EMBO reports

O-GlcNAcylation promotes topoisomerase IIα catalytic activity in breast cancer chemoresistance

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Dear Dr. Liu,

Thank you for the submission of your manuscript to EMBO reports. I have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees indicate that these findings are of interest. However, they have several comments, concerns, and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all the referee concerns need to be addressed, I will not detail them here.

Given the constructive referee comments, I would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

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Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

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Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

In the present manuscript, authors designed and performed experiments to reveal the importance of O-GlcNAcylation of topoisomerase II (TOP2A) in chemotherapy resistance of breast cancer cells. Importantly, authors identified that TOP2A could be O-GlcNAcylated by OGT at Ser1469, and characterized its function in vitro and in vivo. They found that O-GlcNAcylation at Ser1469 promotes TOP2A chromatin DNA binding and catalytic activity, and influences downstream gene expression, which may contribute to breast cancer drug resistance.

This reviewer believe that this is a good example to show the importance of glycosylation, one of PTMs, not only in cancer biology but also in life science, and satisfy the most of data in the present manuscript. However, the following points had better be considered.

1) In Fig. 3I, authors need to clarify the phenomena that the localization of TOP2A-S1469 was diffused in the cytosol in MCF-7/ADR cells, while its was localized in the nucleus of MCF-7 cells.
2) In Fig. 6F, the interactions between TOP2A and OGT were greatly enhanced in the Resistance, compared to the Sensitive. Authors need to explain the reason, what is the underlying molecular mechanism?
3) It is well known that enhancement of drug efflux by overexpressing ABC transporters such as P-glycoprotein is one of main mechanisms involved in cancer drug resistance. Authors need to check whether O-GlcNAcylation or O-GlcNAcylation of TOP2A influences the expression of P-glycoprotein and/or other ABC transporters subfamily members for elucidation of deepening molecular mechanisms.

Referee #2:

Liu et al. identified O-GlcNAcylation as a novel post-translational modification regulating TOP2A activity and provided detailed molecular characterization for the role of O-GlcNAcylation in TOP2A regulation and translational studies on its cancer relevance.

Yet, there are important questions that need to be addressed:

1. Figure 1E states that "TOP2A positively correlates with overall survival in TCGA breast cancer tissues." Instead, the plot suggests that TOP2A expression and survival rate are inversely proportional.

2. One of the primary therapeutic/killing mechanisms of Adm is the stabilization of TOP2-DNA cleavage complexes (TOP2cc). The authors should measure and compare TOP2Acc in breast cancer cells tested in Figure 2A-C to test if Adm resistance is in part due to reduced TOP2Acc in those cell lines. If O-GlcNAcylation enhances TOP2 chromatin binding and cleavage, high O-GlcNAcylation should promote cell proliferation but the other hand, it should also increase Adm-induced TOP2Acc (more targets) and hence Adm-induced cell death. Their results suggest the opposite.

3. To delineate the role O-GlcNAcylation in mitigating Adm cytotoxicity, TOP2Acc and induction of DNA double-strand markers (e.g., gH2AX), and/or cell cycle need to be measured in cells w/ and w/o OGT downregulation and L01 treatment.

4. Figure 2G shows that TOP2A was pulled down in the absence of GalT1 Y289L. Why is that?

5. According to the authors, truncated TOP2A variants were generated in a prokaryotic vector. How did they express in HEK293 cells? Primers and sequencing for generation and validation of all the plasmids including HA-TOP2A must be provided.

6. Figure 2I shows diffusion of TOP2A when O-GlcNAcylation is suppressed. This is strange. TOP2A is a nuclear protein, and it is simply impossible that TOP2A delocalizes to cytoplasm under any circumstances. Please provide explanations.

7. Gels in Figure 4A-D look like cleavage assay rather than relaxation assay. Did the authors add EtBr to agarose gel? Same goes to Figure 6A.

Referee #3:

"O-GlcNAcylation elevates topoisomerase IIa catalytic activity: a role in the chemotherapy resistance of breast cancer" by Liu and colleagues is mostly well written and organized, and touches a very interesting subject: the regulation of TOP2A by O-GlcNAcylation in breast cancer and its influence in resistance to Adriamycin (Adm). The authors successfully demonstrate that TOP2A is functionally regulated by O-GlcNAcylation and describe the site of the modification. They also demonstrate the consequences of preventing this modification in vitro and in vivo in a breast cancer model. They authors make use of adequate and interesting techniques to answer their questions.

Nevertheless there are major and minor concerns about the work as follows:

Major comments:
1. One of the first claims in the paper is that TOP2A expression is increased in cells that are resistant to Adm. Yet, the panel that represents this data (Fig 2A) does not very clearly support this claim in that the levels do not seem to be much different. In fact, in Fig 6F, TOP2A levels in the sensitive and resistant patients cells are not different at all. Those two panels actually point to the levels of TOP2A as not being a decisive factor in Adm resistance. Please comment on that.

