

Distinct and compensatory roles of STAG1 and STAG2 in post-mitotic genome refolding

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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)

This manuscript provides a thorough investigation of the distinct and redundant functions of STAG1 and STAG2 in post-mitotic genome refolding. Using auxin-inducible degron-tagged alleles of STAG1 and STAG2, the authors uncover that STAG2 is the principal driver of shorter-range chromosomal interactions and rapid transcriptional reactivation in early G1, while STAG1 plays a minor, supportive role in longer-range interactions between CTCF sites. Authors propose the distinct roles of STAG1 and STAG2 emerges – at least partly – from the offset in time at which they each accumulate in the nucleus to in early to late G1.

Although the roles of STAG1 and STAG2 in interphase genome folding have been previously investigated, this is the first analysis of their functions during post-mitotic genome refolding. The study is therefore original and of relevance to the field. I believe the study meets the quality standards of Nature Communications. However I think the study would benefit from addressing the following clarifications before publication:

Main suggestions

1. It remains unclear to what extent the different activity of STAG1 and STAG2 (in G1-chromatin binding kinetics and genome folding) are explained by protein sequence divergence or simply by differences in expression levels. Indeed, the absolute and relative abundance of the two paralogs is known to vary extensively across cell types. It would be especially helpful if authors could at least report on relative expression levels.

This could easily be done by separately tagging STAG1 and STAG2 with the same tag (e.g. GFP), verify this tag does not change basal expression level by comparing tagged cells to untagged cells by Western blot with antibodies against endogenous proteins, and then quantifying relative expression using the common tag between STAG1 and STAG2 (e.g. GFP by flow cytometry or Western-blot).

Optionally, if authors wish to gain insight into absolute levels, employing this approach with Halo tagging could enable easy absolute quantification with flow cytometry using reference cell lines that can be distributed by the Handers lab (MIT) or Tjian/Darzacq lab (UC Berkeley) <https://elifesciences.org/articles/40164>

2. The manuscript is lacking key controls for how degron tagging affects total protein levels before triggering degradation. It is common for large tags, such as AID with a fluorescent protein, to destabilize basal protein levels and AID systems can be notoriously leaky (even AID2). To facilitate interpretation of the experiments authors need to report how basal expression in STAG1 and STAG2 tagged cells compare to that of untagged parental cells prior to degradation.

This can be achieved using antibodies against the endogenous proteins and comparing expression levels in WT untagged cells to those in degron tagged cells BEFORE depletion. This require using antibodies against endogenous proteins (not the tag brought with the degron sequence, which is absent from WT cells). My group successfully used the following antibodies for similar purposes, but authors can of course use other antibodies at their discretion:

STAG1 Abcam ab4457

STAG2 Abcam ab4463

Related to this, it would be most helpful to report how AID-tagged but undepleted STAG1 and STAG2 cells compare to WT

untagged cells – for example by overlaying P(s) plots. This could help determine to what extent the AID tagged STAGs are fully functional.

3. The study does not quantify cohesin chromatin occupancy under single Stag2 depletion, leaving open whether small-loop attenuation is Stag2-specific or driven by reduced overall cohesin loading. Figure 2d and 2e show small-loop attenuation upon Stag2 loss, but without direct measurement of RAD21 or SMC1 on chromatin in the single-depletion context, the result could reflect decreased total cohesin occupancy rather than a specific requirement for Stag2. I feel it is important to clarify given that STAG2 knockout can reduce cohesin subunits RAD21 and SMC1 on chromatin, with STAG1 being unable to compensate (Giménez-Llorente et al. EMBO J PMID: 39487368; Fischer et al. Cell Rep PMID: 39084219). Additionally, STAG2 knockdown consistently reduces cohesin occupancy at specific genomic loci without STAG1 compensation. Figure 5f–g addresses occupancy only for the double-depletion scenario, which cannot be extrapolated to single Stag2 loss. Carrying out calibrated cohesin ChIP-seq seems necessary to support the conclusions.

4. Authors base some of their key interpretations from the differences in chromatin loading dynamics between STAG1 and STAG2 reported by the Ellenberg lab in Hela cells (Brunner et al. PMID: 39786339). However these experiments were done in a completely different cell type, where absolute and relative abundances of STAG1 and STAG2 may not be comparable. If generating chromatin rebinding kinetics in (either by imaging or other methods like calibrated ChIP-seq) is not feasible control is beyond what is feasible in the time frame of revisions authors need to acknowledge this in the main text / discussion.

Minor suggestions

5. Authors do not provide osTir2 + 5ph-IAA controls for RNA-seq. If generating this control is beyond what is feasible in the time frame of revisions authors need to acknowledge this in the main text / discussion. While this would not affect the statements about differential genes between STAG1 and STAG2 depletion, genes potentially dysregulated from osTir2 + 5ph-IAA alone may inflate the perception of genes commonly dysregulated after STAG1 or STAG2 depletion.

6. The current loop analyses rely on a single loop-calling framework with fixed parameters, without benchmarking against other callers or assessing parameter sensitivity. Previous work (Parker et al., Bioinform Adv., PMID: PMC10645293) also highlighted that robust detection of differences based on loop calling typically requires extremely deep Hi-C datasets (>6 billion contacts per condition), which may exceed the current sequencing depth. Furthermore, recent studies have shown substantial variation across loop callers (e.g. PMID: 38770419 or PMID: 3264733).

In this context, it would be useful to include a brief power analysis to clarify how sequencing depth and loop-caller choice might influence the reported size- and phase-dependent effects. The APA and fold-change summaries in Figure 2d–2e and Extended Data Figure 6 would also benefit from replicate-level statistics across callers, to help rule out method-specific artifacts.

It would help to acknowledge this power analysis limitation explicitly and explain to what extent it may affect the conclusions (or not?) when contrasting STAG1 to STAG2 Hi-C defects. I suggest considering to explicitly acknowledge computational limitation (e.g., “Loop detection may vary with caller choice and parameterization; cross-caller benchmarking was not performed in this study.”).

7. I found the P(s) graphs very insightful and suggest moving them to main, if space permits.

8. When reporting P(s) graphs it may be helpful to include a secondary panel showing the slope / derivative, as this can help interpret loop extrusion defects. (e.g. as in Fig 1h/i of PMID: 40766525)

9. Mouse protein names are conventionally all capitalized (RAD21, not Rad21 – just like authors use for SMC1/3) – even though not all manuscripts in the field abide to this nomenclature.
<https://www.informatics.jax.org/mgihome/nomen/gene.shtml>

10. Please report the sequence of targeting constructs as annotated genbank in supplementary files. This is important for enabling reproducibility.

11. It would be helpful to readers to explain more about the G1E-ER4 cells: their cell types and tissue of origin, possible transformation states etc...

12. No sample nomenclature in the GEO submission is very difficult to understand. It would be helpful to use a more straightforward nomenclature: chip_S0410_stag1_asyn_no_a_antiSA2 – does this mean STAG1-AID cells? ? What does S0410 mean? Why not use something like chip_stag2_stag1-aid_asyn_no-auxin instead? Or at least include a nomenclature table in the main description of the submission to users can be 100% sure about which sample is what?

