

Top2a complexes with Trim28 to orchestrate stemness of embryonic stem cells and tumorigenesis

Corresponding Author: Professor Mangmang Li

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Combining genomic and proteomic strategies, Wang et. Al have provided lines of evidence that Top2a, together with Trim28 contribute to the embryonic stem cell phenotype and tumorigenesis. Strengths of the paper include: pursuit of an unbiased proteomic approach that identified the Trim28-Top2a complex within the context of mediating competency for sustaining pluripotency; strong evidence for Top2a and Trim28 co-activation of gene expression for supportive metabolic parameters associated with glycolysis in embryonic stem cells and breast tumor; and findings that indicate targeting Top2a and Trim28 have anti-tumor effects.

Results are presented that mechanistically implicate promoter bivalency. Epigenetic control in response to Top2 and Trim28 are based on direct assessments of chromatin accessibility by ATAC-seq and histone modifications that contribute to transcriptional activation and suppression. Results are presented and interpreted in a readily accessible manner with appropriate references to public findings. The mechanistic implications for bivalency have biological and clinical relevance. Here, while not mandatory, the findings can be reinforced by a ChIP and reChIP approach.

Reviewer #2

(Remarks to the Author)

Wang et al describe the association of Top2a with Trim28 to modulate the bivalent state of embryonic stem cells to an active states and promote tumorigenesis in TNBC, enriching the cancer stem cell population. Although the author uses an unbiased approach to identify the association of Top2a and Trim28 in ESCs the relevance to cancer was not very clear. Also the author did not provide any experimental evidence to show recruitment of NuRD complex to the chromatin via the association of Trim28 and Top2a.

Furthermore, there are certain concern that need to be addressed for the betterment of the manuscript.

1. In line 61, it should be "BROMO" instead of BMORO. In line 69, it will be "MDM2". The authors did not clearly mentioned the proteins that they are talking about whether it is human or mouse counterpart, all throughout the manuscript.

2. In all western blot molecular weight marker is missing. Please provide the same. What percentage of input was used in each IP experiment? Please provide the details in Figures and update the same in the method section.

3. For all bar graphs throughout the manuscript, please plot individual data points instead of just showing mean and sem.

4. What statistical analysis method was used to measure the statistical significance. Knockdown of Top2a or Trim28 affecting levels of Nanog, Oct4 at RNA level is not appreciable, however the author shows that it is highly significant. In figure 3B, the author shows Trim28 overexpression, however the extent of overexpression is not evident from the western blots. Please provide quantification of the blots.

5. The IF images are of poor quality in figure 3C, S3F, S3I. Please provide better quality images for supporting the hypothesis. Also provide merge images with DAPI.

The bright field images for colony formation assay is not of good quality. Please provide better quality images.

6. Please provide western blots for H3K4me3, H3K27me3 in figures S3E and S3H.

Reviewer #3

(Remarks to the Author)

The authors explore the function of the Top2a–Trim28 complex in mouse embryonic stem cells (mESCs) and in MDA-MB-231 breast cancer cells. Using native ChIP for Top2a, the authors identified Trim28 as a potential novel cofactor. Subsequent IP and recombinant protein assays further support an interaction between Top2a and Trim28. This interaction appears enriched in mESCs but not in mouse embryonic fibroblasts (MEFs). Additional cellular experiments show that Trim28 modulates Top2a protein levels and that both genes are required for maintaining mESC identity. The authors propose that Trim28 cooperates with Top2a to maintain pluripotency and enhance induced pluripotency through epigenetic regulation. Top2a knockdown results in a global reduction of H3K4me3 and H3K9ac, along with increased levels of the repressive marks H3K27me3 and H3K9me3. However, genome-wide ChIP-seq datasets do not correlate well with the global changes observed by Western blot. The authors propose that the Top2a–Trim28 complex regulates a subset of metabolic genes involved in glycolysis. While a promoter bivalency model is suggested, the evidence is restricted to a few loci and lacks genome-wide co-localization analysis of both proteins and the effects of knockdown on their occupancy. The absence of spike-in controls in ChIP-seq likely limits the ability to quantify global histone modification changes. Although the Top2a–Trim28 interaction is potentially novel, the manuscript has several major weaknesses across cell biology, biochemistry, and genome-wide data analyses. Many results are descriptive, and it remains unclear whether this interaction is functionally significant for the mESC epigenetic landscape. TRIM28's roles in heterochromatin formation and pluripotency are well established, and Top2a is known for its roles in the cell cycle, mitosis, and Pol II regulation. Therefore, it is important to either connect these known activities to the proposed interaction or identify a unique function for this complex in mESCs. Major concerns and minor concerns are listed below.

Major Concerns

1. Interaction specificity in mESCs

The authors claim that the Trim28–Top2a interaction is specific to mESCs, yet both proteins show very low expression in MEFs and differentiated cells. Can the authors confirm whether colocalization frequency on chromatin differs between mESCs, differentiated cells, and MEFs using ChIP-seq data? Additionally, the interaction is later observed in MDA-MB-231 cells, raising the question of what regulates this interaction.

2. Figure 1E

In several blots, Top2a appears as smears, broad bands, or multiple bands, while others show a clean single band. The authors should clarify data quality and explain these inconsistencies.

3. Figure 2: Biochemical analysis

The authors should include Coomassie staining to demonstrate the purity of purified proteins. Details on protein purification (e.g., Ni-NTA purification steps, final buffer composition) are insufficient. It is also unclear whether the observed interactions are DNA-mediated. DNase or Benzonase treatment, or preparation of DNA-free nuclear extracts (e.g., via high salt), should be performed to confirm a direct interaction.

For Figure 2E, the authors state that HP1BD and PHD domains are required. However, Trunc 7 still interacts, and Trunc 2 (lacking HP1BD) also interacts. The logic is unclear. Coomassie staining and replicate analyses are needed, as the input concentrations of recombinant proteins appear highly variable. Methods for the His-tag pulldown assay should be fully described.

4. Figure 3: Expression analyses

Nanog expression in Trim28 NC appears more altered than indicated by the “n.s.” label. The authors should clarify the statistical approach used.

Although Trim28 mRNA increases, the Western blot does not show elevated Trim28 protein. Given that Trim28 overexpression alters Top2a levels, and no kinetic analyses are provided, it is hard to conclude whether Trim28 rescues differentiation phenotypes or whether Top2a knockdown efficiency differs across samples. ICRF-193 treatment produces similar phenotypes, but clarification of experimental timing, knockdown duration, and overexpression windows is needed.

5. Molecular mechanism and upstream function

If Trim28 functions upstream of Top2a, it is unclear when and where the Top2a–Trim28 complex is required. The mechanism by which Trim28 rescues differentiation phenotypes by restoring Top2a expression needs clarification. The conclusion that the complex “maintains open chromatin” is not supported, since Trim28 appears to regulate Top2a levels directly. Evidence of functional cooperation in chromatin accessibility is lacking.

6. Cell-cycle effects

Top2a deletion causes global loss of H3K4me3 and H3K9ac, and increased repressive marks. These changes may be secondary to cell-cycle arrest rather than specific epigenetic regulation.

7. Cell-type-specific interaction

The authors propose that the interaction is cell-type specific, yet it also occurs in recombinant bacterial proteins lacking post-translational modifications. This weakens the argument that cell context regulates the interaction.

8. “Mutually exclusive” conclusion

The statement that “Top2a and Trim28 may be mutually exclusive in MEFs” is unsupported. No results demonstrate mutual exclusivity.

9. Figure 4 – Reprogramming assays

Figures 4C, 4E, and 4G show substantial variability in control iPSC generation efficiency. The authors should provide actual GFP-positive cell counts and clarify these differences.

For Figure 4H, differences between Luc control and Trunc3 or HP1BD-PHD appear significant. The statistical approach and p-value calculations should be more clearly described.

10. Domain requirements

Line 219 claims that HP1BD-PHD is necessary but not sufficient to rescue the phenotype. No data demonstrate necessity. It is unclear why only three mutants were examined, and why other truncations (e.g., Trunc5, Trunc6) were not included.

11. RNA-seq interpretation

Line 235 states that Trim28 directly regulates more genes than Top2a. Given that knockdown efficiencies and off-target effects differ, this conclusion cannot be made from RNA-seq alone.

12. Global histone-mark inconsistencies

Heatmaps show weak correlation between differential expression and histone modification changes. More quantitative analyses (e.g., box plots) are needed. The discrepancy between Western blot results and ChIP-seq signals is likely due to lack of spike-in controls. When global histone levels change, spike-in normalization is often required.

13. ChIP-seq for Trim28 and Top2a

Given the weak overlap between RNA-seq and ChIP-seq, the authors should generate their own Trim28 and Top2a ChIP-seq datasets. Differences from published datasets may reflect culture conditions or cell line variation. It would be useful to examine how Trim28 overexpression or Top2a knockdown affects each protein's genomic occupancy.

14. ATAC-seq analysis

The current analysis focuses only on selected genes. Unbiased differential peak calling is needed to determine whether accessibility changes are global or locus-specific.

15. Figure 6

ChIP-seq and qPCR analyses show locus-specific patterns that differ from the global changes reported earlier. Spike-in controls are necessary for reliable quantification. Genome-wide analyses should assess both global shifts and locus-specific alterations.

16. Clinical data interpretation

Lines 318–319: The subset of samples with high expression of both genes is small. The authors are recommended to analyze patient survival or clinical outcomes relative to expression levels.

17. Breast cancer cell lines vs cancer stem cells

MDA-MB-231 cells are not considered cancer stem cells in general. The authors can further enrich for BCSC populations (e.g., ALDH1 high, 3D culture, sphere assays) and test whether Trim28 or Top2a knockdown specifically affects these cells. The rationale for using a basal-like cancer cell line and connecting these results to mESC biology is insufficient.

Minor concerns

1. Methods

The methods section should be moved to the main text. The figure legends in general do not contain enough details.

2. Fig.1G Top2a label should be adjusted

3. Analysis of CbioPortal should be Analysis of cBioPortal.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Comments have been sufficiently addressed and the manuscript is suitable for publication

Reviewer #2

(Remarks to the Author)

The manuscript have been revised extensively. The authors have tried to address most of the comments. Therefore, the manuscript is suitable for acceptance.

Reviewer #3

(Remarks to the Author)

The revised manuscript has been substantially improved. Although some minor concerns remain, the authors have addressed the majority of the previous issues.

Figure 3E appears to show a similar level of interaction between Trun7 and Top2a. However, the authors do not discuss potential concerns regarding the large variation in the quality and abundance of the recombinant proteins. Some of the truncation mutants are barely detectable by SDS-PAGE. In addition, many of the bands shown in this figure appear likely to be saturated, making reliable quantification difficult. As a result, the conclusions drawn from these data are not fully supported by the presented results.

In Figure 3B, Trim28 overexpression does not appear substantially different from the control by visual inspection. The authors should quantify the western blot band intensities to support this conclusion.