2. Fig 2C presents a cell viability assay of MDA-MB-231 cells upon co-treatment with L01/shTOP2A and Adm, but it is not shown how L01/shTOP2A affects cell viability alone. Thus, it is not known if the impact on cell viability is due to OGT inhibition itself or TOP2A silencing itself or if it is seen only during treatment with Adm. This needs to be clarified with additional data. Please add this information either in the main figure or to the supplementary material.

3. Similarly, in Fig 6G and H is shown the effect of shTOP2A+WT alone but not L01 treatment alone in vivo. Without this control we are not able to know if the effect in the tumor is due to inhibition of TOP2A by the drug or if it is just the effect of the drug treatment itself, since OGT is a known regulator of cell cycle progression by different mechanisms. Without this control the double treatment (shTOP2A+WT+L01) is not informative.

4. The authors state "An increase in cellular O-GlcNAcylation indicated elevated cell survival and colony formation frequency in breast cancer cells under Adm treatment" (line 149-151), however there is no data indicating that those two phenotypes are associated functionally. Please rephrase it.

5. Line 153-154, "Adm-resistant MDA-MB-231 and MCF-7/ADR cells, OGT shRNA transfected cells showed increase sensitivity to Adm.". The text does not refer to any panel in the figures and and these data do not seem to be in the paper. Please clarify.

6. In Fig 5A the authors show a colony formation assay and in the text they claim "The number of colonies was higher in TOP2A-WT group and lower in the TOP2A-S1469A group after Adm treatment, affirming that TOP2A glycosylation promoted Adm resistance in breast cancer cells" (lines 255-258). The authors cannot claim that since the number of colonies in the control condition is also different in the WT and S1469A groups. If the authors want to make this claim they need a quantification of the colony formation experiments to show that even with the difference in control conditions, you still have a significant difference between WT and mutant after treatment with Adm.

7. The authors use p27 as a cell-cycle marker and show that p27 levels go up when OGT is inhibited with L01 (Fig 5E) and go down in patient samples that express higher levels of OGT (#3-5) (Fig 6F). However, p27 has been already described as having its levels regulated by OGT levels/activity (Caldwell et al., 2010) as well as p21 (de Queiroz et al., 2022). Thus, the regulation of p27 shown in the paper is not surprising and is likely not related to TOP2A modification. Because of this, it is suggested that the authors do not use p27 as a marker in the abovementioned figures. If the authors wish to keep it in the figure the should add cite the published evidence about p27 and OGT in the discussion session.

8. In Fig 6 C-E the authors show a blot for the overexpression of TOP2A. They do not specify in the figure legend in which cell line the blot is from. Also, the authors should show blots for all 3 cell lines to show that the expression levels of the plasmids were similar, like in the one blot shown in the panel.

9. Still regarding the in vivo treatment with L01, we have had experience with in vivo treatment with OSMI-1 and we do not see an effect in mice, meaning O-GlcNAc levels are not changed in mouse tissues (neither tumor nor normal tissue) after treatment with OSMI-1 for 27 days. To prove your treatment with L01 is indeed inducing OGT inhibition in mice, please add a GlcNAc blot of your mouse tissue samples to Fig 6.

10. The discussion needs a lot of work. This section of the paper, that is supposed to discuss matters involving the subject of the work, is not really discussing anything. Most of the discussion is facts about TOP2A, that could even be included in the introduction, or description of the results shown in the Results section. Please work on this section.

Minor comments:

1. Throughout the entire paper the "O" in O-GlcNAcylation need to be italicized as well as the term "in vivo".
2. Throughout the entire paper the authors use the word "obviously" to describe their results (e.g. line 191 and 201). This word is not appropriate to describe results, please change it to a different word.
3. In the synopsis figure please add a legend for the blue and gray ellipses in it. It is not very clear what they represent.
4. Line 64, 69, substitute the word "poison" for a better fitting one.
5. Line 101, substitute the word "enigmatic" for a better fitting one.
6. Line 126, the data described has no indication of a Figure and panel. Please add.
7. Line 284, substitute the word "poisoning" for inhibiting.
8. Line 309-327, there are multiple references to a Figure 7 that does not exist in the manuscript, but instead they look like they should refer to later panels on Figure 6. Please rename the figure references in this part of the manuscript.
9. In Figure legend 1, panel C please clarify what are the green and blue bars.
10. In Figure 2, panel B shows two different experiments using two different techniques so they should be two different panels. Please separate them.
11. In Figure legend 2 (line 81) panel H, shTOP2A should be shOGT.
12. In Figure 4 panel A, the lane designations say "MCA-MB-231", please correct it to MDA-MB-231.
13. In Figure 4 panel C, the O-GlcNAc blot is not well aligned with the others.
14. In Figure 5 panel B and C, the G2 bars cannot be seen. Please change the color of it for better visualization.
15. In Figure legend 5, the designation of panels D and E are a bit confusing. Please change it for clarification.
16. In Figure legend 6 panels C-E the authors mention T test was used for statistical analysis but they do not say what conditions were compared. Since T test is for comparison of 2 groups and they have 3 in each graph, please clarify.

Referee #1

1. In Fig. 3I, authors need to clarify the phenomena that the localization of TOP2A-S1469 was diffused in the cytosol in MCF-7/ADR cells, while its was localized in the nucleus of MCF-7 cells.