13. The ChIP-seq protocol did not use spike ins or calibration. Please acknowledge this clearly and explain that global changes may therefore be missed. Please explain whether conclusions from fig. ext 1 and 2 need to be tampered accordingly.

14. Please clarify if the Hi-C heatmaps are presented in linear or log10 scale? This could be done by adding tick marks on the scale bar

Reviewer #2

(Remarks to the Author)

In the current manuscript, Zhang et al. colleagues dissect the roles of Stag1 and Stag2 cohesin complexes in the re-establishment of genome architecture at the exit from mitosis. For that, they perform Hi-C analyses of clones derived from the mouse G1E-ER4 erythroblast line carrying AID degrons for Stag1 or Stag2, alone or combined. The consequences of ablating each complex for reactivation of gene expression is also explored. The authors conclude that despite a large degree of redundancy between the two complexes, Stag2 is the principal regulator of post-mitotic genome reorganization while Stag1 provides compensatory support.

This study follows a previous one by the corresponding author in the Blobel group that used the same experimental setup to address and compare the roles of CTCF and (total) cohesin in genome refolding after mitosis. Major findings reported here are not surprising given that previous study together with previous reports on the specific contributions of Stag1 and Stag2-cohesin complexes to genome folding (Kojic 2017 NSMB, Cuadrado 2019 Cell Rep, Wutz 2020 eLife, van der Weide NAR 2021, Brunner 2025) but provide additional clues about the regulation and function of Stag2-cohesin. Some of the analyses presented are interesting and creative, like the one addressing context-specific interactions (euchromatin versus heterochromatin). Other conclusions, however, like the existence of an "intrinsic loop extrusion activity" of a core cohesin complex -without a Stag subunit- require further validation.

A. Major points.

1. Total amount of cohesin on chromatin as well as relative abundance of Stag1 and Stag2 in the parental cell lines and AID clones.

One important issue that is not addressed and can impact the conclusions made by the authors is the relative abundance of the two paralogs in the cell line used. Depending on this abundance and the upregulation of a Stag paralog upon depletion of the other, cells will have more or less cohesin on chromatin. Collisions between cohesin complexes and availability of cohesin regulators affect the processivity of loop extruding cohesin and thereby genome folding. The authors write about the "status of Stag2 as the dominant paralog, accounting for ~75% of the total cohesin pool³⁶.", but the study cited is HeLa cells, and different cell lines have different ratios (see for instance Kojic 2018 NSMB).

Moreover, tagging with AID may reduce the stability of the protein. If the amount of Stag1-mAID is low (compared with untagged Stag1 in parental cell line) then differences plus/minus auxin will be attenuated. This may explain the "unexpected insensitivity of structural loops to Stag1 loss".

I therefore believe that interpretation of the data requires the authors to present chromatin fractionation data showing how much cohesin is present on chromatin in the different cell lines used, with and without auxin, in early and late G1. The parental cell line should also be included for comparison and blots for Stag1 and Stag2 (instead of GFP or mCherry) in addition to Rad21 or Smc1.

2. Early versus late G1.

The authors conclude (abstract) that "Stag2 competitively displaces Stag1" but no experimental evidence support this displacement. The authors should show by quantitative chromatin immunoprecipitation a change in the amounts of Stag1 and Stag2, genome wide or at defined genomic locations, between early and late G1.

The chromatin fractionation requested in point 1, will serve also to show changes in the amounts of Stag1 and Stag2 in early versus late G1.

3. Loop extrusion without Stag subunit

At least in vitro, loop extrusion requires the Stag subunits in addition to NIPBL. The presence of a small number of loops in the absence of Stags in vivo is a surprising result. However, it is difficult to demonstrate that the depletion of the Stags is complete, particularly with the western blots shown in Extended Figure 1. Even in the ChIPs shown in that Figure, there are Stag2 signals in the Stag2-AID+auxin conditions.

Please show Stag1 and Stag2 blots for the chromatin fractionation in Figure 5d (this is in fact part of the request made in point 1) Moreover, the authors should immunoprecipitate SMC1 in extracts prepared from the same sorted cells used for the Hi-C (double Stag1AID; Stag2 AID and Smc3AID) and check for the presence of Stags in the immunoprecipitates. Likewise, it would be important to demonstrate the presence of cohesin and CTCF, but no Stag1/2, at the borders of (at least some of) these loops that remain after removal of the two Stag proteins.

4. What happens with non-structural cohesin loops? These would include loops that have cohesin, CTCF, and CRE-element on both sites of the loop, but also other loops that may have cohesin but no CTCF.

B. Additional issues:

5. The quality of the ChIPs shown in Extended Fig 1 is rather variable, with low enrichment for Stag1 in Stag1mAID clone, for instance. It would be better to show also a common subunit (Rad21 or SMC1/3). Please indicate number of called peaks in each condition, whether they colocalize with CTCF or not. Previous reports have shown that cohesin positions without

CTCF are enriched in Stag2.

6. I am confused with the saddle plots presented throughout the manuscript

(1) The values for late G1 in Stag1(+) and Stag2(+) control conditions are quite different (4.56 and 5.24, respectively), when they should be the same.

(2) The two replicates of the Stag1-AID cell lines show changes in opposite directions (4.12 to 3.96 in rep1; 5.80 to 5.94 in rep2 (Extended Figure 3d)

(3) The Smc3(+) has a much lower score than the Stag1(+) or Stag2(+): 2.44

What is the reason for these differences?

C. Additional suggestions.

7. For the analyses of contact probability, using the derivative of $P(s)$ provides a better way to assess the changes related to contacts in the range of either cohesin loops or compartments.

8. Figure 5g, the Y axis should be “protein levels” instead of “expression”

D. Citations

9-Line 437 “This result mildly contrasts with prior reports of transcriptional alterations upon Stag1 loss, a discrepancy that may reflect cell type specific differences or technical variations^{34,36}”.

Ref 34 (Rao 2017) examines total cohesin loss; ref 36 (Holzman 2019) is a proteomic study that quantifies cohesin and regulators in HeLa cells. Thus, they are not “reports of transcriptional alterations upon Stag1 loss”. Instead, maybe cite, Remeseiro 2012 (PMID: 22415368), Cuadrado 2019 (PMID: 31216471) or Pezic 2023 (PMID: 37802073).

10-Line 487 “Cohesin loading is widely attributed to Nipbl, which exhibits high DNA-binding affinity ref 8,45-48. It is therefore surprising that depletion of Stag proteins extensively reduces cohesin loading and loop formation”

Previous reports have shown in vivo and in vitro the importance of Stag subunits for cohesin loading, for instance:

-in vivo: Alonso Gil 2023 (PMID: 36898992) shows presence of Stag1 and Stag2 cohesins on chromatin after strong downregulation of NIPBL;

- in vitro: Murayama 2014 (PMID: 24291789) shows the requirement of Scc3/Stag for DNA entrapment; the cited ref 8 shows that Stag subunit is required for in vitro loop formation, in addition to NIPBL; ref. 46 and 47 also suggest an important role for Stag1 subunit is binding DNA.