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Point-by-point responses to Reviewers' comments

Reviewer #1 (Remarks to the Author):

Combining genomic and proteomic strategies, Wang et. Al have provided lines of evidence that Top2a, together with Trim28 contribute to the embryonic stem cell phenotype and tumorigenesis. Strengths of the paper include: pursuit of an unbiased proteomic approach that identified the Trim28-Top2a complex within the context of mediating competency for sustaining pluripotency; strong evidence for Top2a and Trim28 co-activation of gene expression for supportive metabolic parameters associated with glycolysis in embryonic stem cells and breast tumor; and findings that indicate targeting Top2a and Trim28 have anti-tumor effects. Results are presented that mechanistically implicate promoter bivalency. Epigenetic control in response to Top2 and Trim28 are based on direct assessments of chromatin accessibility by ATAC-seq and histone modifications that contribute to transcriptional activation and suppression. Results are presented and interpreted in a readily accessible manner with appropriate references to public findings. The mechanistic implications for bivalency have biological and clinical relevance. Here, while not mandatory, the findings can be reinforced by a ChIP and reChIP approach.

We greatly appreciate the reviewer's positive assessment of our work. We fully agree that performing reChIP experiments would further reinforce the evidence. Unfortunately, since the first author has already graduated, we are unable to conduct these experiments. We sincerely ask for the reviewer's understanding. We have noted this limitation in the revised Discussion section (**revised Discussion, lines 426-432**) as follows:

“However, due to the current technical challenges in obtaining high-quality ChIP-seq data for Top2a, Trim28 and core factors of PRC2 and NuRD in mESCs with modified levels of Top2a and Trim28, we were unable to depict the genome-wide association among Top2a, Trim28, and bivalency or to fully elucidate how PRC2 and NuRD are involved in this regulatory axis. Nevertheless, future ChIP-qPCR or reChIP experiments following Top2a or Trim28 perturbation will be needed to directly test whether these complexes are recruited via the Top2a-Trim28 complex.”

Reviewer #2 (Remarks to the Author):

Wang et al describe the association of Top2a with Trim28 to modulate the bivalent state of embryonic stem cells to an active states and promote tumorigenesis in TNBC, enriching the cancer stem cell population. Although the author uses an unbiased approach to identify the association of Top2a and Trim28 in ESCs the relevance to cancer was not very clear. Also the author did not provide any experimental evidence to show recruitment of NuRD complex to the chromatin via the association of Trim28 and Top2a.

We thank the reviewer for these important comments.

Regarding the relevance to cancer: Although we have provided multiple lines of evidence linking the Top2a-Trim28 complex to human triple negative breast cancer line MDA-MB-231 (Fig. 7, S5A, S5B), we agree with the reviewer that data derived from only one TNBC cell line are insufficient to establish broad relevance to cancer. We have therefore toned down our claims in the revised Discussion and explicitly acknowledged this limitation. The revised text now reads as follows (**revised Discussion, lines 492-495**):

“Nevertheless, we acknowledge that our cancer-related data are derived exclusively from a single TNBC cell line, providing preliminary evidence linking the Top2a-Trim28 complex to breast cancer. Future studies using additional models are required to extend these findings to generalized cancer types.”

Regarding no experimental evidence showing recruitment of NuRD complex to chromatin via Top2a-Trim28 association: We fully agree with the reviewer that, due to current technical challenges, in this study we did not provide direct evidence for the recruitment of the NuRD (or other chromatin modifier complexes) to chromatin via the Top2a-Trim28 complex. To acknowledge this limitation, we have now added a clearer statement and future direction in the revised Discussion as follows (**revised Discussion, lines 426-432**):

“However, due to the current technical challenges in obtaining high-quality ChIP-seq data for Top2a, Trim28 and core factors of PRC2 and NuRD in mESCs with modified levels of Top2a and Trim28, we were unable to depict the genome-wide association among Top2a, Trim28, and bivalency or to fully elucidate how PRC2 and NuRD are involved in this regulatory axis. Nevertheless, future ChIP-qPCR or reChIP

experiments following Top2a or Trim28 perturbation will be needed to directly test whether these complexes are recruited via the Top2a-Trim28 complex.”

Furthermore, there are certain concern that need to be addressed for the betterment of the manuscript.

1. In line 61, it should be “BROMO” instead of BMORO. In line 69, it will be “MDM2”. The authors did not clearly mentioned the proteins that they are talking about whether it is human or mouse counterpart, all throughout the manuscript.

We thank the reviewer for pointing out the spelling errors in the manuscript. We have made the corresponding corrections accordingly.

Regarding the species of the proteins discussed throughout the manuscript, we sincerely apologize for any confusion caused. Our study investigated the functions of Top2a and Trim28 in multiple model systems, including mouse embryonic stem cells (mESCs), mouse embryonic fibroblasts (MEFs), and the human breast cancer cell line MDA-MB-231. Accordingly, the protein sequences used in each experimental context correspond to the respective species (mouse or human). We have also ensured the consistent use of appropriate species-specific gene and protein symbols throughout the text for clarity.

2. In all western blot molecular weight marker is missing. Please provide the same. What percentage of input was used in each IP experiment? Please provide the details in Figures and update the same in the method section.

We thank the reviewer for these important points. We have now included molecular weight markers in all relevant western blot panels, as requested.

Regarding the input amount for each IP experiment: For endogenous IP, 50 µg out of 1 mg total lysate (5% input) was used as input; for chromatin-associated complexes, 15 µg out of 1 mg (1.5% input); and for recombinant protein co-IP, 3 µL out of 20 µL (15% input). These details have been clearly described in the co-IP section of the revised Materials and Methods as follows (lines 590-601):

“Co-Immunoprecipitation (co-IP)

For endogenous IP, 1 mg of cell lysate prepared in RIP150 buffer was used. A 50 µg aliquot of lysate (or 15 µg for chromatin-associated complexes) was reserved as the input control. The remaining lysate (950 µg) was incubated with 1 µg of rabbit IgG (control) or target antibody overnight at 4°C with gentle rotation. For recombinant

protein co-IP, purified His-Top2a (1 μ g) and His-Trim28 (1 μ g) were combined in a total volume of 20 μ L. A 3 μ L aliquot (15% of total protein) was reserved as the input control. The mixture was incubated with 1 μ g anti-His tag antibody (Thermo Fisher Scientific, MA1-21315) for 1 hour at 4°C. After antigen-antibody incubation, Protein A/G magnetic beads (Bio-Rad, 161-4013), pre-blocked with 5% BSA, were then added and incubated for 2 hours at 4°C. The beads were washed five times with wash buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 0.1% NP-40). Bound proteins were eluted by boiling in 2 $\times$ SDS sample buffer for 5 minutes at 95°C.”

3. For all bar graphs throughout the manuscript, please plot individual data points instead of just showing mean and sem.

We thank the reviewer for this valuable suggestion. We have revised all bar graphs throughout the manuscript as requested. They are now presented as dot plots (showing individual data points for each independent replicate) with the mean and standard error of the mean (SEM) indicated.

4. What statistical analysis method was used to measure the statistical significance. Knockdown of Top2a or Trim28 affecting levels of Nanog, Oct4 at RNA level is not appreciable , however the author shows that it is highly significant. In figure 3B, the author shows Trim28 overexpression, however the extent of overexpression is not evident from the western blots. Please provide quantification of the blots.

We thank the reviewer for raising these points.

Statistical analysis method: All statistical analyses were performed using GraphPad Prism. The specific test applied depended on the experimental design: one-way ANOVA was used for comparisons involving a single categorical variable, and two-way ANOVA was used for experiments involving two categorical variables. These details have been explicitly stated in the figure legends and the revised Methods section as follows (lines 799-807):

“Statistical analysis

Data are presented as mean $\pm$ SEM ($n \geq 3$ independent experiments). All statistical analyses were performed using GraphPad (version 9.5). For comparisons involving a single categorical variable, one-way ANOVA was used, followed by appropriate post hoc multiple comparisons tests (Dunnett’s test for comparisons against a single control

group, or Tukey's tests for pairwise comparisons). For experiments involving two categorical variables, two-way ANOVA was used followed by Tukey's post hoc test. Statistical significance was defined as follows: ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$."

Nanog and Oct4 mRNA levels after Top2a knockdown (Fig. 3A): The effects of Top2a knockdown (KD) on mRNA levels of Nanog and Oct4 in mESCs are shown as red bars. One-way ANOVA revealed that Top2a depletion reduced Nanog and Oct4 mRNA to approximately 25% and 40% of control (EV-NC si), respectively. The reduction in Nanog was statistically significant ($p < 0.001$), whereas the reduction in Oct4 did not achieve statistical significance. Raw data for the statistical analyses are provided in the [new Supplementary Excel file](#).

Nanog and Oct4 mRNA levels after Trim28 knockdown (Fig. S2A): Lentiviral-shRNA mediated Trim28 KD led to more pronounced and statistically more significant decreases in Nanog and Oct4 mRNA levels than Top2a KD. Raw data for the statistical analyses are provided in the [new Supplementary Excel file](#).

Regarding the Western blot for Trim28 overexpression (OE) in Fig. 3B: We appreciate the opportunity to clarify. The perception of a subtle effect was likely caused by the uniform scaling applied to all blot panels to maintain consistent dimensions across the figure, which inadvertently compressed some lanes and may have diminished the visual contrast. We have now replaced the panel with one that better reflects its original proportions [in the revised Figure 3B](#), and the difference between the Trim28-OE and control groups is visually more apparent.

Finally, we would like to clarify that the quantification for the Western blots in Fig. 3B is actually presented in the bar graphs on the right side of the Figure, as also noted in the figure legend. We apologize if this was not immediately clear.

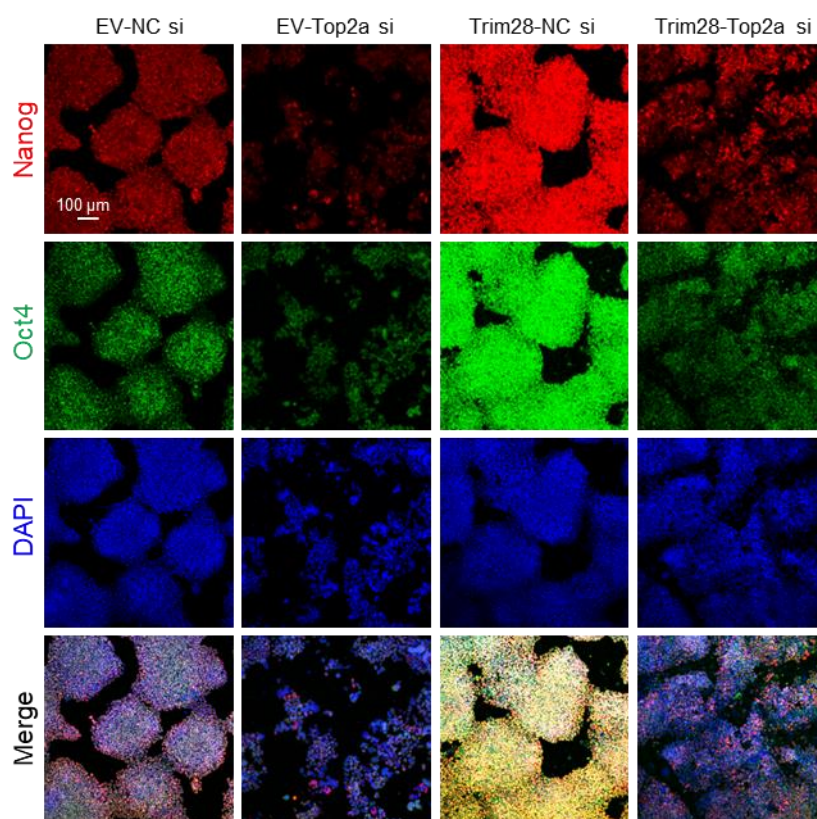
5. The IF images are of poor quality in figure 3C, S3F, S3I. Please provide better quality images for supporting the hypothesis. Also provide merge images with DAPI. The bright field images for colony formation assay is not of good quality. Please provide better quality images.