Reply

We made a mistake about the cell lines we used in Figure 3I. In fact, the cells we used were MDA-MB-231 and MCF-7/ADR, as presented in Figure 3F-H. We have revised this image and described the cells we used in the figure legends.

This reviewer also expressed concerns regarding mislocalization of TOP2A-S1469A in Figure 3I. Although TOP2A is a well-known nuclear protein, this enzyme has been reported to diffuse in the cytoplasm under particular circumstances. The literature as early as 1997 revealed that TOP2A was detected in both the cell nucleus and cytoplasm of cell lines and normal and tumor tissues (British Journal of Cancer 1997, 75(9), 1340-1346). Further studies have demonstrated that the nuclear localization signal (NLS) sequence in TOP2A is located between amino acids 1454-1497 (Nucleic Acids Research, 45(10), 2017, 5995–6010) in the C-terminal domain (CTD). The CTD is important for the proper subcellular localization of TOP2A, and the loss or alteration of the CTD could lead to TOP2A cytoplasmic mislocalization in yeast and mammalian cells (Nucleic Acids Research, 25(15), 1997, 3135-3142; Nucleic Acids Research, 35(11), 2007, 3810-3822). Deletion of TOP2A residues 1174-1446, which overlap with the CTD, disrupts nuclear localization and mitotic chromatin association despite retaining the major NLS, suggesting additional contributions from other residues within the CTD (Int. J. Mol. Sci. 2019, 20, 1238). It was also reported that a truncated cytoplasmic TOP2A (deficiency of amino acids 1423-1489) in lung cancer cells (H209/VP) contributes to VP-16 drug resistance (International journal of cancer, 2000, 85(4), 534-539). Overall, these results suggest that multiple sequences, including the NLS within the CTD, contribute to the robust nuclear localization of TOP2A. The TOP2A CTD is rich in residues subject to posttranslational modifications (PTMs) such as phosphorylation, SUMOylation,

ubiquitination and acetylation, which have been found to influence its catalytic activity, protein stability and subcellular distribution. We reported that TOP2A *O*-GlcNAcylation occurs at S1469, which is located in the TOP2A NLS. We speculate that the *O*-GlcNAcylation and other PTMs of particular residues within the CTD, especially in the NLS of TOP2A, could impact TOP2A subcellular localization and then control its ability to bind chromatin, with a rapid exchange between molecules in the chromatin and cytosolic pools apparently governing TOP2A's biological function. The precise mechanisms underlying this regulation remain to be further identified.

To further demonstrate the cytoplasmic localization of non-glycosylated TOP2A, additional experiments were performed (Figure 3H). The cytoplasm was isolated from HA-TOP2A-WT- or HA-TOP2A-S1469A-overexpressing MDA-MB-231 and MCF-7/ADR cells, and cytoplasmic TOP2A was detected using an anti-HA tag antibody. Consistent with immunofluorescence assays, recombinant TOP2A-S1469A revealed more cytoplasmic localization than TOP2A-WT. The suppression of TOP2A-WT glycosylation by the OGT inhibitor increased the cytoplasmic localization of this enzyme. We also observed that more TOP2A-S1469A was diffused in the cytosol in MCF-7/ADR cells than in MDA-MB-231 cells. In Figure 3G, the threonine phosphorylation level of TOP2A-S1469A in MCF-7/ADR cells was lower than that in MDA-MB-231 cells, indicating that the interplay between *O*-GlcNAcylation and phosphorylation may also participate in the nuclear localization of TOP2A. However, our data also showed that deletion of TOP2A *O*-GlcNAcylation in MCF-7/ADR cells still does not fully restore the cell's sensitivity to Adm, suggesting that TOP2A glycosylation is not the only factor that can influence the drug resistance of MCF-7/ADR cells. We added clear descriptions of these issues in the revised manuscript and modified some of the text and certain figures.

2. In Fig. 6F, the interactions between TOP2A and OGT were greatly enhanced in the Resistance, compared to the Sensitive. Authors need to explain the reason, what is the underlying molecular mechanism?

Reply

This reviewer is concerned about the interactions between TOP2A and OGT in different drug sensitivity samples. In both breast cancer cells and patient samples, we observed consistent results (Figure 2F and 6F). TOP2A-OGT interactions were enhanced in drug-resistant cells/samples compared to drug-sensitive cells/samples. This is why elevated levels of *O*-GlcNAc-modified TOP2A were detected in resistant cells/samples. The underlying molecular mechanism can be explained in two ways.

First, it has been reported that OGT is largely dependent on the cellular free sugar substrate UDP-GlcNAc levels for enzymatic activity (Journal of Biological Chemistry, 2012, 287(19), 15395-15408). Our previous research (Cell Death and Disease, 2018, 9:485) revealed that the synthesis of UDP-GlcNAc through the hexosamine biosynthetic pathway is upregulated in cancer cells with intrinsic and acquired chemoresistance (MCF-7/ADR cells). This accumulation of the pool of sugar substrate might activate OGT and lead to elevated cellular *O*-GlcNAcylation. In the present study, not only TOP2A *O*-GlcNAcylation but also global *O*-GlcNAcylation levels showed obvious increases in Adm-resistant cells (Figure 2A and Appendix Figure S3), while OGT expression levels were comparable in Adm-sensitive and Adm-resistant breast cancer cells, indicating that the upregulation of UDP-GlcNAc might be pivotal in regulating OGT activation and the OGT-TOP2A interaction.