Reviewer #3

(Remarks to the Author)

The manuscript by Wang et al. reveals distinct and compensatory roles for STAG1 and STAG2 across early- and late-G1. The authors show that STAG2 plays an important role in forming short loops—including enhancer–promoter (E-P) loops—and in suppressing large loops by competing with STAG1, whereas STAG1 depletion has more modest effects including attenuation of large loops during post-mitotic genome refolding in the G1E-ER4 cell line. The authors further suggest that STAG2-mediated loop extrusion is constrained by CTCF-bound barriers rather than by genomic travel distance. Finally, co-depletion of STAG1 and STAG2 results in a synergistic loss of virtually all structural loops and a more severe disruption of transcription than either single deletion.

While some results are simply consistent with prior observations, this study also reports novel and interesting findings, including a potential mechanism for STAG2-dependent short-loop formation (CTCF barrier vs. travel-distance limitation) and the possibility of cohesin-mediated loop extrusion without STAG subunits—both of which may require further experimental validation. In addition, the balance/abundance between STAG1 and STAG2 in the G1E-ER4 cell line is not discussed and may be an important confounding factor. By addressing the concerns outlined below, this manuscript could be reconsidered for publication. Overall, this read to me like an interesting and important contribution to the field.

Major points

1. Line 120: Fig. 1a shows STAG1 tagged with mAID–GFP and STAG2 with mAID–mCherry, but this is not clearly stated in the main text. Please state this explicitly, as large tags can affect protein stability. In addition, please provide immunoblots for STAG1 and STAG2 (using isoform-specific antibodies) alongside the parental cell line to confirm that tagging does not markedly reduce protein levels; STAG proteins are prone to downregulation after tagging.

2. Line 123-125: It is better to include data from the parental cell line to demonstrate that these tagging do not compromise cell proliferation.

3. Line 125-127: It is unclear whether spike-in normalization was used. Spike-in controls are necessary to discuss this statement. Please also discuss whether depletion of one STAG subunit leads to compensatory increases in the other, as this is important for interpreting downstream results.

4. Line 128-132: Although the synchronization approach has been validated previously, please include representative

FACS plots demonstrating effective cell-cycle control and provide appropriate citations^{1,2}.

5. Line 151-158: While cohesin abundance influences compartmentalization, the more relevant citations for loop-extrusion-dependent effects should be 3–5. If you intend to argue for differential loop extrusion rather than altered cohesin abundance after depletion, the authors should include RAD21 (or SMC1/3) western blots to show that total cohesin levels are comparable between conditions; otherwise, differential abundance cannot be excluded.

6. Line 179: The purpose of Fig. 5d is unclear. If the intended message is that larger loops are more affected by STAG1 loss, consider showing differential loop strength as box plots stratified by genomic distance quantiles.

7. Line 187-188: This is previously shown in HeLa cells⁶ and prior work shows that STAG1/STAG2 ratios are cell-type specific^{5,7}. It is better to quantify STAG1- and STAG2-cohesin in G1E-ER4 cells (e.g., by mass spectrometry). This is an important prerequisite for interpreting variant-specific effects throughout the manuscript.

8. Line 209-215: This idea could further be supported by western blots comparing STAG1 and STAG2 levels in early- vs. late-G1 to investigate changes in their relative abundance.

9. Line 217-218: This section is potentially interesting but difficult to follow and should be rewritten for clarity. The hypothesis could be tested more directly by conditional CTCF depletion (e.g., using a dTAG system)⁸.

10. Line 281-286: This is very interesting point. Although many studies associate STAG2 with (super)enhancers, to this reviewer's knowledge there is no direct evidence of preferential loading at these regions. It may be better to consider adding NIPBL ChIP-seq with and without STAG2 to test this. If comparing early vs. late G1, does NIPBL loading shift from euchromatin (early G1) to heterochromatin (late G1)?

11. Line 297-299: the same with comment 1.

12. Line 348-49: This is also novel and intriguing finding. Ideally, it would be tested by an in vitro loop-extrusion assay or an ATPase assay while it is a lot of work.

13. Line 376-378: How many of the affected loops are putative E-P loops?

14. Line 381-384: What are the sets of 'non-sig'? In this type of analysis, the background sets must be selected carefully. It might be suitable to select the genes in which the expression levels are similar.

15. Line 404-406: How is "association with E-P" defined in this analysis? Please specify the criteria.

Discussion: Please revise the Discussion section after addressing the points above.

Minor points

1. Line 74, 303, etc: "SMC3" is capitalized whereas "Rad21" appears mixed-case. Similar inconsistencies occur elsewhere. Please use consistent nomenclature: proteins in all capitals (e.g., SMC3, RAD21) and genes in italicized lowercase with an initial capital (e.g., Stag1, Stag2, Rad21) where appropriate.

2. Line 123 (Extended Data Figure 1C): Please indicate the scale for the color map (e.g., CPM, FPKM). More broadly, ensure that all figures specify units and normalization for color scales either in the panel or in the legend.

3. Recent work (PMID: 41107486) suggests that some CRE loops are maintained in mitosis, but this was not discussed here. This seems relevant and should be discussed.

Reference

1. Zhao, H. et al. Extensive mutual influences of SMC complexes shape 3D genome folding. *Nature* 640, 543–553 (2025).
2. Zhang, H. et al. Chromatin structure dynamics during the mitosis-to-G1 phase transition. *Nature* 576, 158–162 (2019).
3. Schwarzer, W. et al. Two independent modes of chromatin organization revealed by cohesin removal. *Nature* 551, 51–56 (2017).
4. Haarhuis, J. H. I. et al. The Cohesin Release Factor WAPL Restricts Chromatin Loop Extension. *Cell* 169, 693-707.e14 (2017).
5. Nagano, M. et al. The mitotic STAG3-cohesin complex shapes male germline nucleome. *Nat. Struct. Mol. Biol.* 1–16 (2025).
6. Holzmänn, J. et al. Absolute quantification of cohesin, CTCF and their regulators in human cells. *Elife* 8, (2019).
7. Kojic, A. et al. Distinct roles of cohesin-SA1 and cohesin-SA2 in 3D chromosome organization. *Nat. Struct. Mol. Biol.* 25, 496–504 (2018).
8. Narducci, D. N. & Hansen, A. S. Putative looping factor ZNF143/ZFP143 is an essential transcriptional regulator with no looping function. *Mol. Cell* 85, 9-23.e9 (2025).

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Authors have addressed my initial comments with care. I only have two small standing comments below and recommend timely publication.

- Extended figure 4 there seems to be a typo: below the bargraphs quantifying the western blots the labels of clone #34 read S36. Please double check.