We thank the reviewer for raising these concerns regarding image quality. We sincerely

apologize that the resolution of the images in the original submitted PDF file may have been compromised during file compression.

Regarding Figures 3C, S3F, and S3I: We have now provided high-resolution TIFF files including DAPI merge channels as requested **in the revised Figs. 3C, S4F, and S4I.**

Specifically for Figure 3C, we intentionally used low-magnification imaging to capture the overall morphological changes in ESC colonies following Top2a KD (which impairs stemness) and Trim28 overexpression (which enhances stemness). Therefore, single-cell resolution was not the objective for this figure. Below is the enlarged Figure 3C.



For revised Figures S4F and S4I, in contrast, we used high-magnification imaging to visualize the increased number of H3K9me3 foci within the nuclei of individual cells following Top2a or Trim28 KD. These images were acquired at sufficient resolution to support our conclusion.

Regarding the colony formation assay images: We agree that the original images lacked sufficient contrast and clarity. As requested, we have replaced the representative bright-

field images with improved, higher-quality versions in **the revised Figures. S1F, S2D, and S4C.**

6. Please provide western blots for H3K4me3, H3K27me3 in figures S3E and S3H.

We thank the reviewer for this comment. We have now added the WB for H3K4me3 and H3K27me3 in **the revised Figures S4E and S4H**, as requested.

Reviewer #3 (Remarks to the Author):

The authors explore the function of the Top2a–Trim28 complex in mouse embryonic stem cells (mESCs) and in MDA-MB-231 breast cancer cells. Using native ChIP for Top2a, the authors identified Trim28 as a potential novel cofactor. Subsequent IP and recombinant protein assays further support an interaction between Top2a and Trim28. This interaction appears enriched in mESCs but not in mouse embryonic fibroblasts (MEFs). Additional cellular experiments show that Trim28 modulates Top2a protein levels and that both genes are required for maintaining mESC identity. The authors propose that Trim28 cooperates with Top2a to maintain pluripotency and enhance induced pluripotency through epigenetic regulation. Top2a knockdown results in a global reduction of H3K4me3 and H3K9ac, along with increased levels of the repressive marks H3K27me3 and H3K9me3. However, genome-wide ChIP-seq datasets do not correlate well with the global changes observed by Western blot. The authors propose that the Top2a–Trim28 complex regulates a subset of metabolic genes involved in glycolysis. While a promoter bivalency model is suggested, the evidence is restricted to a few loci and lacks genome-wide co-localization analysis of both proteins and the effects of knockdown on their occupancy. The absence of spike-in controls in ChIP-seq likely limits the ability to quantify global histone modification changes. Although the Top2a–Trim28 interaction is potentially novel, the manuscript has several major weaknesses across cell biology, biochemistry, and genome-wide data analyses. Many results are descriptive, and it remains unclear whether this interaction is functionally significant for the mESC epigenetic landscape. TRIM28's roles in heterochromatin formation and pluripotency are well established, and Top2a is known for its roles in the cell cycle, mitosis, and Pol II regulation. Therefore, it is important to either connect these known activities to the proposed interaction or identify a unique function for this complex in mESCs. Major concerns and minor concerns are listed below.

We sincerely thank the reviewer for the thorough and insightful assessment of our manuscript. We appreciate the recognition that the Top2a-Trim28 interaction is potentially novel. We also acknowledge that major weaknesses pointed out by the reviewer, and we have carefully considered each concern in revising the manuscript. Below we provide point-by-point response to all major and minor concerns.

Major Concerns

1. Interaction specificity in mESCs

The authors claim that the Trim28–Top2a interaction is specific to mESCs, yet both proteins show very low expression in MEFs and differentiated cells. Can the authors confirm whether colocalization frequency on chromatin differs between mESCs, differentiated cells, and MEFs using ChIP-seq data? Additionally, the interaction is later observed in MDA-MB-231 cells, raising the question of what regulates this interaction.

Regarding the specificity of Top2a-Trim28 interaction: We sincerely thank the reviewer for raising the crucial point regarding the claim of specificity of the Trim28–Top2a interaction for mESCs. We agree with the reviewer's observation that the expression levels of both Top2a and Trim28 are lower in MEFs compared to ESCs (as seen in the input). However, our IP-WB experiments show that using Top2a or Trim28 antibodies, we pulled down nearly equivalent amounts of the respective proteins from both mESCs and MEFs. This suggests that the antibody concentration used was sufficient to immunoprecipitate the majority of the target protein present in MEF lysates, but was likely sub-saturating for the more abundant target in ESC chromatin lysates. Importantly, despite these differences in starting protein levels, comparable total amounts of the bait protein (Top2a or Trim28) were pulled down from both cell types. Therefore, if an equivalent Trim28–Top2a interaction existed in MEFs, one would theoretically expect to co-precipitate a similar amount of the interacting partner. Our results, however, show that Top2a pulled down by the Trim28 antibody, and Trim28 pulled down by the Top2a antibody, were detectable only in mESCs and not in MEFs. This indicates that the Trim28–Top2a interaction is likely either absent or significantly weaker in MEFs compared to ESCs. For better precision, we have rewritten the abstract as follows (**revised Abstract, lines 22-24**):

“Specifically, Top2a directly interacts with Trim28 in mESCs but only weakly, if at all, in mouse embryonic fibroblasts (MEFs).”

Regarding the ChIP-seq experiment in MEFs: We fully agree with the reviewer that performing Top2a and Trim28 ChIP-seq in MEFs would provide valuable insight into their genomic co-localization. We acknowledge this as an important experiment. However, even in mESCs, obtaining high-quality ChIP-seq data for Top2a and Trim28 was challenging; we attempted the experiment multiple times but were unable to achieve satisfactory quality. As a result, we had to rely on publicly available ChIP-seq datasets for these two proteins. For MEFs, given that the expression levels of both proteins are even lower than those in ESCs, obtaining their ChIP-seq data would be substantially more difficult. Therefore, we regret that we are unable to fulfill the reviewer's request at this time. We sincerely hope for the reviewer's understanding.

Regarding the Top2a-Trim28 interaction in MDA-MB-231 cells: Our detection of this

interaction in MDA-MB-231 cells led us to hypothesize that it might be associated with a stem-like cellular state. In support of this, we characterized our MDA-MB-231 cells using the established breast cancer stem cell (CSC) markers CD44 and CD24. We found that approximately 80% of the cells exhibited the CD44⁺/CD24⁻/low phenotype (revised Figures 7E and S7F), indicating a highly enriched CSC population. This finding supports our hypothesis that the Top2a-Trim28 interaction, which we originally identified in mESCs, may be a feature shared by pluripotent embryonic stem cells and certain cancer stem cells, potentially contributing to a common regulatory mechanism for maintaining a stem-like chromatin architecture.

2. Figure 1E

In several blots, Top2a appears as smears, broad bands, or multiple bands, while others show a clean single band. The authors should clarify data quality and explain these inconsistencies.

We thank the reviewer for raising this point. The smear is likely due to overexposure or band distortion during X-ray film development caused by an excessively strong signal. To address this concern, we have replaced the Top2a blots with alternative ones showing clear, sharp bands (revised Figure 1E, Top2a blots in the first line).

3. Figure 2: Biochemical analysis

The authors should include Coomassie staining to demonstrate the purity of purified proteins. Details on protein purification (e.g., Ni-NTA purification steps, final buffer composition) are insufficient. It is also unclear whether the observed interactions are DNA-mediated. DNase or Benzonase treatment, or preparation of DNA-free nuclear extracts (e.g., via high salt), should be performed to confirm a direct interaction.

For Figure 2E, the authors state that HP1BD and PHD domains are required. However, Trunc 7 still interacts, and Trunc 2 (lacking HP1BD) also interacts. The logic is unclear. Coomassie staining and replicate analyses are needed, as the input concentrations of recombinant proteins appear highly variable. Methods for the His-tag pulldown assay should be fully described.

We thank the reviewer for these comments.

Regarding the recombinant protein expression and purification: We have added revised Figure S3 showing Coomassie Brilliant Blue staining of purified His-tagged recombinant full-length (FL) and truncated versions of Top2a (Figure S3A) and Trim28 (Figure S3B) separated on SDS-PAGE. We have also provided a detailed description of protein expression and purification procedures in *E. coli* in the revised Materials and

Methods (lines 571-589).

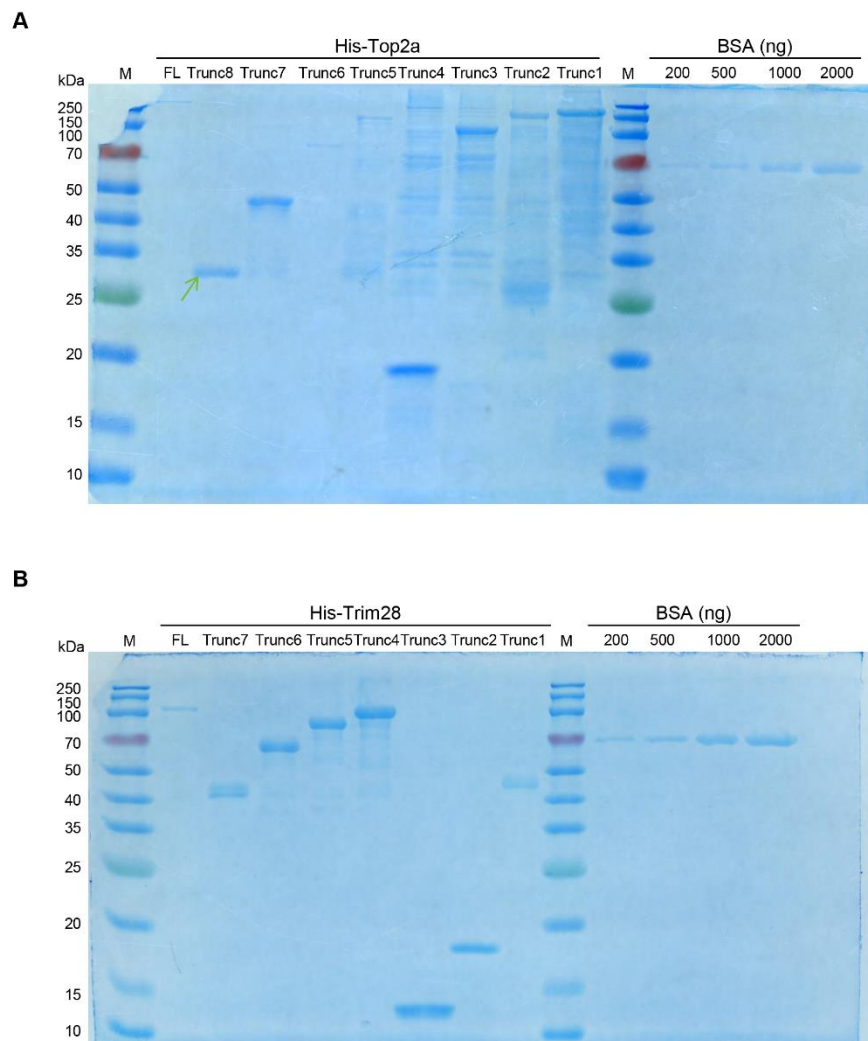


Figure S3

Figure S3. Purification of recombinant full-length (FL) and truncated Top2a and Trim28 proteins. (A-B) Coomassie Brilliant Blue staining of purified His-tagged recombinant FL and truncated versions of Top2a (A) and Trim28 (B) separated on SDS-PAGE. Different amounts of BSA were used as standards to measure the concentration of purified proteins. M indicates protein marker.