Second, previous OGT interactome analyses indicate that dysregulated OGT may serve as a hub protein to mediate the interaction of multiple proteins (International Journal of Molecular Sciences, 2021, 22, 9620). In the present study, the TOP2A *O*-GlcNAcylation state regulated the interaction of this enzyme with cell cycle regulators, suggesting that OGT might participate in TOP2A-involved complex assembly. In this sense, changes in the composition of the complex may also affect the interaction between OGT and TOP2A. It is believed that approximately half of nuclear OGT exists in a complex with the auxiliary factor HCFC1 (Proceedings of the National Academy of Sciences, 2011, 108, 2747-2752), which may link OGT to chromatin and TOP2A. We performed additional experiments, and the results showed that the associations between TOP2A, HCFC1 and OGT were increased in

drug-resistant breast cancer cells and patient samples (Figure 2F and 6F). This result suggested that HCFC1 may act as an influential partner to recruit OGT to chromatin-bound TOP2A. We have added a discussion of this issue and revised the related text in the revised manuscript.

3. It is well known that enhancement of drug efflux by overexpressing ABC transporters such as P-glycoprotein is one of main mechanisms involved in cancer drug resistance. Authors need to check whether *O*-GlcNAcylation or *O*-GlcNAcylation of TOP2A influences the expression of P-glycoprotein and/or other ABC transporters subfamily members for elucidation of deepening molecular mechanisms.

Reply

We agree with this reviewer's comment and performed additional experiments to check whether cellular *O*-GlcNAcylation influences the expression of P-gp in drug-resistant breast cancer cells. The expression of this multidrug resistance-related ABC transporter was associated with the cellular response to Adm in breast cancer cells (Figure 2A). Regardless of the *O*-GlcNAcylation levels, P-gp expression levels remained unchanged in MDA-MB-231 and MCF-7/ADR cells. Given that inhibition of *O*-GlcNAcylation with L01 increased sensitivity to Adm (Figure 2D and E), we suggested that *O*-GlcNAcylation potentiates drug resistance in a P-gp-independent manner. We added descriptions of these results in the revised manuscript and modified some of the text and certain figures.

Referee #2

1. Figure 1E states that "TOP2A positively correlates with overall survival in TCGA breast cancer tissues." Instead, the plot suggests that TOP2A expression and survival rate are inversely proportional.

Reply

We accept this reviewer's comment. We made a mistake in the figure legend of Figure 1E. TOP2A expression and survival rate are indeed inversely proportional. We

revised the figure legend as “In TCGA breast cancer tissues, the samples with higher *TOP2A* mRNA levels had shorter overall survival times than those with lower *TOP2A* mRNA levels”.

2. One of the primary therapeutic/killing mechanisms of Adm is the stabilization of TOP2-DNA cleavage complexes (TOP2cc). The authors should measure and compare TOP2Acc in breast cancer cells tested in Figure 2A-C to test if Adm resistance is in part due to reduced TOP2Acc in those cell lines. If *O*-GlcNAcylation enhances TOP2 chromatin binding and cleavage, high *O*-GlcNAcylation should promote cell proliferation but the other hand, it should also increase Adm-induced TOP2Acc (more targets) and hence Adm-induced cell death. Their results suggest the opposite.

Reply

This reviewer suggested that we measure TOP2A-DNA covalent complexes (TOP2Acc) in breast cancer cells to elucidate the relationship between TOP2Acc and TOP2A *O*-GlcNAcylation-related Adm resistance. We agree with this point and performed additional experiments. The amount of TOP2Acc in Adm-treated cells was measured with a band depletion assay. As shown in Figure EV2A, Adm trapped TOP2A into TOP2Acc in Adm-sensitive MCF-7 cells. On the other hand, the amount of Adm-trapped TOP2Acc was reduced in Adm-resistant MDA-MB-231 and MCF-7/ADR cells, indicating that the Adm resistance in these cells is at least in part due to reduced TOP2Acc. We further tested whether site-specific *O*-GlcNAcylation reflects the formation of Adm-induced TOP2Acc (Figure EV2C). *O*-GlcNAcylation site mutation (TOP2A-S1469A) or L01 treatment increased the amount of TOP2A covalently trapped on DNA compared with wild-type glycosylated TOP2A-WT in Adm-resistant breast cancer cells, suggesting that TOP2A *O*-GlcNAcylation impeded the formation of Adm-induced TOP2Acc and reduced cytotoxicity. One possible explanation for this phenomenon is that *O*-GlcNAcylation of TOP2A S1469 might affect the conformation of TOP2A-DNA complexes and antagonize Adm binding and the formation of TOP2Acc. In this sense, although *O*-GlcNAcylation was found to

enhance TOP2A chromatin binding and cleavage in this study, Adm might lose its drug target in resistant cells. We added descriptions of these issues in the revised results and discussion sections. Certain figures have also been revised.