- Extended Figure 6: I am a little puzzled how chromatin binding of cohesin RAD21 and SMC1 can remain unaffected after removal of STAG2, especially given STAG2 is the most abundant STAG paralog. Does it mean STAGs may not be required for chromatin binding of cohesin? Isn't that surprising? Also is the conclusion of Extended Figure 6 that cohesin binding by ChIP-seq is not altered? How can this be reconciled with altered Hi-C? It would be helpful to discuss these two points briefly.

Reviewer #2

(Remarks to the Author)

The authors have made a thorough revision of the manuscript, addressing many of the reviewers' comments, and the Discussion has also improved. I still have some problems with the section regarding the double depletion of STAG1/2.

- Figure 5f shows a chromatin fractionation experiment using cells depleted from both STAGs in which despite STAG absence, 50% of SMC1-Rad21 (and presumably SMC3) remains on chromatin. The authors refer to this result in their rebuttal saying "Our data hence strongly suggest that STAG proteins are not critically required for the initial association of the cohesin ring with chromatin."

However, the experiment has been performed with asynchronously growing cells, and not cells that exit from mitosis in the absence of STAGs, as is the case for the Hi-C in Figure 5. Thus, these (presumably) trimeric complexes that are left on chromatin in the absence of STAGs may correspond to complexes loaded before STAG depletion, which maintain their association with chromatin. This possibility should be mentioned,

Related to that:

The abstract says, referring to the double depletion of STAG1/2: "approximately 50% of cohesin remains chromatin-associated and retains active extrusion capacity".

As it is written, it seems that the cohesin trimer does loop extrusion as well as the tetramer. I suggest the authors to tone down this statement and say instead "approximately 50% of cohesin remains chromatin-associated and retains SOME (or MEASURABLE) extrusion capacity".

-Regarding amounts of STAGs left after depletion: it is not possible to determine these amounts without having an idea of the dynamic range of the antibody used for immunoblotting (Extended data 1a-d; Extended data 11a-b). It is essential to load decreasing amounts of the control condition to check the limit of detection, i.e., can the antibody detect a reduction of 80, 90 or 99%? Likewise, in the IP shown in new Extended Data Figure 12 c-d: (c) The signal for STAG1 and STAG2 in 0h are already low (compare with those of Smc1 or Rad21); better signals are needed for quantifications; (d) it is unclear what exactly is being quantified. For STAG1, in 0h condition, relative protein level is 0.4, but relative to what?

Reviewer #3

(Remarks to the Author)

As I noted in my evaluation of the previous version of this manuscript, I consider this to be an important study that reports several novel and highly interesting findings, including a potential mechanism underlying STAG2-dependent short-loop formation (i.e., a CTCF barrier versus travel-distance limitation) and the possibility that cohesin-mediated loop extrusion can occur even in the absence of STAG subunits.

In the revised manuscript, I was particularly impressed by the extraordinary efforts the authors have made to address my previous concerns. They have responded in a highly rigorous and comprehensive manner through a series of well-designed and informative additional experiments, including calibrated ChIP-seq, Western blotting, and quantitative mass spectrometry to assess STAG variant dosage and the chromatin-associated fraction of the cohesin complex, as well as biochemical experiments evaluating the integrity of the cohesin complex. In addition, the authors have substantially improved the presentation of the manuscript through thoughtful reorganization and clarification of the text.

In my view, all of my major concerns have been fully resolved. The revised manuscript is now highly comprehensive, rigorous, and convincing. I therefore congratulate the authors on this excellent study and their outstanding revision, and I fully support its publication.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

I am satisfied with the response to my comments.

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Reviewer comments:

Reviewer #1:

This manuscript provides a thorough investigation of the distinct and redundant functions of STAG1 and STAG2 in post-mitotic genome refolding. Using auxin-inducible degron-tagged alleles of STAG1 and STAG2, the authors uncover that STAG2 is the principal driver of shorter-range chromosomal interactions and rapid transcriptional reactivation in early G1, while STAG1 plays a minor, supportive role in longer-range interactions between CTCF sites. Authors propose the distinct roles of STAG1 and STAG2 emerges – at least partly – from the offset in time at which they each accumulate in the nucleus to in early to late G1.

Although the roles of STAG1 and STAG2 in interphase genome folding have been previously investigated, this is the first analysis of their functions during post-mitotic genome refolding. The study is therefore original and of relevance to the field. I believe the study meets the quality standards of Nature Communications. However I think the study would benefit from addressing the following clarifications before publication:

We thank the reviewer for the thorough and constructive comments. We have performed additional experiments and data analyses to address the reviewer's concerns. We also optimized our text to better present this work to a broader readership.

Major comments

1. It remains unclear to what extent the different activity of STAG1 and STAG2 (in G1-chromatin binding kinetics and genome folding) are explained by protein sequence divergence or simply by differences in expression levels. Indeed, the absolute and relative abundance of the two paralogs is known to vary extensively across cell types. It would be especially helpful if authors could at least report on relative expression levels.

This could easily be done by separately tagging STAG1 and STAG2 with the same tag (e.g. GFP), verify this tag does not change basal expression level by comparing tagged cells to untagged cells by Western blot with antibodies against endogenous proteins, and then quantifying relative expression using the common tag between STAG1 and STAG2 (e.g. GFP by flow cytometry or Western-blot).

Optionally, if authors wish to gain insight into absolute levels, employing this approach with Halo tagging could enable easy absolute quantification with flow cytometry using reference cell lines that can be distributed by the Handers lab (MIT) or Tjian/Darzacq lab (UC Berkeley) <https://elifesciences.org/articles/40164>

RESPONSE: We thank the reviewer for raising this important point.

To directly compare the endogenous abundance of STAG1 and STAG2 in our G1E-ER4 cells, we performed the following two experiments.

(1) Endogenous FLAG knock-in and Western blot quantification.

We employed CRISPR-Cas9 genome editing to generate knock-in cell lines where either STAG1 or STAG2 was endogenously fused to an identical FLAG-P2A-HALO epitope tag (new Extended Data Fig. 4a). We confirmed that this knock-in did not alter the basal expression of each protein by comparing each clone with the parental G1E-ER4 cell line (new Extended Data Fig.4b-e). Using a common FLAG antibody, we quantified the relative abundance of STAG1 and STAG2 across multiple clones (2 clones for STAG1 and 2 clones for STAG2) and biological replicates. These

experiments revealed that in G1E-ER4 cells, STAG2 is significantly more abundant than STAG1, with a ratio of approximately 5:1. (new Extended Data Fig.4f, g)

(2) Mass spectrometry.

To further validate the above result using an antibody-independent approach, we performed mass spectrometry. We used paralog-specific unique peptides to estimate the relative abundance of STAG1 and STAG2 across biological replicates. Consistent with our immunoblotting data, the MS-based quantification showed that the STAG2/STAG1 ratio is approximately 5:1 (new Extended Data Fig. 4h, i; Supplementary Table 3).

In summary, both common-epitope (FLAG) immunoblotting and mass spectrometry converge on the same conclusion that STAG2 is the predominant paralog in G1E-ER4 cells. This ratio (~5:1) is also consistent with previous quantitative proteomic measurements of cohesin subunits (PMID: 31204999).