Importantly, because the pulldown experiments were performed using purified recombinant proteins, the system lacks cellular components such as chromatin. Therefore, the observed interactions represent direct physical interactions between the

two proteins and are not dependent on DNA or chromatin.

Regarding the domains of Trim28 that interact with Top2a: Our conclusions regarding the Trim28 domains required for Top2a interaction are based on the following observations from truncation mapping (Figure 2E): 1. Trim28 Trunc 1, which lacks the entire Trunc 7 region, interacts with Top2a; 2. Trim28 Trunc 2, which is further truncated from Trunc 1 by deleting the HP1BD domain but retains the PHD, still interacts with Top2a; 3. Trim28 Trunc 3, which retains only the C-terminal Bromo domain, completely loses the ability to interact with Top2a; 4. All truncations containing either the HP1BD or PHD domain (Trunc 1,2,4,5, and 6) can interact with Top2a. From these results, we concluded that the HP1BD and PHD domains of Trim28 are required for the interaction with Top2a. We did not exclude the possibility that Trunc 7 can also interact with Top2a, however, its role in the interaction appears to be less critical compared to the HP1BD and PHD domains.

We hope these clarifications address the reviewer's concerns.

4. Figure 3: Expression analyses

Nanog expression in Trim28 NC appears more altered than indicated by the “n.s.” label. The authors should clarify the statistical approach used.

Although Trim28 mRNA increases, the Western blot does not show elevated Trim28 protein. Given that Trim28 overexpression alters Top2a levels, and no kinetic analyses are provided, it is hard to conclude whether Trim28 rescues differentiation phenotypes or whether Top2a knockdown efficiency differs across samples. ICRF-193 treatment produces similar phenotypes, but clarification of experimental timing, knockdown duration, and overexpression windows is needed.

We appreciate the reviewer's careful observation. For Nanog mRNA levels in Figure 3A, we re-examined our statistical analysis using GraphPad and found that the significance labels for Trim28-NC si (green column) and Trim28-Top2a si (purple column) relative to EV-NC si control had been inadvertently swapped. We have now corrected the labels: Trim28-NC si shows statistical significance ($p < 0.01$), whereas Trim28-Top2a si shows no significant difference (ns) compared to the EV-NC si control.

Regarding the Western blot for Trim28 overexpression (OE) in Figure 3B: We appreciate the opportunity to clarify. The perception of a subtle effect was likely caused by the uniform scaling applied to all blot panels to maintain consistent dimensions

across the figure, which inadvertently compressed some lanes and may have diminished the visual contrast. We have now replaced the panel with one that better reflects its original proportions in **the revised Figure 3B**, and the difference between the Trim28-OE and control groups is visually more apparent.

Regarding the reviewer's concern about experimental timing, knockdown duration, and overexpression windows: We have now clearly described these details in the revised Materials and Methods section. First, a Trim28-overexpressing mESC line was established using the transposon system followed by G418 selection, with an empty vector (EV) as the negative control (**revised Materials and Methods, lines 567-570**). To achieve efficient Top2a knockdown, both EV and Trim28 OE mESCs were transfected daily with siRNA targeting Top2a for 2–4 consecutive days (**revised Materials and Methods, lines 614-615**). For ICRF-193 treatment, both EV and Trim28 OE mESCs were treated with 10 nM ICRF-193 for two days to inhibit Top2a activity, with DMSO as a negative control (**revised Materials and Methods, lines 514-516**). We hope these clarifications address the reviewer's concerns.

5. Molecular mechanism and upstream function

If Trim28 functions upstream of Top2a, it is unclear when and where the Top2a–Trim28 complex is required. The mechanism by which Trim28 rescues differentiation phenotypes by restoring Top2a expression needs clarification.

The conclusion that the complex “maintains open chromatin” is not supported, since Trim28 appears to regulate Top2a levels directly. Evidence of functional cooperation in chromatin accessibility is lacking.

We thank the reviewer for this critical mechanistic question. We have carefully considered the relationship between Top2a and Trim28.

Regarding genetic hierarchy and the limitation of the rescue experiment: We acknowledge that Top2a itself is a Top2a-Trim28 co-activated gene (Figure 5B) and Trim28 acts upstream of Top2a at the expression level. Therefore, we cannot exclude the possibility that Trim28 OE rescues the Top2a KD phenotype, at least in part, by inducing Top2a expression. This is a genuine limitation of our rescue experiment.

Despite this limitation, several independent lines of evidence support a cooperative,

non-redundant function beyond a simple linear pathway:

- Trim28 redistribution upon Top2a KD (Figure 6): Our ChIP-qPCR data show that Top2a KD alone significantly reduces Trim28 enrichment at co-activated gene promoters while increasing it at co-repressed gene promoters, accompanied by corresponding changes in transcription and the H3K4me3/H3K27me3 ratio. This indicates that Top2a directly influences Trim28's genomic distribution, independent of Trim28's effect on Top2a expression.
- Synergistic effects of double KD (Figure 7): In MDA-MB-231 cells (~80% CD44+/CD24-/low CSCs), double KD of TOP2A and TRIM28 exhibits significantly stronger anti-tumor effects than single KDs (Figures 7B-7I), suggesting synergistic cooperation in CSCs that cannot be explained solely by Trim28's regulation of Top2a expression.
- No synergistic effects in MEFs: In contrast to mESCs and CSCs, the absence of Top2a-Trim28 interaction in MEFs (Figures 1G and 1H) correlates with the absence of cooperative functional effects (Figures 3H-3K). These data serve as a negative control.

Taken together, while Trim28 acts upstream of Top2a expression, the ESC-specific interaction, redistribution of Trim28 upon Top2a loss, synergistic anti-tumor effects of double KD, and the absence of these effects in MEFs collectively support a cooperative, non-redundant function of the Top2a-Trim28 complex.

Revised conclusion regarding chromatin accessibility: According to the reviewer's comment #14 on ATAC-seq analysis, we performed additional unbiased genome-wide differential accessibility analyses as requested (see detailed response to Q14). Based on these analyses, we have revised our interpretation of the ATAC-seq data: accessibility changes are not global for both perturbations equally; rather, Top2a depletion causes widespread and structured chromatin accessibility changes, whereas Trim28 depletion has a much more limited, locus-specific effect in this assay. We have now revised the Results accordingly as follows (lines 283-296):

“To determine whether chromatin accessibility changes upon Top2a or Trim28 depletion, we performed transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) followed by unbiased genome-wide differential accessibility analysis. The results revealed a clear difference between the two perturbations (Figure S6I). Top2a KD produced a substantial accessibility phenotype, with 5,666 significantly increased peaks and 2,688 significantly decreased peaks out of 101,143 tested peaks. In contrast, Trim28 KD showed only 52 increased and 52 decreased peaks. Annotation of differential peaks showed that Top2a KD-associated increased peaks were predominantly promoter-associated, whereas decreased peaks were enriched in distal regions (Figure S6J). Consistently, Top2a KD, but not Trim28 KD, modestly decreased chromatin accessibility within defined gene regions for both co-activated and co-repressed genes, supporting Top2a’s role in maintaining open chromatin in ESCs (Figure 5F). These results suggest that other mechanisms may contribute to the activation or repression of Top2a-Trim28 targets.”

6. Cell-cycle effects

Top2a deletion causes global loss of H3K4me3 and H3K9ac, and increased repressive marks. These changes may be secondary to cell-cycle arrest rather than specific epigenetic regulation.

We thank the reviewer for this important mechanistic consideration. We fully agree that Top2a plays multiple roles in cells, including its well-established function in resolving topological stress during DNA replication and chromosome segregation. Therefore, we cannot exclude the possibility that these functions also contribute to epigenetic changes. We have now explicitly stated this limitation in the revised Discussion (**lines 443-446**):

“It is worth noting that, given Top2a’s multiple cellular roles in cell cycle progression (e.g., DNA replication and chromosome segregation), we cannot exclude the possibility that some of the observed phenotypic outcomes may also reflect these additional functions.”

7. Cell-type-specific interaction

The authors propose that the interaction is cell-type specific, yet it also occurs in

recombinant bacterial proteins lacking post-translational modifications. This weakens the argument that cell context regulates the interaction.

We thank the reviewer for this critical observation. We agree that the *in vitro* interaction between recombinant Top2a and Trim28 expressed in *E. coli* suggests that the two proteins have an intrinsic capacity to physically associate. However, we respectfully note that this finding does not exclude the possibility of cell-type-specific interaction. In living cells, protein-protein interactions are dynamically regulated by factors such as expression levels, subcellular localization, post-translational modifications, and competing binding partners. Indeed, our co-IP data show that the Top2a-Trim28 interaction is readily detectable in mESCs but not in MEFs under identical conditions, suggesting that the cellular context determines whether the interaction is established or maintained.

To describe our results more accurately and address the reviewer's concern, we have toned down our results and conclusion in the revised manuscript (**revised Results, lines 108-118**) by using more cautious language as follows:

“To figure out whether Top2a-Trim28 interaction is conserved in other cell types, we compared mouse embryonic fibroblasts (MEFs) with mESCs. In contrast to mESCs, Top2a and Trim28 did not co-immunoprecipitate from native chromatin of MEFs (Figure 1G), suggesting that this association may be influenced by cell-type-specific factors. Immunofluorescence (IF) staining of Top2a and Trim28 in undifferentiated and differentiated mESCs, induced by either leukemia inhibitory factor withdrawal (-LIF) or RA treatment without LIF (-LIF+RA), as well as in MEFs, further showed that Top2a colocalizes with Trim28 in the nucleoplasm of mESCs, but was less evident in differentiated cells (Figure 1H). Collectively, our study identifies the chromatin interactome of Top2a in mESCs and reveals a robust Top2a-Trim28 association on ESC chromatin.”

8. “Mutually exclusive” conclusion

The statement that “Top2a and Trim28 may be mutually exclusive in MEFs” is

unsupported. No results demonstrate mutual exclusivity.

We thank the reviewer for this critical comment. We agree that the “mutually exclusive” statement is not suitable. In fact, our results only showed that in MEFs, Top2a KD further increased Trim28 expression, and Trim28 KD increased Top2a expression, whereas the reciprocal rescue effects observed in mESCs were absent. We now have revised the text to accurately reflect these observations as follows (revised Results, lines 189-190):

“These results suggest that the regulatory relationship between Top2a and Trim28 in MEFs differs from that in mESCs.”