3. To delineate the role *O*-GlcNAcylation in mitigating Adm cytotoxicity, TOP2Acc and induction of DNA double-strand markers (e.g., gH2AX), and/or cell cycle need to be measured in cells w/ and w/o OGT downregulation and L01 treatment.

Reply

This reviewer also suggested that the induction of DNA strand break markers should be measured in cells with/without OGT downregulation and L01 treatment. We agree with this point and performed additional experiments. One of the major Adm activities is poisoning TOP2 and causing DNA damage. In the current study, we tested whether *O*-GlcNAcylation could prevent Adm-induced DNA damage in drug-resistant cells (Figure EV2B). Both OGT knockdown and L01 treatment enhanced DNA damage in MDA-MB-231 and MCF-7/ADR cells, confirming the role of *O*-GlcNAcylation in mitigating Adm cytotoxicity. We have revised the text and figures in the new manuscript.

4. Figure 2G shows that TOP2A was pulled down in the absence of GalT1 Y289L. Why is that?

Reply

This reviewer expressed concerns regarding experiments using GalT1 Y289L in Figure 2G. In this experiment, TOP2A was immunoprecipitated using an anti-TOP2A antibody. Subsequently, the immunoprecipitated fractions were subjected to *O*-GlcNAc labeling with GalT1 Y289L using UDP-GalNAz (labeled with azide), followed by a click reaction with alkyne-biotin. The labeled proteins were analyzed using streptavidin-HRP. Thus, TOP2A immunoprecipitation was performed before GalT1 Y289L-induced labeling. In the revised manuscript, we added clear descriptions of the procedures of this experiment.

5. According to the authors, truncated TOP2A variants were generated in a prokaryotic vector. How did they express in HEK293 cells? Primers and sequencing for generation and validation of all the plasmids including HA-TOP2A must be provided.

Reply

This reviewer was concerned about the expression of truncated TOP2A variants in HEK293T cells. In fact, we expressed these TOP2A variants in OGT-overexpressing HEK293T cells. The results showed that TOP2A-C but no other TOP2A variants could be *O*-GlcNAcylated in HEK293T cells. However, there were some nonspecific bands in immunoprecipitation assays. We provided this result in Appendix Figure S4.

The primer sequences used in this study are provided in Appendix Table S5 in the revised manuscript. The raw data of wild-type and *O*-GlcNAcylation site mutated TOP2A sequencing were provided in the Source data files.

6. Figure 2I shows diffusion of TOP2A when *O*-GlcNAcylation is suppressed. This is strange. TOP2A is a nuclear protein, and it is simply impossible that TOP2A delocalizes to cytoplasm under any circumstances. Please provide explanations.

Reply

This reviewer also expressed concerns regarding mislocalization of TOP2A-S1469A in Figure 3I. Although TOP2A is a well-known nuclear protein, this enzyme has been reported to diffuse in the cytoplasm under particular circumstances. The literature as early as 1997 revealed that TOP2A was detected in both the cell nucleus and cytoplasm of cell lines and normal and tumor tissues (British Journal of Cancer (1997) 75(9), 1340-1346). Further studies have demonstrated that the nuclear localization signal (NLS) sequence in TOP2A is located between amino acids 1454-1497 (Nucleic Acids Research, 45(10), 2017, 5995–6010) in the C-terminal domain (CTD). The CTD is important for the proper subcellular localization of

TOP2A, and the loss or alteration of the CTD could lead to TOP2A cytoplasmic mislocalization in yeast and mammalian cells (Nucleic Acids Research, 25(15), 1997, 3135-3142; Nucleic Acids Research, 35(11), 2007, 3810-3822). Deletion of TOP2A residues 1174-1446, which overlap with the CTD, disrupts nuclear localization and mitotic chromatin association despite retaining the major NLS, suggesting additional contributions from other residues within the CTD (Int. J. Mol. Sci. 2019, 20, 1238). It was also reported that a truncated cytoplasmic TOP2A (deficiency of amino acids 1423-1489) in lung cancer cells (H209/VP) contributes to VP-16 drug resistance (International journal of cancer, 2000, 85(4), 534-539). Overall, these results suggest that multiple sequences, including the NLS within the CTD, contribute to the robust nuclear localization of TOP2A. The TOP2A CTD is rich in residues subject to posttranslational modifications (PTMs) such as phosphorylation, SUMOylation, ubiquitination and acetylation, which have been found to influence its catalytic activity, protein stability and subcellular distribution. We report that TOP2A *O*-GlcNAcylation occurs at S1469, which is located in the TOP2A NLS. We speculate that the *O*-GlcNAcylation and other PTMs of particular residues within the CTD, especially in the NLS of TOP2A, could control the enzyme's ability to bind chromatin and then impact its subcellular localization, with a rapid exchange between molecules in the chromatin and cytosolic pools apparently governing TOP2A's biological function. The precise mechanisms underlying this regulation remain to be further identified.