We reason that if the two paralogs were only differing in abundance but not function, we would expect a global reduction of loop strength across all size ranges upon loss of the more abundant paralog (STAG2). Instead, the observation that STAG2 deletion triggers a non-uniform shift with the enhancement of large-scale loops and the loss of small-scale loops indicates that STAG1 and STAG2 possess diverse functional roles in genome architecture.

We have incorporated discussion into the revised manuscript (discussion, lines 568–575 of PDF file).

2. The manuscript is lacking key controls for how degron tagging affects total protein levels before triggering degradation. It is common for large tags, such as AID with a fluorescent protein, to destabilize basal protein levels and AID systems can be notoriously leaky (even AID2). To facilitate interpretation of the experiments authors need to report how basal expression in STAG1 and STAG2 tagged cells compare to that of untagged parental cells prior to degradation.

This can be achieved using antibodies against the endogenous proteins and comparing expression levels in WT untagged cells to those in degron tagged cells BEFORE depletion. This requires using antibodies against endogenous proteins (not the tag brought with the degron sequence, which is absent from WT cells). My group successfully used the following antibodies for similar purposes, but authors can of course use other antibodies at their discretion:

STAG1 Abcam ab4457

STAG2 Abcam ab4463

Related to this, it would be most helpful to report how AID-tagged but undepleted STAG1 and STAG2 cells compare to WT untagged cells – for example by overlaying P(s) plots. This could help determine to what extent the AID tagged STAGs are fully functional.

RESPONSE: We thank the reviewer for this constructive comment. To address this, we have included two new sets of control data.

(1) Validation of basal protein levels.

We performed Western blotting on untagged parental G1E-ER4 cells alongside the *Stag1*^{mAID}, *Stag2*^{mAID}, and the double-tagged *Stag1*^{mAID}*Stag2*^{mAID} lines using antibodies against endogenous STAG1 and STAG2. We confirm that total protein levels of STAG1 or STAG2 were comparable between the AID-tagged lines and the untagged parental line in the absence of 5-Ph-IAA treatment (new Extended Data Fig. 1a-d; new Extended Data Fig. 11a, b). Upon 5-Ph-IAA treatment, these

proteins became undetectable. These results demonstrate that the addition of AID-tag does not trigger premature degradation of STAG1 or STAG2.

(2) Functional assessment via P_S curves:

To determine if AID-tagging interferes with the biological functions of STAG proteins, we compared the P_S curves of the unperturbed *Stag1^{mAID}* and *Stag2^{mAID}* cells with that of the untagged parental G1E-ER4 cells (Zhang et al. Nature 2019) in both early- and late-G1 phase. The P_S curves are highly comparable in both time points across the full range of genomic separations (new Extended Data Fig. 1m), suggesting that AID-tagging to STAG proteins does not measurably affect the loop extrusion activity of cohesin.

In summary, our new experiments and analyses indicate that demonstrate that AID-tagging preserves both the endogenous abundance and the physiological function of STAG proteins prior to depletion.

3. The study does not quantify cohesin chromatin occupancy under single Stag2 depletion, leaving open whether small-loop attenuation is Stag2-specific or driven by reduced overall cohesin loading. Figure 2d and 2e show small-loop attenuation upon Stag2 loss, but without direct measurement of RAD21 or SMC1 on chromatin in the single-depletion context, the result could reflect decreased total cohesin occupancy rather than a specific requirement for Stag2. I feel it is important to clarify given that STAG2 knockout can reduce cohesin subunits RAD21 and SMC1 on chromatin, with STAG1 being unable to compensate (Giménez-Llorente et al. EMBO J PMID: 39487368; Fischer et al. Cell Rep PMID: 39084219). Additionally, STAG2 knockdown consistently reduces cohesin occupancy at specific genomic loci without STAG1 compensation. Figure 5f–g addresses occupancy only for the double-depletion scenario, which cannot be extrapolated to single Stag2 loss. Carrying out calibrated cohesin ChIP-seq seems necessary to support the conclusions.

RESPONSE:

We agree that it is important to distinguish between a specific requirement for STAG2 or a global reduction in cohesin loading. To investigate whether cohesin occupancy was affected by STAG2 loss, we performed the following experiments:

(1) Calibrated RAD21 ChIP-seq under single STAG2 depletion.

We performed calibrated RAD21 ChIP-seq in late-G1 phase *Stag2^{mAID}* cells following 5-Ph-IAA treatment. Our results demonstrate that total cohesin occupancy on the genome was NOT affected by STAG2 depletion (new Extended Data Fig. 6c, d).

(2) Chromatin-fractionation and immunoblotting of cohesin subunits.

To further substantiate our Calibrated ChIP-seq results, we performed chromatin fractionation in asynchronous cells, followed by immunoblotting for the core cohesin subunits SMC1 and RAD1. While STAG2 was efficiently depleted from the chromatin-bound fraction within 4 h of 5-Ph-IAA treatment, the levels of chromatin-associated SMC1 and RAD21 remained comparable to untreated controls (new Extended Data Fig. 6g, h).

We note that previous studies (Giménez-Llorente et al., EMBO J PMID: 39487368; Fischer et al., Cell Rep PMID: 39084219) have reported reduced cohesin occupancy upon STAG2 knockout. We attribute this difference to the experimental timescale: those studies utilized long-term genetic knockouts, which may lead to secondary effects on cohesin stability or loading through cellular adaptation. In contrast, our approach employs acute protein degradation (4 h), allowing us to capture the primary consequences of STAG2 loss while minimizing indirect effects or long-term compensatory changes.

In summary, the calibrated RAD21 ChIP-seq together with chromatin-fractionation immunoblotting experiments show that acute STAG2 depletion does not reduce overall cohesin occupancy on chromatin. This supports the idea that the short-range loop attenuation observed upon STAG2 loss reflects a STAG2-dependent function rather than a global decrease in cohesin loading. These data are incorporated in the manuscript (Results, lines 283–285 and lines 292–294 of PDF file).

4. Authors base some of their key interpretations from the differences in chromatin loading dynamics between STAG1 and STAG2 reported by the Ellenberg lab in HeLa cells (Brunner et al. PMID: 39786339). However these experiments were done in a completely different cell type, where absolute and relative abundances of STAG1 and STAG2 may not be comparable. If generating chromatin rebinding kinetics in (either by imaging or other methods like calibrated ChIP-seq) is not feasible control is beyond what is feasible in the time frame of revisions authors need to acknowledge this in the main text / discussion .

RESPONSE: We thank the reviewer for this important point. We agree that the absolute and relative abundances of STAG1 and STAG2, as well as their specific chromatin loading kinetics, may vary across cell types. We have updated the manuscript to explicitly acknowledge these potential cell-type-specific differences and have moderated our interpretations accordingly (discussion, line:590–607 of PDF file).