We appreciate the reviewer’s careful reading and thank them for helping us improve the accuracy of our statements.

9. Figure 4 – Reprogramming assays

Figures 4C, 4E, and 4G show substantial variability in control iPSC generation efficiency. The authors should provide actual GFP-positive cell counts and clarify these differences.

We thank the reviewer for this comment. We also noticed that the control iPSC generation efficiencies vary across Figures 4C, 4E, and 4G. This is because each figure panel represents a different experimental setup: all experiments were performed with retroviral transduction of Yamanaka four factors, but 4C involved additional siRNA transfection, 4E involved transduction of a fifth retrovirus, and 4G involved both siRNA and retrovirus. Therefore, the baseline reprogramming efficiencies in the control groups differ accordingly.

To address the reviewer’s concern, we have added **New Figure S5** in the revised manuscript, showing flow cytometry analysis of the percentage of reprogrammed GFP+ cells at the experimental endpoint. These results are highly consistent with the GFP+ colony count data in Figure 5.

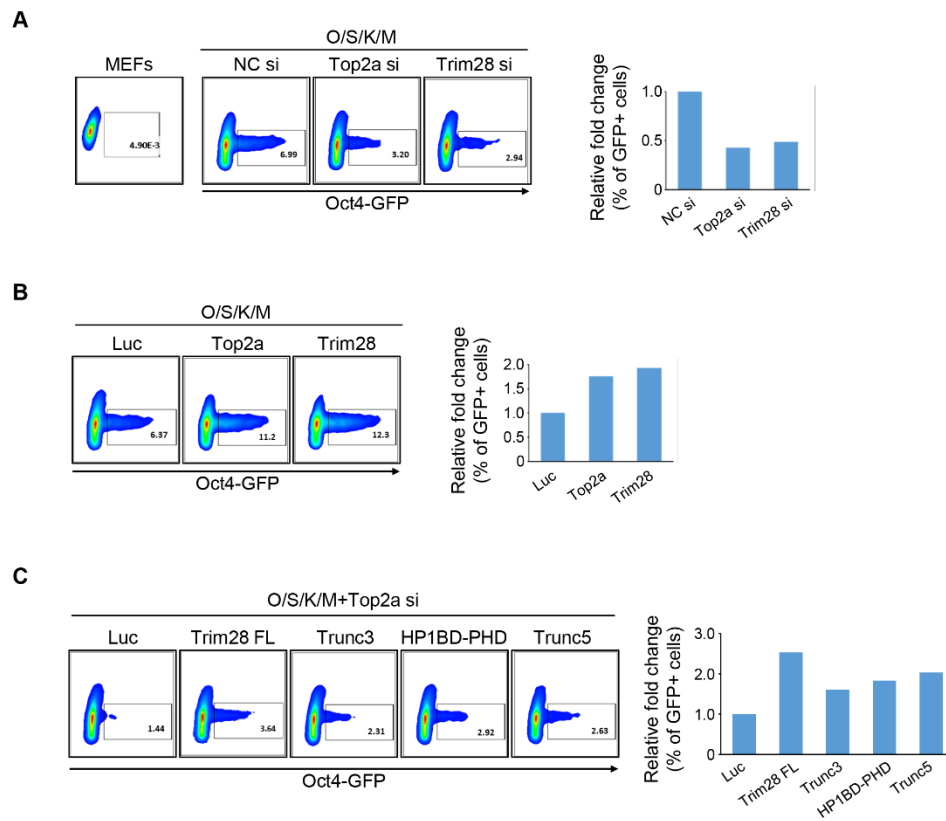


Figure S5

Figure S5. Flow cytometry analysis of iPSC reprogramming efficiency under different conditions. (A) GFP+ cells generated from Oct4-GFP MEFs transduced with Yamanaka factors (O/S/K/M) and subjected to either Top2a KD or Trim28 KD after 12 days of culture in iSF1 medium. Oct4-GFP MEFs with no genetic modification served as a negative control for iPSC generation. (B) GFP+ cells generated from Oct4-GFP MEFs transduced with Yamanaka factors together with either Top2a or Trim28 after 12 days of culture in iSF1 medium. (C) GFP+ cells generated from Oct4-GFP MEFs with continuous Top2a KD and ectopic expression of different forms of Trim28 on top of Yamanaka factors.

For Figure 4H, differences between Luc control and Trunc3 or HP1BD-PHD appear significant. The statistical approach and p-value calculations should be more clearly described.

We thank the reviewer for this comment. For Figure 4H, statistical significance was

determined using one-way ANOVA followed by appropriate post hoc multiple comparisons tests. Specifically, Dunnett's test was used for comparisons of each group against the Luc control, yielding adjusted p-values of 0.0008 (Trim28 FL), 0.1334 (Trunc 3), 0.0755 (HP1BD-PHD), and 0.0118 (Trunc 5). Therefore, Trim28 FL and Trunc 5 showed statistically significant differences from control group ($p < 0.05$), whereas Trunc3 and HP1BD-PHD did not.

10. Domain requirements

Line 219 claims that HP1BD-PHD is necessary but not sufficient to rescue the phenotype. No data demonstrate necessity. It is unclear why only three mutants were examined, and why other truncations (e.g., Trunc5, Trunc6) were not included.

We thank the reviewer for this comment. Based on our truncation mapping (Figure 2E), all constructs containing either HP1BD or PHD (Trim28 Trunc 1, 2, 4, 5, 6) interact with full-length Top2a, while Trunc 3 lacking both fails to interact. Thus, we concluded that HP1BD-PHD is required for the interaction. We agree that our data do not address sufficiency. We have removed the claim of “not sufficient” from the revised text. Regarding the selection of Trim28 truncations for the rescue assay of iPSC generation (Figure 4H), we intended to test whether the minimal Top2a-interacting domain HP1BD-PHD is functionally equivalent to FL. Therefore, we focused on full length (positive control), Trunc 3 (lacking HP1BD-PHD), HP1BD-PHD (minimal interacting region), and Trunc 5 (containing HP1BD-PHD but lacking the C-terminal Bromo domain).

Accordingly, we have revised the text as follows (**revised Results, lines 212-222**):

“To study the functional relevance of the Top2a-Trim28 interaction in reprogramming, we investigated whether Trim28 could rescue the diminished iPSC formation caused by Top2a depletion. Transduction with Trim28 FL at d3 and d6 post-O/S/K/M infection remarkably rescued the compromised reprogramming upon continuous Top2a KD (Figures 4G and 4H). Based on our domain mapping (Figure 2F), we selected three truncations for functional testing: HP1BD-PHD (the minimal Top2a-interacting

domain), Trunc 3 (lacking HP1BD-PHD), and Trunc 5 (containing HP1BD-PHD). In contrast, although all three truncations showed increased efficiency, only Trim28 trunc5 exhibited a statistically significant difference compared to the Luc control. Thus, multiple domains within Trim28 collectively, rather than any single truncation, contribute to its cooperation with Top2a in improving iPSC generation.”

11. RNA-seq interpretation

Line 235 states that Trim28 directly regulates more genes than Top2a. Given that knockdown efficiencies and off-target effects differ, this conclusion cannot be made from RNA-seq alone.

We thank the reviewer for this careful comment. We agree that, despite our integrated analysis of ChIP-seq and RNA-seq data (revised Figures S6B and S6C), potential off-target effects of shRNA or siRNA knockdown, as well as differences in knockdown efficiency or ChIP-seq data quality between Top2a and Trim28, could confound a direct comparison of the number of their directly regulated genes. Therefore, we have removed the comparative conclusion (“indicating that Trim28 directly regulates more genes than Top2a”) from the revised manuscript. We now simply report the numbers of Top2a- and Trim28-bound genes with expression changes upon knockdown without making a direct comparison.

12. Global histone-mark inconsistencies

Heatmaps show weak correlation between differential expression and histone modification changes. More quantitative analyses (e.g., box plots) are needed. The discrepancy between Western blot results and ChIP-seq signals is likely due to lack of spike-in controls. When global histone levels change, spike-in normalization is often required.

We thank the reviewer for this important point. We agree that when global histone modification levels change, spike-in–based normalization is the preferred approach for assessing absolute changes by ChIP-seq. In the absence of spike-in controls, our ChIP-seq data cannot be used to directly quantify absolute genome-wide gains or losses of histone marks and should instead be interpreted in terms of relative genomic enrichment patterns. We have revised the Results as follows (**revised Results, lines 250-252**).

“In the absence of spike-in controls, our ChIP-seq data cannot be used to directly quantify absolute genome-wide gains or losses of histone marks; instead, we interpret these data in terms of relative enrichment patterns.”

To address the reviewer’s request for more quantitative analysis, we now include two complementary levels of boxplot-based quantification. First, we performed genome-wide boxplot analyses of SES-normalized ChIP-seq signal across uniform 10 kb genomic bins (**revised Figure S6F**). Signal Extraction Scaling (SES) normalization aligns background signal distributions between samples rather than equalizing total read counts, thereby reducing bias introduced by library-size differences and providing a more robust basis for comparing relative enrichment patterns than total read-count normalization alone, although it does not enable absolute quantification in the absence of spike-in controls (Diaz et al., 2012; Ramirez et al., 2016). Our analyses show that, after SES normalization, median genome-wide $\log_2(\text{ChIP}/\text{control})$ values remain close to zero for most histone marks, with only modest shifts across conditions, indicating that the ChIP-seq data do not support strong conclusions about large, uniform genome-wide abundance changes in the absence of spike-in normalization.

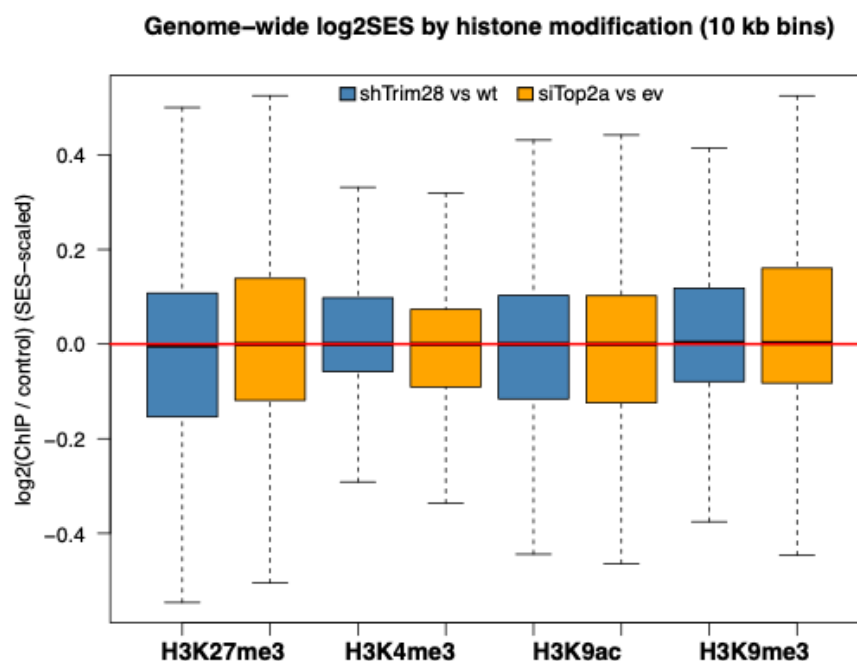


Figure S6F. Genome-wide histone modification changes following Trim28 or Top2a depletion. Distribution of SES-normalized ChIP-seq signals across 10-kb genomic bins for H3K27me3, H3K4me3, H3K9ac, and H3K9me3 in shTrim28 vs WT and siTop2a vs EV comparisons. Boxplots represent $\log_2(\text{ChIP}/\text{Input})$ values across the genome. The red line indicates no change ($\log_2 = 0$).