To further demonstrate the cytoplasmic localization of non-glycosylated TOP2A, additional experiments were performed (Figure 3H). The cytoplasm was isolated from HA-TOP2A-WT- or HA-TOP2A-S1469A-overexpressing MDA-MB-231 and MCF-7/ADR cells, and cytoplasmic TOP2A was detected using an anti-HA tag antibody. Consistent with immunofluorescence assays, recombinant TOP2A-S1469A revealed more cytoplasmic localization than TOP2A-WT. The suppression of TOP2A-WT glycosylation by the OGT inhibitor increased the cytoplasmic localization of this enzyme. We also observed that more TOP2A-S1469A was diffused in the cytosol in MCF-7/ADR cells than in MDA-MB-231 cells. In Figure 3G, the

threonine phosphorylation level of TOP2A-S1469A in MCF-7/ADR cells was lower than that in MDA-MB-231 cells, indicating that the interplay between O-GlcNAcylation and phosphorylation may also participate in the nuclear localization of TOP2A. However, our data also showed that deletion of TOP2A *O*-GlcNAcylation in MCF-7/ADR cells still does not fully restore the cell's sensitivity to Adm, suggesting that TOP2A glycosylation is not the only factor that can influence the drug resistance of MCF-7/ADR cells. We added clear descriptions of these issues in the revised manuscript and modified some of the text and certain figures.

7. Gels in Figure 4A-D look like cleavage assay rather than relaxation assay. Did the authors add EtBr to agarose gel? Same goes to Figure 6A.

Reply

We agree with this reviewer's comment. We confused the DNA cleavage assay and relaxation assay. In fact, the experimental procedure we used is a DNA cleavage assay. In the revised manuscript, we added clear descriptions of the procedure used in this study and corrected some of the text and certain figures.

Referee #3

Major comments:

1. One of the first claims in the paper is that TOP2A expression is increased in cells that are resistant to Adm. Yet, the panel that represents this data (Fig 2A) does not very clearly support this claim in that the levels do not seem to be much different. In fact, in Fig 6F, TOP2A levels in the sensitive and resistant patients cells are not different at all. Those two panels actually point to the levels of TOP2A as not being a decisive factor in Adm resistance. Please comment on that.

Reply

This reviewer expressed concerns regarding the exact role of TOP2A expression in Adm resistance. We accept that our data does not support that TOP2A expression is increased in breast cancer cells that are resistant to Adm. In this study, we provide evidence that TOP2A is hyper *O*-GlcNAcylated by OGT in Adm-resistant breast

cancer cells. Although increased TOP2A expression was associated with poor clinical outcomes in breast cancer tissues (TCGA database and tissue microarrays), we could not claim that TOP2A expression governs Adm resistance as a single decisive factor. Instead, our results showed that not only TOP2A but also cellular *O*-GlcNAcylation levels increased correspondingly with the aggressiveness of breast cancer (Figure 1G and H). Moreover, a positive correlation was found between TOP2A and cellular *O*-GlcNAcylation in breast cancer tissues, indicating that the interplay between TOP2A and *O*-GlcNAcylation might be an important point in drug resistance. Furthermore, in cell models, we revealed that TOP2A is hyper *O*-GlcNAcylated in Adm-resistant breast cancer cells. Although knockdown of TOP2A expression reduced breast cancer malignancy, we ascribed it to the fact that while TOP2A expression is inhibited, *O*-GlcNAcylated TOP2A is also inhibited. By comparing the function of *O*-GlcNAcylated TOP2A-WT and non-glycosylated TOP2A-S1469A, we confirmed that TOP2A *O*-GlcNAcylation but not TOP2A expression itself protects cells from Adm-induced cell death. In both Adm-resistant breast cancer cells and tumor tissues, TOP2A expression did not seem to be much different from that in Adm-sensitive cells, while TOP2A *O*-GlcNAcylation was obviously upregulated, suggesting that TOP2A was activated by *O*-GlcNAcylation and that *O*-GlcNAcylated TOP2A antagonizes Adm-induced cell death. We have amended the role of TOP2A expression and corrected the related text in the new manuscript.

2. Fig 2C presents a cell viability assay of MDA-MB-231 cells upon co-treatment with L01/shTOP2A and Adm, but it is not shown how L01/shTOP2A affects cell viability alone. Thus, it is not known if the impact on cell viability is due to OGT inhibition itself or TOP2A silencing itself or if it is seen only during treatment with Adm. This needs to be clarified with additional data. Please add this information either in the main figure or to the supplementary material.

Reply

We agree with this point and performed additional experiments. As shown in Appendix Figure S2 in the revised manuscript, OGT inhibition itself (50 μM L01 for

48 h) or TOP2A shRNA transfection itself did not have a significant impact on the viability of MDA-MB-231 and MCF-7/ADR cells. We have revised the text in the new manuscript.

3. Similarly, in Fig 6G and H is shown the effect of shTOP2A+WT alone but not L01 treatment alone in vivo. Without this control we are not able to know if the effect in the tumor is due to inhibition of TOP2A by the drug or if it is just the effect of the drug treatment itself, since OGT is a known regulator of cell cycle progression by different mechanisms. Without this control the double treatment (shTOP2A+WT+L01) is not informative.