Other comments

5. Authors do not provide osTir2 + 5ph-IAA controls for RNA-seq. If generating this control is beyond what is feasible in the time frame of revisions authors need to acknowledge this in the main text / discussion. While this would not affect the statements about differential genes between STAG1 and STAG2 depletion, genes potentially dysregulated from osTir2 + 5ph-IAA alone may inflate the perception of genes commonly dysregulated after STAG1 or STAG2 depletion.

RESPONSE: We thank the reviewer for this important suggestion. To rigorously control for potential transcriptional artifacts induced by the AID system or 5-Ph-IAA treatment itself, we performed the following experiments and analyses:

We generated a control cell line expressing osTIR2 without any AID-tagged endogenous proteins. We then performed RNA-seq to compare gene expression profiles before and after 4 hours of 5-Ph-IAA treatment (n=3 biological replicates). Differential expression analysis revealed that 5-Ph-IAA treatment alone induces only negligible transcriptomic changes (new Extended Data Fig. 15c).

To further ensure that our primary findings are not treatment-driven artifacts, we cross-referenced the differentially expressed genes (DEGs) identified in our STAG1 or STAG1/2 co-depletion TT-seq data with the expression profiles of cells treated with 5-Ph-IAA alone. We found that the vast majority of DEGs identified after STAG depletion do not show significant changes in the 5-Ph-IAA-only control, with only a single gene overlapping between the two datasets (new Extended Data Fig. 15c). This lack of overlap confirms that the transcriptional responses reported in our study are a direct result of STAG protein loss and are not confounded by 5-Ph-IAA or the TIR2 system.

6. The current loop analyses rely on a single loop-calling framework with fixed parameters, without benchmarking against other callers or assessing parameter sensitivity. Previous work (Parker et al., Bioinform Adv., PMID: PMC10645293) also highlighted that robust detection of differences based on loop calling typically requires extremely deep Hi-C datasets (>6 billion contacts per condition), which

may exceed the current sequencing depth. Furthermore, recent studies have shown substantial variation across loop callers (e.g. PMID: 38770419 or PMID: 3264733).

In this context, it would be useful to include a brief power analysis to clarify how sequencing depth and loop-caller choice might influence the reported size- and phase-dependent effects. The APA and fold-change summaries in Figure 2d–2e and Extended Data Figure 6 would also benefit from replicate-level statistics across callers, to help rule out method-specific artifacts.

It would help to acknowledge this power analysis limitation explicitly and explain to what extent it may affect the conclusions (or not?) when contrasting STAG1 to STAG2 Hi-C defects. I suggest considering to explicitly acknowledge computational limitation (e.g., “Loop detection may vary with caller choice and parameterization; cross-caller benchmarking was not performed in this study.”).

RESPONSE:

We thank the reviewer for raising this important point regarding the robustness of loop-calling analyses. To avoid the possibility that our conclusions are method-biased, we performed additional analysis using a different loop caller, cooltools-dots (cooldots), which is widely used as an alternative to HICCUPS. Although cooltools identified a more conservative set of loops (for example, loops in the 0–100 kb range were rarely detected), the overall trend was consistent with our original analysis. In particular, upon STAG2 depletion, larger loops showed increased strength, with a similar cell-cycle-stage-dependent pattern (new Extended Data Fig. 5c). These results support that our main conclusions are reproducible across different loop-calling methods.

7. I found the $P(s)$ graphs very insightful and suggest moving them to main, if space permits.

RESPONSE: We thank the reviewer for this helpful suggestion. In the revised manuscript, we have moved the most informative P_s analyses from the Extended Data to the main figures (new Fig. 5h, i). Specifically, we moved the SMC3(+/-) late-G1 P_s curves and corresponding slope plots to Fig. 5h, where they serve as a benchmark for the near-complete loss of cohesin-mediated loop extrusion. We also moved the STAG1/2 double-depletion P_s curves from early-G1 and late-G1 cells, together with their slope plots, to Fig. 5i. Presenting these datasets in the main text makes the key comparison more explicit: although STAG1/STAG2 co-depletion reduces loop-extrusion signatures, the reduction is substantially weaker than that observed upon SMC3 depletion. These data therefore support the conclusion that residual loop-extrusion activity persists after loss of both STAG paralogs.

8. When reporting $P(s)$ graphs it may be helpful to include a secondary panel showing the slope / derivative, as this can help interpret loop extrusion defects. (e.g. as in Fig 1h/i of PMID: 40766525)

RESPONSE: We thank the reviewer for this suggestion. We have now added a secondary panel showing the slope/derivative of the P_s curves for all relevant conditions and included these plots in the revised figures (new Fig. 5h, i; Extended Data Fig. 13g-i).

9. Mouse protein names are conventionally all capitalized (RAD21, not Rad21 – just like authors use for SMC1/3) – even though not all manuscripts in the field abide to this nomenclature. <https://www.informatics.jax.org/mgihome/nomen/gene.shtml>

RESPONSE: We apologize for this oversight. We have carefully checked the manuscript and corrected mouse protein nomenclature, using standard all-capital formatting consistently.

10. Please report the sequence of targeting constructs as annotated genbank in supplementary files. This is important for enabling reproducibility.

RESPONSE: We thank the reviewer for this suggestion. We have now provided the sequences of all targeting constructs as annotated GenBank files exported from SnapGene, including *Stag1^{mAID}*, *Stag2^{mAID}*, *Stag1^{FLAG}* and *Stag2^{FLAG}*, to facilitate reproducibility (Supplementary Files 1-4).

11. It would be helpful to readers to explain more about the G1E-ER4 cells: their cell types and tissue of origin, possible transformation states etc...

RESPONSE: We thank the reviewer for this suggestion. In the revised manuscript, we have expanded the description of the G1E-ER4 cell line in the Methods section (Methods, line:725–728 of PDF file). We have also provided the appropriate citations to give a clearer context for readers.

12. No sample nomenclature in the GEO submission is very difficult to understand. It would be helpful to use a more straightforward nomenclature: chip_S0410_stag1_asyn_no_a_antiSA2 – does this mean STAG1-AID cells? ? What does S0410 mean? Why not use something like chip_stag2_stag1-aid_asyn_no-auxin instead? Or at least include a nomenclature table in the main description of the submission to users can be 100% sure about which sample is what?

RESPONSE: We apologize for the confusing sample nomenclature in our GEO submission. We have now thoroughly reviewed all submitted sample names and metadata and added a clear sample nomenclature table that maps each GEO accession/sample to its exact cell line, treatment condition (e.g., ± 5 -Ph-IAA), time point, and assay.

13. The ChIP-seq protocol did not use spike ins or calibration. Please acknowledge this clearly and explain that global changes may therefore be missed. Please explain whether conclusions from fig. ext 1 and 2 need to be tampered accordingly.

RESPONSE: We thank the reviewer for this important point. Our original ChIP-seq analysis was primarily presented using CPM normalization and did not explicitly include spike-in calibration.