Second, and more directly relevant to the reviewer's concern, we quantitatively summarized TSS-centered histone-mark changes by calculating the mean SES-normalized ChIP-seq signal across the same TSS window used for the heatmaps (TSS ± 2 kb) on a per-gene basis. We first compared these distributions among upregulated, downregulated, and unchanged genes (**revised Figure S6G**), which provides a direct quantitative counterpart to the heatmaps and shows that the relationship between chromatin changes and transcriptional response is mark-dependent rather than uniform across all histone marks. In particular, clearer gene-class-associated differences are observed for H3K4me3, H3K9ac, and H3K27me3, whereas H3K9me3 shows comparatively weak separation. In addition, we further stratified differentially expressed genes into target and non-target groups and quantified the same TSS-centered signals within upregulated and downregulated subsets (**revised Figure S6H**). This additional analysis shows that, in several cases, the TSS-centered chromatin differences are sharpened further within the direct target subset, thereby linking the broader gene-class-level trends to the more selective target-associated promoter changes.

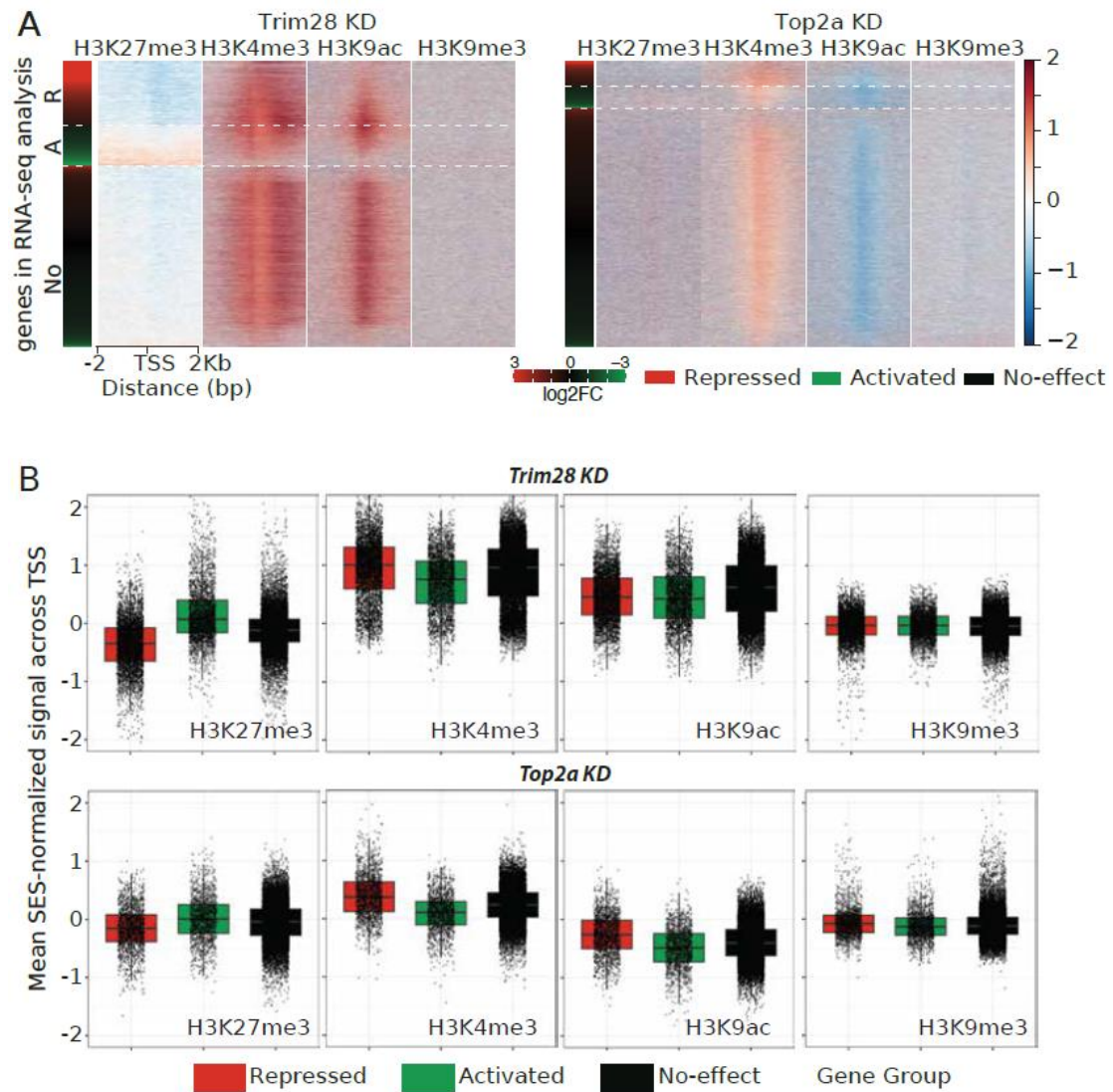


Figure S6G. Quantitative TSS-centered histone-mark profiles across differentially expressed gene classes after Trim28 or Top2a depletion. A) Heatmaps showing SES-normalized ChIP-seq signals for H3K27me3, H3K4me3, H3K9ac, and H3K9me3 across the TSS-centered region (± 2 kb) of genes included in the RNA-seq analysis following shTrim28 or siTop2a perturbation. Genes are ordered by RNA-seq log2 fold change, as indicated by the side color bar. B) Boxplots summarizing the mean SES-normalized ChIP-seq signal across the same TSS-centered window (± 2 kb) for upregulated, downregulated, and unchanged genes in shTrim28 and siTop2a samples. Each point represents one gene.

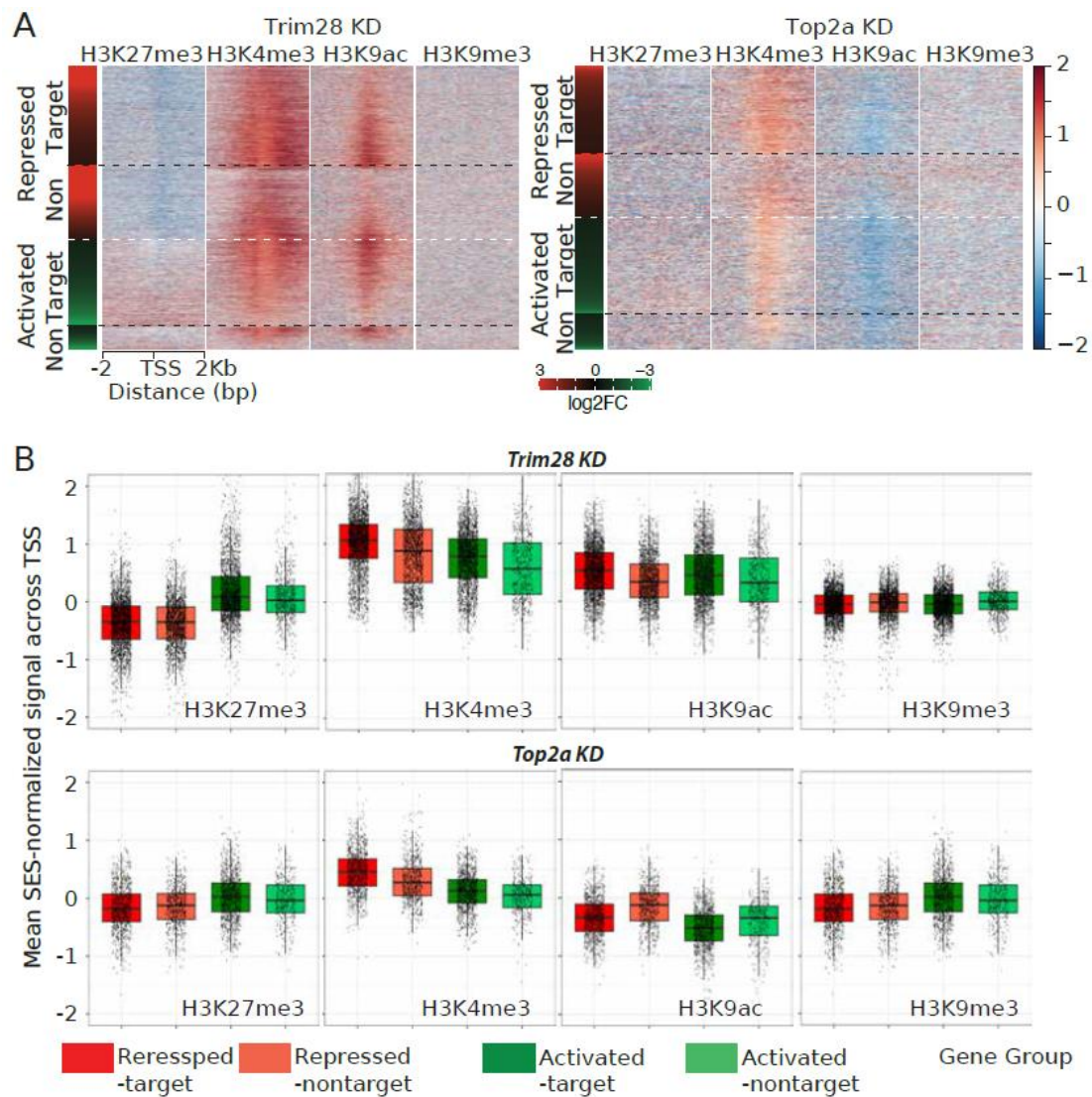


Figure S6H. Quantitative TSS-centered histone-mark profiles in target and non-target differentially expressed genes after Trim28 or Top2a depletion. A) Heatmaps showing SES-normalized ChIP-seq signals for H3K27me3, H3K4me3, H3K9ac, and H3K9me3 across the TSS-centered region (± 2 kb) of upregulated and downregulated genes following shTrim28 or siTop2a perturbation. Within each transcriptional group, genes are further separated into target and non-target classes, corresponding to the gene grouping used for the heatmap organization. Genes are ordered by RNA-seq log2 fold change, as indicated by the side color bar. B) Boxplots summarizing the mean SES-normalized ChIP-seq signal across the same TSS-centered window (± 2 kb) for up-target, up-nontarget, down-target, and down-nontarget genes in shTrim28 and siTop2a samples. Each point represents one gene.