Reply

We agree with this point and performed additional experiments. As shown in Appendix Figure S5 in the revised manuscript, L01 treatment (1 mg/kg, tail vein injection every other day for 20 days) alone reduced the tumor volumes for MDA-MB-231 and MCF-7/ADR xenografts. This result was consistent with that in the shTOP2A+WT+L01 group, as shown in Figure 6G and H. The rescue of TOP2A-WT expression in shTOP2A cells could be considered to recover the biological function of *O*-GlcNAcylated TOP2A. Corresponding to results in the glycosylation site-specific deleted group (TOP2A-S1469A), we believe that both TOP2A *O*-GlcNAcylation deletion and L01-induced cellular *O*-GlcNAcylation inhibition could reduce breast cancer cell proliferation and drug resistance. Although we did not observe that L01 induced cell death in breast cancer cells in our in vitro cell model presented in Appendix Figure S2, continuous administration of L01 *in vivo* resulted in the inhibition of O-GlcNAcylation, which could suppress tumor growth in our *in vivo* model. We have revised the text in the new manuscript.

4. The authors state "An increase in cellular *O*-GlcNAcylation indicated elevated cell survival and colony formation frequency in breast cancer cells under Adm treatment" (line 149-151), however there is no data indicating that those two phenotypes are associated functionally. Please rephrase it.

Reply

We agree with this reviewer's comment and rephrase this sentence as "An increase in cellular O-GlcNAcylation was found in Adm resistant cells with elevated cell survival and colony formation frequency under Adm treatment."

5. Line 153-154, "Adm-resistant MDA-MB-231 and MCF-7/ADR cells, OGT shRNA transfected cells showed increase sensitivity to Adm.". The text does not refer to any panel in the figures and these data do not seem to be in the paper. Please clarify.

Reply

We accept this reviewer's comment. We made a mistake in this sentence. In fact, we used TOP2A shRNA in this experiment. We have revised the text in the new manuscript.

6. In Fig 5A the authors show a colony formation assay and in the text they claim "The number of colonies was higher in TOP2A-WT group and lower in the TOP2A-S1469A group after Adm treatment, affirming that TOP2A glycosylation promoted Adm resistance in breast cancer cells" (lines 255-258). The authors cannot claim that since the number of colonies in the control condition is also different in the WT and S1469A groups. If the authors want to make this claim they need a quantification of the colony formation experiments to show that even with the difference in control conditions, you still have a significant difference between WT and mutant after treatment with Adm.

Reply

We accept this reviewer's comment and performed quantitative analysis in the colony formation experiments. The results revealed that even with the difference in control conditions, the colony formation rate (Adm treatment/control) still have a significant difference between TOP2A-WT and TOP2A-S1469A groups after treatment with Adm. We have revised the text and figure in the new manuscript.

7. The authors use p27 as a cell-cycle marker and show that p27 levels go up when OGT is inhibited with L01 (Fig 5E) and go down in patient samples that express higher levels of OGT (#3-5) (Fig 6F). However, p27 has been already described as having its levels regulated by OGT levels/activity (Caldwell et al., 2010) as well as p21 (de Queiroz et al., 2022). Thus, the regulation of p27 shown in the paper is not surprising and is likely not related to TOP2A modification. Because of this, it is suggested that the authors do not use p27 as a marker in the abovementioned figures. If the authors wish to keep it in the figure the should add cite the published evidence about p27 and OGT in the discussion session.

Reply

This reviewer is concerned about the role of p27 in TOP2A *O*-GlcNAcylation-related drug resistance. We accept that OGT inhibition by L01 treatment could affect p27 expression as previous reports described. However, in the present study, we showed that *O*-GlcNAcylated TOP2A (TOP2A-WT) reduced p27 expression, while site-specific *O*-GlcNAcylation deletion (non-glycosylated TOP2A-S1469A) led to opposite result (Figure 5E). In Figure 6F, no clear correlation was found between OGT expression levels and Adm sensitivity. Instead, drug resistant samples with much higher TOP2A *O*-GlcNAcylation levels reduced p27 expression compared with that of sensitive samples. These data suggested that TOP2A *O*-GlcNAcylation plays a role in regulating p27 expression. To further illustrate the role of TOP2A *O*-GlcNAcylation in cell-cycle related gene expression, the expression of another negative regulator of the cell cycle p57 was analyzed in the revised manuscript. Similar to p27, hyper TOP2A *O*-GlcNAcylation reduced the expression of p57 in breast cancer cells and tissues (Figure 5E and 6F). We have cited the published evidence about p27 and OGT in the revised manuscript and added a discussion of this issue.

8. In Fig 6 C-E the authors show a blot for the overexpression of TOP2A. They do not specify in the figure legend in which cell line the blot is from. Also, the authors should show blots for all 3 cell lines to show that the expression levels of

the plasmids were similar, like in the one blot shown in the panel.

Reply

We accept this reviewer's comment. In the revised Figure 6C-E, we have showed the results of Western blots for all 3 cell lines and modified the figure legends.