To address the reviewer's concern regarding potential global changes in ChIP-seq signal, we performed new ChIP-seq experiments with spike-in controls and analyzed these datasets using spike-in-derived scaling factors. We then compared the spike-in-calibrated results with the CPM-normalized analyses (new Extended Data Fig. 1i, j; new Extended Data Fig. 3f, g; new Extended Data Fig. 6a). Importantly, the spike-in-calibrated data are highly consistent with the original results, indicating that our original conclusions are robust.

Based on these analyses, CPM- and spike-in normalized signals are strongly correlated in our datasets. Consistent with previous work, for narrow-peak binding factors and histone marks (e.g., CTCF, RAD21, Pol II, H3K27ac), CPM normalization can be informative for global quantification because the majority of ChIP-seq reads map to non-peak/background regions, which effectively provides an internal reference across libraries; in our data, these background reads typically constitute >80% of total mapped reads (Zhang et al., PMID: 31776509; Zhao et al., PMID: 38802567; Hsiung et al., PMID: 27340175). This point has been discussed previously (Hsiung et al., PMID: 27340175).

In summary, we now provide spike-in calibrated ChIP-seq quantification and show that it agrees with CPM-based results; therefore, the main conclusions remain unchanged.

14. Please clarify if the Hi-C heatmaps are presented in linear or log10 scale? This could be done by adding tick marks on the scale bar.

RESPONSE: We thank the reviewer for this helpful suggestion. The Hi-C heatmaps are displayed using a log₁₀-transformed contact frequency scale. To make this clearer, we have revised the color bar labels to explicitly indicate log₁₀ scaling.

Reviewer #2:

In the current manuscript, Zhang and colleagues dissect the roles of Stag1 and Stag2 cohesin complexes in the re-establishment of genome architecture at the exit from mitosis. For that, they perform Hi-C analyses of clones derived from the mouse G1E-ER4 erythroblast line carrying AID degrons for Stag1 or Stag2, alone or combined. The consequences of ablating each complex for reactivation of gene expression is also explored. The authors conclude that despite a large degree of redundancy between the two complexes, Stag2 is the principal regulator of post-mitotic genome reorganization while Stag1 provides compensatory support.

This study follows a previous one by the corresponding author in the Blobel group that used the same experimental setup to address and compare the roles of CTCF and (total) cohesin in genome refolding after mitosis. Major findings reported here are not surprising given that previous study together with previous reports on the specific contributions of Stag1 and Stag2-cohesin complexes to genome folding (Kojic 2017 NSMB, Cuadrado 2019 Cell Rep, Wutz 2020 eLife, van der Weide NAR 2021, Brunner 2025) but provide additional clues about the regulation and function of Stag2-cohesin. Some of the analyses presented are interesting and creative, like the one addressing context-specific interactions (euchromatin versus heterochromatin). Other conclusions, however, like the existence of an “intrinsic loop extrusion activity” of a core cohesin complex -without a Stag subunit- require further validation.

RESPONSE: We thank the reviewer for the thoughtful comments and for recognizing the strengths of our study. We appreciate the reviewer’s concern regarding the statement on an “intrinsic loop extrusion activity” of a core cohesin complex lacking STAG subunits. In the revised manuscript, we have clarified and tempered this interpretation to ensure the conclusions are appropriately framed and supported.

1. Total amount of cohesin on chromatin as well as relative abundance of Stag1 and Stag2 in the parental cell lines and AID clones.

One important issue that is not addressed and can impact the conclusions made by the authors is the relative abundance of the two paralogs in the cell line used. Depending on this abundance and the upregulation of a Stag paralog upon depletion of the other, cells will have more or less cohesin on chromatin. Collisions between cohesin complexes and availability of cohesin regulators affect the processivity of loop extruding cohesin and thereby genome folding. The authors write about the “status of Stag2 as the dominant paralog, accounting for ~75% of the total cohesin pool³⁶.”, but the study cited is HeLa cells, and different cell lines have different ratios (see for instance Kojic 2018 NSMB).

Moreover, tagging with AID may reduce the stability of the protein. If the amount of Stag1-mAID is low (compared with untagged Stag1 in parental cell line) then differences plus/minus auxin will be attenuated. This may explain the “unexpected insensitivity of structural loops to Stag1 loss”.

I therefore believe that interpretation of the data requires the authors to present chromatin fractionation data showing how much cohesin is present on chromatin in the different cell lines used, with and without auxin, in early and late G1. The parental cell line should also be included for comparison and blots for Stag1 and Stag2 (instead of GFP or mCherry) in addition to Rad21 or Smc1.

RESPONSE: We thank the reviewer for their thoughtful and constructive critique. Following these suggestions, we have categorized the comments into three primary issues and addressed them with new experimental data and updated manuscript text.

(1) Relative abundance of STAG1 and STAG2

To directly quantify the relative abundance of STAG1 and STAG2 in G1E-ER4 cells, we performed two independent measurements.

- We first employed CRISPR-Cas9 genome editing and generated endogenous FLAG-P2A-HALO knock-in lines for STAG1 and STAG2. We confirmed that this tagging does not affect the endogenous levels of these two proteins (new Extended Data Fig. 4 a-e). By immunoblotting with a common anti-FLAG antibody, we eliminated differences in antibody affinity. This analysis reveals a STAG1:STAG2 ratio of approximately 1:5 (new Extended Data Fig. 4 f-g).
- In addition, we also performed Mass-spectrometry analysis. Using STAG1- and STAG2-specific unique peptides for relative abundance estimation, we independently confirmed the ~1:5 ratio of STAG1 and STAG2 (new Extended Data Fig. 4h, i). This ratio is also consistent with previous quantitative proteomic measurements of cohesin subunits (PMID: 31204999).

Our findings are consistent with previous quantitative proteomic measurements in other systems (PMID: 31204999) and supports our interpretation of STAG2 as the dominant paralog in these cells.

(2) Stability of STAG proteins after mAID-tagging

- To address whether mAID-tagging affects the stability of STAG proteins, we performed immunoblotting with antibodies recognizing endogenous STAG1 or STAG2 proteins. Our experiments demonstrate that AID-tagging does not cause a significant reduction in steady-state protein levels compared to the untagged parental control (new Extended Data Fig. 1a-d; new Extended Data Fig. 11a, b).
- As a functional control, we compared the contact probability P_S curves (indicative of loop extrusion status) of undepleted *Stag1*^{mAID} and *Stag2*^{mAID} cells with parental G1E-ER4 cells at early- and late-G1 phase. The profiles were nearly identical across all genomic separations, indicating that the presence of the AID tag does not impair loop-extrusion-dependent genome folding in these cells, suggesting functional integrity (new Extended Data Fig. 1m).

(3) Chromatin bound cohesin levels for different cell lines

We agree that chromatin fractionation in early- versus late-G1 would be informative. However, in our experimental workflow, these distinct populations are isolated via FACS sorting following formaldehyde fixation. Because formaldehyde cross-linking covalently anchors the proteome, it is biochemically incompatible with detergent-based chromatin fractionation, which requires unfixed, native cells to separate soluble and chromatin-bound pools.