Thus, the updated analyses support a more nuanced interpretation than our original presentation. Rather than indicating a simple global gain or loss of histone marks across the genome, the data are more consistent with a model in which global abundance changes detected by Western blot are accompanied by non-uniform, promoter-centered relative redistribution of specific chromatin marks, with the strongest relationships to transcriptional output observed at TSS-proximal regions and for specific activating or repressive marks. This revised interpretation is now reflected in the manuscript text as follows (**revised Results, lines 267-282**):

“To provide a more quantitative assessment, we performed genome-wide boxplot analyses of signal extraction scaling (SES)-normalized ChIP-seq signals across uniform 10 kb genomic bins, which showed that median genome-wide enrichment shifts were generally modest (Figure S6F). Furthermore, TSS-centered per-gene quantification revealed that the relationship between chromatin changes and transcription response was mark-dependent, with clear gene-class-associated differences observed for H3K4me3, H3K9ac, and H3K27me3 (Figures S6G and S6H). Together, these analyses indicate that the observed changes are not indicative of simple global gains or losses of histone marks across the genome. Rather, the data are more consistent with a model in which global abundance changes detected by Western blot are accompanied by non-uniform, promoter-centered relative redistribution of specific chromatin marks, with the strongest relationships to transcriptional output observed at TSS-proximal regions and for specific activating or repressive marks. Specifically, upon depletion of either Top2a or Trim28, transcriptional repression of co-activated genes was associated with an increase in H3K27me3 levels, while transcriptional activation of co-repressed genes was associated with a decrease in H3K27me3 levels. ”

13. ChIP-seq for Trim28 and Top2a

Given the weak overlap between RNA-seq and ChIP-seq, the authors should generate their own Trim28 and Top2a ChIP-seq datasets. Differences from published datasets may reflect culture conditions or cell line variation. It would be useful to examine how Trim28 overexpression or Top2a knockdown affects each protein's genomic occupancy.

We thank the reviewer for this constructive suggestion. We fully agree that generating our own ChIP-seq datasets for Top2a and Trim28 under control, Top2a KD, and Trim28 OE would be valuable for deciphering the mechanism. However, despite multiple attempts, we were unable to obtain high-quality ChIP-seq data for endogenous Top2a and Trim28 in mESCs. We have therefore noted this as a limitation of our study in the revised Discussion (lines 426-432). We hope the reviewer understands the technical difficulties.

“However, due to the current technical challenges in obtaining high-quality ChIP-seq data for Top2a, Trim28 and core factors of PRC2 and NuRD in mESCs with modified levels of Top2a and Trim28, we were unable to depict the genome-wide association among Top2a, Trim28, and bivalency or to fully elucidate how PRC2 and NuRD are involved in this regulatory axis. Nevertheless, future ChIP-qPCR or reChIP experiments following Top2a or Trim28 perturbation will be needed to directly test whether these complexes are recruited via the Top2a-Trim28 complex.”

14. ATAC-seq analysis

The current analysis focuses only on selected genes. Unbiased differential peak calling is needed to determine whether accessibility changes are global or locus-specific.

We thank the reviewer for this important suggestion. We agree that the original ATAC-seq presentation, which focused on defined co-regulated genes, did not fully address whether accessibility changes are broad or restricted to a subset of loci. To address this, we have now performed an unbiased genome-wide differential accessibility analysis using biological replicates.

Briefly, we first called ATAC peaks genome-wide in each condition and constructed a union peak universe across control and knockdown samples. To ensure that our

conclusions were not dependent on a single peak definition, we also performed parallel analyses using two control-grounded peak universes: a relaxed baseline control consensus and a strict baseline control consensus derived from the overlap between EV and WT control peaks. We then quantified read counts for each retained replicate across these peak universes and performed replicate-based differential accessibility analysis using DESeq2.

This analysis showed a clear difference between the two perturbations (Fig rebuttal supplementary 4). In the union peak universe, Top2a depletion produced a substantial accessibility phenotype, with 5,666 significantly increased peaks and 2,688 significantly decreased peaks out of 101,143 tested peaks. Importantly, this pattern remained robust in both the relaxed and strict baseline peak universes, indicating that the effect is not driven by a particular peak-set definition. In contrast, Trim28 depletion showed only 52 increased and 52 decreased peaks in the union universe, and no significant differential peaks in either the relaxed or strict baseline universes. Thus, the unbiased genome-wide analysis indicates that Top2a depletion is associated with broad accessibility remodeling, whereas Trim28 depletion has little to no robust genome-wide accessibility effect and appears much more locus-restricted.

all_universes_DESeq2_summary

universe	contrast	n_tested	n_increased	n_decreased
union	shTrim28_vs_EV	103233	52	52
union	siTop2a_vs_WT	101143	5666	2688
relaxed	shTrim28_vs_EV	45742	0	0
relaxed	siTop2a_vs_WT	45578	5399	1971
strict	shTrim28_vs_EV	34995	0	0
strict	siTop2a_vs_WT	34928	4763	2013

Figure S6I. Genome-wide differential ATAC peak summary across union and baseline-consensus peak universes. Summary of replicate-based differential ATAC-seq analysis across three peak universes: the union peak universe, the relaxed baseline

control consensus universe, and the strict baseline control consensus universe. For each universe, peaks were tested by DESeq2 in two contrasts: shTrim28 vs EV and siTop2a vs WT. The table shows the total number of peaks tested (n_tested) and the number of significantly increased (n_increased) or decreased (n_decreased) peaks, defined by adjusted P value < 0.05 and the sign of log2 fold change.

We further annotated the significant differential peaks by genomic category using a TSS $\pm$ 2 kb promoter definition (Figure rebuttal supplementary 5). This revealed that Top2a-depletion-associated increased peaks were predominantly promoter-associated, whereas decreased peaks were enriched in distal/intergenic regions, indicating that the accessibility changes are broad but not random across the genome. By contrast, the very limited number of differential peaks detected after Trim28 depletion did not support a robust genome-wide accessibility shift.

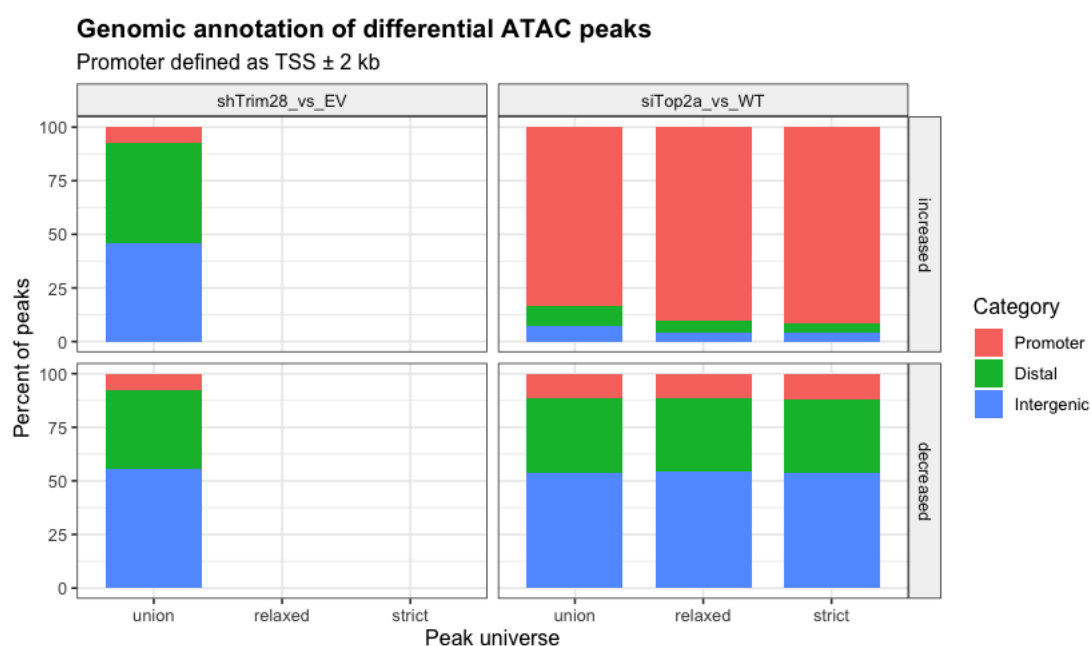


Figure S6J. Genomic distribution of differential ATAC peaks across union and baseline-consensus peak universes. Stacked bar plot showing the percentage of significantly differential ATAC peaks assigned to Promoter, Distal, or Intergenic categories in the union, relaxed baseline control consensus, and strict baseline control consensus peak universes. Peaks were identified by replicate-based DESeq2 analysis in

two contrasts: shTrim28 vs EV and siTop2a vs WT, and were separated into increased and decreased peak sets. Genomic annotation was performed using ChIPseeker, with promoters defined as transcription start site (TSS) $\pm$ 2 kb.

Finally, we examined the overlap between Trim28- and Top2a-associated differential ATAC peaks. Among the 52 Trim28-increased peaks, only 2 overlapped Top2a-increased peaks, and among the 52 Trim28-decreased peaks, only 21 overlapped Top2a-decreased peaks; no overlap was observed in the opposite direction. These results further indicate that the weak ATAC phenotype observed upon Trim28 depletion is only minimally shared with the broader Top2a-associated accessibility program.

Together, these additional analyses directly address the reviewer's concern and revise our interpretation of the ATAC-seq data: accessibility changes are not global for both perturbations equally; rather, Top2a depletion causes widespread and structured chromatin accessibility changes, whereas Trim28 depletion has a much more limited, locus-specific effect in this assay. We have now revised the Results accordingly as follows (lines 283-296):

“To determine whether chromatin accessibility changes upon Top2a or Trim28 depletion, we performed transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) followed by unbiased genome-wide differential accessibility analysis. The results revealed a clear difference between the two perturbations (Figure S6I). Top2a KD produced a substantial accessibility phenotype, with 5,666 significantly increased peaks and 2,688 significantly decreased peaks out of 101,143 tested peaks. In contrast, Trim28 KD showed only 52 increased and 52 decreased peaks. Annotation of differential peaks showed that Top2a KD-associated increased peaks were predominantly promoter-associated, whereas decreased peaks were enriched in distal regions (Figure S6J). Consistently, Top2a KD, but not Trim28 KD, modestly decreased chromatin accessibility within defined gene regions for both co-activated and co-repressed genes, supporting Top2a's role in maintaining open chromatin in ESCs (Figure 5F). These results suggest that other mechanisms may contribute to the

activation or repression of Top2a-Trim28 targets.”

15. Figure 6

ChIP-seq and qPCR analyses show locus-specific patterns that differ from the global changes reported earlier. Spike-in controls are necessary for reliable quantification. Genome-wide analyses should assess both global shifts and locus-specific alterations.

We thank the reviewer for this important point. We agree that the relationship between global, gene-class-level, and locus-specific chromatin changes should be interpreted carefully, particularly in the absence of spike-in normalization. As noted in response to comment 12, without spike-in controls, our ChIP-seq data cannot provide direct absolute quantification of genome-wide histone-mark gain or loss, and we therefore interpret these data in terms of relative enrichment patterns, while using Western blotting to inform conclusions regarding total histone-mark abundance.

To address the reviewer’s concern more directly, we now distinguish three analytical scales in the revised analysis. First, we quantified SES-normalized ChIP-seq signal genome-wide across uniform 10 kb bins, which showed that median genome-wide enrichment shifts are generally modest after normalization (revised Figure S6F). Second, we performed per-gene TSS-centered quantitative analyses by summarizing mean SES-normalized ChIP-seq signal across TSS ± 2 kb and comparing these distributions among upregulated, downregulated, and unchanged genes (revised Figure S6G). Third, we further stratified transcriptionally responsive genes into target and non-target groups and quantified the same TSS-centered signal distributions within these subsets (new target/non-target boxplot analysis; revised Figure S6H).