9. Still regarding the in vivo treatment with L01, we have had experience with in vivo treatment with OSMI-1 and we do not see an effect in mice, meaning *O*-GlcNAc levels are not changed in mouse tissues (neither tumor nor normal tissue) after treatment with OSMI-1 for 27 days. To prove your treatment with L01 is indeed inducing OGT inhibition in mice, please add a GlcNAc blot of your mouse tissue samples to Fig 6.

Reply

We accept this reviewer's comment and performed additional *O*-GlcNAcylation Western blot using mouse tumor samples. The results further confirmed that L01 injection (1 mg/kg every other day, for 22 days) could reduce the cellular *O*-GlcNAcylation in mouse tumor tissues (Figure 6J). Similar results were reported by research using OSMI-1/OSMI-4 in mouse model (Journal of Pineal Research, 2021, 71(3): e12765; Proceedings of the National Academy of Sciences, 2023, 120 (13), e2216796120). We have revised the text and figure in the new manuscript.

10. The discussion needs a lot of work. This section of the paper, that is supposed to discuss matters involving the subject of the work, is not really discussing anything. Most of the discussion is facts about TOP2A, that could even be included in the introduction, or description of the results shown in the Results section. Please work on this section.

Reply

We accept this reviewer's comment and the discussion section have been rewritten.

Minor comments:

1. Throughout the entire paper the "O" in *O*-GlcNAcylation need to be italicized as well as the term "in vivo".

Reply

We accept this reviewer's comment and the section have been corrected.

2. Throughout the entire paper the authors use the word "obviously" to describe their results (e.g. line 191 and 201). This word is not appropriate to describe results, please change it to a different word.

Reply

We agree with this reviewer's comment and rephrase this word as "clearly".

3. In the synopsis figure please add a legend for the blue and gray ellipses in it. It is not very clear what they represent.

Reply

We agree with this reviewer's comment and added legends in this figure.

4. Line 64, 69, substitute the word "poison" for a better fitting one.

Reply

We agree with this reviewer's comment and rephrase this word as "chemotherapy drug".

5. Line 101, substitute the word "enigmatic" for a better fitting one.

Reply

We agree with this reviewer's comment and rephrase this word as "unclear".

6. Line 126, the data described has no indication of a Figure and panel. Please add.

Reply

We agree with this reviewer's comment. This data is a description of Figure 1E. We have revised the text in the new manuscript.

7. Line 284, substitute the word "poisoning" for inhibiting.

Reply

We agree with this reviewer's comment and this word have been corrected.

8. Line 309-327, there are multiple references to a Figure 7 that does not exist in the manuscript, but instead they look like they should refer to later panels on Figure 6. Please rename the figure references in this part of the manuscript.

Reply

We made a mistake in these sentences. We correct this mistake in the revised manuscript.

9. In Figure legend 1, panel C please clarify what are the green and blue bars.

Reply

The blue bar represents log_2FC is greater than 1. The green bar represents that log_2FC is less than 1. The descriptions have been added in revised figure.

10. In Figure 2, panel B shows two different experiments using two different techniques so they should be two different panels. Please separate them.

Reply

We agree with this reviewer's comment, and Figure 2B have been separated into two figures.

11. In Figure legend 2 (line 81) panel H, shTOP2A should be shOGT.

Reply

We agree with this reviewer's comment, and correct it in the revised manuscript.

12. In Figure 4 panel A, the lane designations say "MCA-MB-231", please correct it to MDA-MB-231.

Reply

We sincerely thank the reviewer for careful reading. We have corrected the "MCA-MB-231" into "MDA-MB-231" in the revised manuscript.

13. In Figure 4 panel C, the *O*-GlcNAc blot is not well aligned with the others.

Reply

We agree with this reviewer's comment and the Figure 4C have been revised.

14. In Figure 5 panel B and C, the G2 bars cannot be seen. Please change the color of it for better visualization.

Reply

The G2 bars in Figure 5B and C have been revised.

15. In Figure legend 5, the designation of panels D and E are a bit confusing. Please change it for clarification.

Reply

We agree with this reviewer's comment, and the legends for Figure 5D and E have been revised.

16. In Figure legend 6 panels C-E the authors mention T test was used for statistical analysis but they do not say what conditions were compared. Since T test is for comparison of 2 groups and they have 3 in each graph, please clarify.

Reply

We agree with this reviewer's comment, and add clearly descriptions in the revised figures to show which groups we compared.

Dear Dr. Liu,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that I asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study in EMBO reports.

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If n<5, please show single datapoints for diagrams.

- Please make sure that all figure panels are called out separately and sequentially (main, EV and Appendix figures). Presently, there are no separate callouts for Fig. EV5A-C. Please check.
- Please add scale bars of similar style and thickness to the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, several scale bars are too small or hardly visible (e.g. in Fig. 6K).

- The two panels (upper and lower) in Appendix Fig. S1 seem to be identical. Should this be the case?

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Prof. Yubo Liu
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The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
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Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Materials and Methods
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods Figures legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figures legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figures legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	Materials and Methods
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Yes	Materials and Methods
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Materials and Methods
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Yes	Materials and Methods Reference