analysis, we showed that galectin-3, but not Znrf3/Rnf43, binding to T antigen on Frizzled-5 mediates its endocytosis in ESCs (Fig. 7A-F; Movie 7; Fig. S7A-D). The Results and Discussion sections were modified as follows: "Galectins (Lgals) belong to a family of carbohydrate-binding proteins that bind to β-galactose-containing glycoproteins (Johannes et al., 2018). Previous studies have reported that Lgals, in particular Lgals3, have a stimulatory effect on endocytosis (Merlin et al., 2011; Gao et al., 2012; Lepur et al., 2012; Lakshminarayan et al., 2014). Moreover, frontal affinity chromatography analysis has demonstrated that T antigen is a ligand for Lgals9, followed by Lgals2, Lgals3, and Lgals4 (Iwaki and Hirabayashi, 2018). Therefore, we hypothesized that Lgals are involved in Fzd5 endocytosis. Immunostaining using an anti-Fzd5 Ab in permeabilized ESCs showed a marked reduction of internalized Fzd5 in ESCs treated with a high concentration of lactose, demonstrating

that Lgals regulate Fzd5 endocytosis (Fig. 7A,B). RNA-seq analysis showed that, among the Lgals that can bind T antigen, *Lgals3* was the most highly expressed in ESCs (Fig. 7C). Consistently, addition of Lgals3 resulted in an enhancement of internalized Fzd5, confirming that Lgals3 is involved in Fzd5 endocytosis in ESCs (Fig. 7D,E; Movie 7). The cell-surface E3 ubiquitin ligase Znrf3 and its homologous Rnf43 inhibit Wnt signaling by targeting surface-expressed Fzd to lysosomes in human embryonic kidney cells and mouse intestinal stem cells (Hao et al, 2012; Koo et al, 2012). Thus, reduction of T antigen on Fzd5 may alter its interaction with Znrf3/Rnf43, prolonging Wnt receptor availability at the cell surface. RNA-seq analysis showed that *Znrf3* is highly expressed in ESCs compared to *Rnf43* (Fig. S7A). However, *Znrf3* knockdown in ESCs did not affect Fzd5 endocytosis (Fig. S7B-D). In conclusion, our findings demonstrate that reduction of T antigen results in disruption of Lgals-3-mediated endocytosis of the Wnt receptor Fzd5. As a result, Fzd5 is retained at the plasma membrane, thereby prolonging the activation of Wnt signaling (Fig. 7F)." (pages 8-9, lines 254-277), and: "In this study, we demonstrated that T antigen on the Wnt receptor Fzd5 modulates endocytosis of Fzd5 via Lgals3 (Fig. 7E)" (page 10, lines 331-333). Details of the methodology for galectin dissociation, galectin-3 addition, and RNA-seq were added to the Materials and Methods section "Cell culture" and "Real-time PCR and RNA-seq analysis" (page 13, lines 408-411; page 13, lines 425-430). *Znrf3* KD 1 and KD 2 sequences, and *Znrf3* Real-Time PCR primers were added to the Materials and Methods section "Cell culture" (page 12, lines 384-389) and Table S1, respectively. RNA-seq data generated for this study has been deposited in the GEO repository under accession number GSE152688.

Second decision letter

MS ID#: JOCES/2020/245845

MS TITLE: Mucin-type *O*-glycosylation controls pluripotency in mouse embryonic stem cells via Wnt receptor endocytosis

AUTHORS: Federico Pecori, Yoshihiro Akimoto, Hisatoshi Hanamatsu, Jun-ichi Furukawa, Yasuro Shinohara, Yuzuru Ikehara, and Shoko Nishihara
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.