These additional analyses show that the apparent difference between global and locus-specific measurements is not a contradiction, but instead reflects the fact that different analyses answer different questions. The 10 kb genome-wide boxplots address whether there is a large, uniform shift across the genome and indicate that such shifts are limited after normalization. By contrast, the TSS-centered per-gene analyses show that histone-mark changes are more evident at transcriptionally responsive genes and are mark-

dependent, with clearer separation for H3K4me3, H3K9ac, and H3K27me3 than for H3K9me3. Moreover, the target/non-target stratified analyses show that, in several cases, these TSS-centered differences are sharpened further within the direct target subset, providing an intermediate scale between whole-genome summaries and the selected loci shown in Figure 6.

We therefore interpret the locus-specific ChIP-seq and ChIP-qPCR results in Fig. 6 not as direct proxies for absolute genome-wide changes, but as representative promoter-proximal examples drawn from a broader, non-uniform and target-associated chromatin response. In this revised framework, Western blotting captures total histone-mark abundance, genome-wide ChIP-seq boxplots capture relative overall enrichment distributions, TSS-centered per-gene boxplots capture gene-class-associated chromatin behavior, and Fig. 6 highlights selected regulatory loci where these changes are most clearly illustrated. We have revised the Results and Discussion accordingly to make this scale-dependent interpretation explicit.

16. Clinical data interpretation

Lines 318–319: The subset of samples with high expression of both genes is small. The authors are recommended to analyze patient survival or clinical outcomes relative to expression levels.

We thank the reviewer for this comment, which prompted us to re-evaluate our data. The major finding of our study is the role of the Top2a-Trim28 interaction in mESCs and its extension to cancer stem cells (CSCs). The MDA-MB-231 cell line used in our study contains approximately 80% CD44+/CD24-/low CSCs. However, the clinical sequencing data in public databases are derived from tumor bulk RNA-seq. While both *TOP2A* and *TRIM28* are, on average, more highly expressed in breast cancer compared to normal tissues, these bulk data cannot reflect the true expression levels of these two genes specifically within CSCs. Therefore, survival curve analysis based on bulk RNA-seq would not be relevant to our findings.

Based on the above considerations, we have removed the phrase ‘with a subset of

samples exhibiting high expression of both genes’ from the revised Results. We sincerely thank the reviewer for helping us improve our study.

17. Breast cancer cell lines vs cancer stem cells

MDA-MB-231 cells are not considered cancer stem cells in general. The authors can further enrich for BCSC populations (e.g., ALDH1 high, 3D culture, sphere assays) and test whether Trim28 or Top2a knockdown specifically affects these cells. The rationale for using a basal-like cancer cell line and connecting these results to mESC biology is insufficient.

We thank the reviewer for this important comment. We agree that MDA-MB-231 cells are not conventionally considered pure cancer stem cells (CSCs); rather, they are a basal-like triple-negative breast cancer (TNBC) cell line known to contain a subpopulation of CSCs. However, it has been extensively documented that MDA-MB-231 cells can be efficiently enriched for CSCs through the serum/serum-free alternation method (PMID: 34123797). Using this approach, the authors reported that the proportion of CD44⁺/CD24⁻/low BCSCs reached 70.5%. The MDA-MB-231 cell line used in our study was generously provided by from our collaborator. In our study, we characterized the CSC content of this MDA-MB-231 cell line using the established CSC surface marker CD44⁺/CD24⁻/low. Flow cytometry analysis revealed that approximately 80% of the cells exhibited the CD44⁺/CD24⁻/low phenotype (Figures 7E and S7F), indicating a highly enriched CSC population under our experimental conditions. Thus, although MDA-MB-231 cells are not pure CSCs per se, the high proportion of CD44⁺/CD24⁻/low cells in our culture allows us to use this cell line as a surrogate model to investigate CSC-related biology. This high CSC content, together with our functional CSC assays, mammosphere formation and ALDH1 expression in xenografts (Figures 7F and 7H), supports the conclusion that the TOP2A-TRIM28 complex contributes to CSC maintenance.

However, we agree with the reviewer that data derived from only one TNBC cell line are insufficient to establish broad relevance to cancer. We have therefore toned down our claims in the revised Discussion and explicitly acknowledged this limitation. The revised text now reads as follows (**revised Discussion, lines 492-495**):

“Nevertheless, we acknowledge that our cancer-related data are derived exclusively from a single TNBC cell line, providing preliminary evidence linking the Top2a-Trim28 complex to breast cancer. Future studies using additional models are required to extend these findings to generalized cancer types.”

Minor concerns

1. Methods

The methods section should be moved to the main text. The figure legends in general do not contain enough details.

We thank the reviewer for this suggestion. The Materials and Methods section has been moved to the main text and the figure legends have been expanded as requested.

2. Fig.1G Top2a label should be adjusted

We have adjusted Top2a label in revised Figure 1G.

3. Analysis of CbioPortal should be Analysis of cBioPorta l.

We thank reviewer for pointing out the typo. We have corrected it in the revised Materials and Methods section.

Reference

Ramírez, F., Ryan, D. P., Grüning, B., Bhardwaj, V., Kilpert, F., Richter, A. S., Heyne, S., Dündar, F., & Manke, T. (2016). deepTools2: a next generation web server for deep-sequencing data analysis. *Nucleic Acids Research*, 44(W1), W160–W165. <https://doi.org/10.1093/nar/gkw257>

Diaz, A., Nellore, A., & Song, J. S. (2012). CHANCE: comprehensive software for quality control and standardization of ChIP-seq data. *Statistical Applications in Genetics and Molecular Biology*, 11(3). <https://doi.org/10.1515/1544-6115.1750>

Point-by-point responses to Reviewers' comments

Reviewer #1 (Remarks to the Author):

Comments have been sufficiently addressed and the manuscript is suitable for publication

We thank Reviewer for the positive evaluation and for the constructive comments throughout the review process, which have significantly improved our work.

Reviewer #2 (Remarks to the Author):

The manuscript have been revised extensively. The authors have tried to address most of the comments. Therefore, the manuscript is suitable for acceptance.

We greatly appreciate the reviewer's expertise in reviewing our manuscript. We are pleased that our revision has addressed most of the reviewer's comments, and the insightful suggestions have greatly strengthened our manuscript.

Reviewer #3 (Remarks to the Author):

The revised manuscript has been substantially improved. Although some minor concerns remain, the authors have addressed the majority of the previous issues.

We thank the reviewer for the positive assessment and for the insightful comments which have greatly strengthen our work. We will address the remaining minor concerns in the revision.

Figure 3E appears to show a similar level of interaction between Trun7 and Top2a. However, the authors do not discuss potential concerns regarding the large variation in the quality and abundance of the recombinant proteins. Some of the truncation mutants are barely detectable by SDS-PAGE. In addition, many of the bands shown in this figure appear likely to be saturated, making reliable quantification difficult. As a result, the conclusions drawn from these data are not fully supported by the presented results.

Based on the reviewer's comments, we assume that the reviewer is referring to the results presented in Figure 2E. We thank the reviewer for this careful and critical observation.

As shown in Figure S3, the purity and relative concentration of each truncation

mutant were assessed by Coomassie Brilliant Blue staining using different amounts of BSA as standards for quantification. We acknowledge that in our pull-down assays, equal masses (1ug) of each Trim28 truncation and Top2a FL were used, rather than equimolar amounts. As shown in the table below, the molar amounts of different truncations vary considerably. Therefore, the band intensities shown in the Figure 2E should be interpreted qualitatively (presence or absence of interaction) rather than quantitatively (comparison of binding strength).

Table. Calculated molar amounts of recombinant proteins used in pull-down assays.

Protein	cDNA length (bp)	Number of amino acids ($\approx$ bp/3)	Estimated MW (kDa)	input (ng)	input (pmol)	IP (ng)	IP (pmol)
Top2a FL	4587	1529.0	~168.2			1000	5.95
Trim28 Trunc1	1114	371.0	~40.8	25	0.613	1000	24.5
Trim28 Trunc2	660	220.0	~24.2	25	1.03	1000	41.30
Trim28 Trunc3	454	151.0	~16.6	25	1.510	1000	60.2
Trim28 Trunc4	2431	810.0	~89.1	25	0.28	1000	11.20
Trim28 Trunc5	2032	677.0	~74.5	25	0.34	1000	13.40
Trim28 Trunc6	1564	521.0	~57.3	25	0.44	1000	17.40
Trim28 Trunc7	1156	385.0	~42.4	25	0.59	1000	23.6

Molecular weights were estimated using the formula: MW (kDa) $\approx$ (cDNA length in bp $\div$ 3) $\times$ 110 Da $\div$ 1000. Molar amounts were calculated as pmol = ng $\div$ kDa.

As shown in Figure 2E, Trunc 7 shows relatively lower input levels and pull-down efficiency than other truncations, probably due to its lower purity. Despite this, a specific interaction between Trunc 7 and Top2a FL was still detectable compared to the IgG control. Therefore, we no longer claim that HP1BD-PHD are the primary domains, nor do we downplay the interaction of Trunc 7. The data support that Trim28 Trunc 1, 2, 4, 5, 6, and 7 all interact with Top2a, whereas Trunc 3 (Bromo domain alone) does not.

We have therefore revised our results and conclusions as follows (**revised Results, lines 124-136**), and have also updated the schematic illustration of the interaction between Top2a and Trim28 accordingly (**revised Figure 2F**).

“To further identify the functional domains of Top2a and Trim28 required for their physical interaction, we generated a series of truncations of Top2a (trunc1-8) and

Trim28 (trunc1-7), respectively (Figures 2B and 2D) [1,19]. The purity and relative abundance of each recombinant truncation mutant were assessed by Coomassie Brilliant Blue staining (Figure S3). The results showed that the ATPase, DNA gyrase B and topoisomerase/primase domains (TOPRIM) of Top2a are required for the direct interaction with Trim28 FL (Figure 2C). For Trim28, with the sole exception of the C-terminal Bromo domain, multiple regions within Trim28 contribute to Top2a binding (Figure 2E). This implies that the PHD-bromodomain cassette of Trim28, which is responsible for scaffolding chromatin modifiers and binding histone modifications, comes into contact with the functional domains of Top2a that are involved in relieving topological stress in DNA [9,20] (Figure 2F). Together, these data demonstrate a direct physical interaction between Top2a and Trim28 in vitro.”

We thank the reviewer for this comment. As noted in our previous response for reviewer's comment #4, the quantification of the WB band intensities for Trim28 overexpression (OE) in Figure 3B is provided in the bar graphs on the right side of the figure. The statistical analysis (one-way ANOVA) shows that Trim28 OE-NC si is increased approximately 1.89-fold compared to EV-NC si control (1.890 ± 0.514), which is statistically higher ($p < 0.0001$). We have now replaced the panel with one that better reflects its original proportions in the revised Figure 3B. For clarity, we have now presented the corresponding bar graph below. We hope this resolves the reviewer's concern.