We reason that the vast majority of cells in an asynchronous population have progressed past early-G1. These cells have therefore established full cohesin loading and a mature architectural state. Consequently, asynchronous cells serve as a reliable proxy for the late-G1 phase, which is precisely where we observe the most significant structural changes upon STAG2 loss.

We therefore performed chromatin fractionation (cytoplasmic / soluble nuclear / chromatin fractions) in asynchronous cells using the *Stag1*^{mAID} line, the *Stag2*^{mAID} line, and the *Stag1*^{mAID}*Stag2*^{mAID} line, with (4h) and without (0h) 5-Ph-IAA treatment, and immunoblotted endogenous STAG1, STAG2, RAD21, and SMC1 (new Fig.5f, g; new Extended Data Fig. 3d, e; new Extended Data Fig. 6g, h).

Our key findings include:

- In the *Stag1^{mAID}* cell line, we observed that while total STAG1 levels in whole-cell lysates remain stable (new Extended Data Fig. 1a, b), the detectable chromatin-bound fraction of STAG1 is lower than in the parental control (new Extended Data Fig. 3d, e). We suspect this difference arises from minor degradation of the mAID-tagged STAG1 during sample preparation.

Crucially, however, the chromatin occupancy of all other subunits, including RAD21, SMC1, and STAG2, remained unaffected, demonstrating that the total cohesin pool on DNA is stable and comparable to parental levels (new Extended Data Fig. 3d, e).

Following 4 hours of 5-Ph-IAA treatment, STAG1 was completely eliminated without reducing the levels of other core subunits (new Extended Data Fig. 3d, e). The complete preservation of core cohesin subunits is highly consistent with our Hi-C findings that STAG1 loss failed to trigger major changes in post-mitotic chromatin folding, confirming that the "unexpected insensitivity" to STAG1 loss is a genuine biological observation

- In the *Stag2^{mAID}* line, we found that the basal levels of all cohesin subunits, including STAG1, STAG2, RAD21, and SMC1, were comparable to the parental control prior to depletion, confirming that mAID-tagging did not affect chromatin bound cohesin abundance (new Extended Data Fig. 1c, d; new Extended Data Fig. 6g, h).

Following 4 h of 5-Ph-IAA treatment, chromatin-associated STAG2 was efficiently eliminated, yet the levels of chromatin-bound RAD21, SMC1 and STAG1 remained stable (new Extended Data Fig. 6g, h). These data confirm that the total amount of cohesin on chromatin is not reduced by STAG2 loss

- In the *Stag1^{mAID}Stag2^{mAID}* double-degrogen line, we confirmed that the simultaneous tagging of both paralogs did not affect the basal abundance of chromatin-bound cohesin (new Fig. 5f, g). Following 4 h of 5-Ph-IAA treatment, both STAG1 and STAG2 were efficiently depleted from the chromatin fraction. We also observed a significant reduction in chromatin-bound RAD21 and SMC1 (new Fig. 5f, g). Notably, however, core cohesin was not completely eliminated from the chromatin, suggesting that a residual pool remains associated with DNA even in the absence of both STAG subunits.

In summary, we now provide a reviewer-guided set of controls addressing (1) STAG1/STAG2 relative abundance, (2) STAG protein stability after AID labeling, and (3) chromatin-associated cohesin levels before and after 5-Ph-IAA treatment. Together, these data support that STAG2 is more abundant than STAG1 in G1E cells (approximately 5-fold), that AID tagging does not detectably impair basal protein stability or loop-extrusion-dependent folding in undepleted cells, and that acute single-paralog depletion (*Stag1^{mAID}* or *Stag2^{mAID}*) does not detectably reduce chromatin-associated RAD21/SMC1 in our asynchronous chromatin-fractionation assays, whereas double depletion (*Stag1^{mAID}Stag2^{mAID}*) does partially reduce chromatin-associated RAD21/SMC1.

2. Early versus late G1.

The authors conclude (abstract) that "Stag2 competitively displaces Stag1" but no experimental evidence support this displacement. The authors should show by quantitative chromatin immunoprecipitation a change in the amounts of Stag1 and Stag2, genome wide or at defined genomic locations, between early and late G1.

The chromatin fractionation requested in point 1, will serve also to show changes in the amounts of Stag1 and Stag2 in early versus late G1.

RESPONSE: We thank the reviewer for this critical insight. We agree that our original hypothesis—that STAG2 competitively displaces STAG1—was inferential and lacked direct experimental support. To rigorously test this, we performed three independent lines of investigation. Our results indicate that STAG1 chromatin occupancy does not increase upon STAG2 loss, leading us to substantially revise our conclusions in the updated manuscript.

(1) Chromatin fractionation in *Stag2*^{mAID} cells

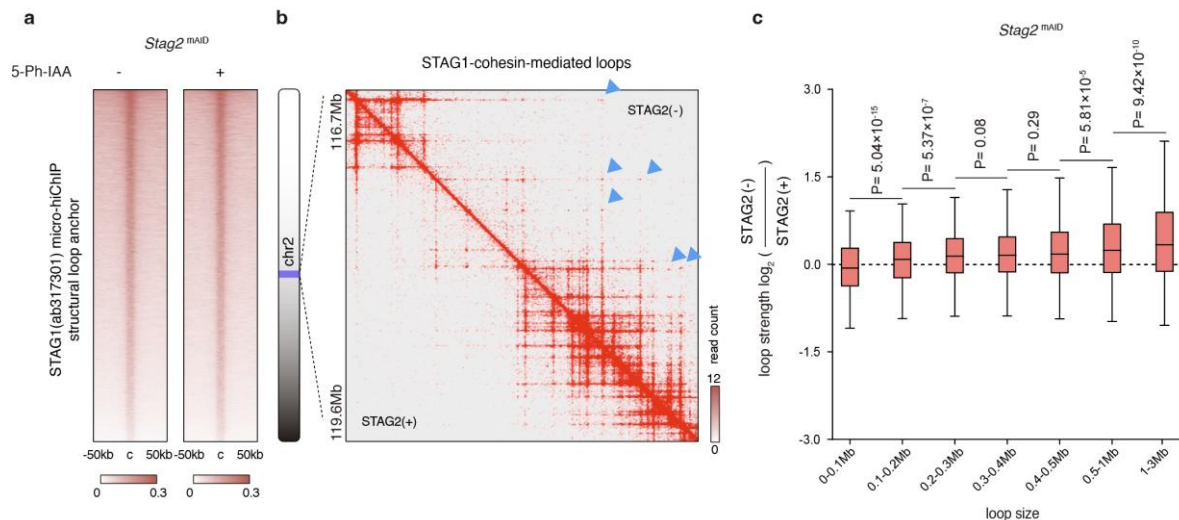
As discussed in our response to Comment 1, performing chromatin fractionation on sorted early- vs. late-G1 cells is biochemically incompatible with our workflow, as the required formaldehyde fixation prevents the clean separation of soluble and chromatin-bound pools. Instead, we used asynchronous cells as a high-quality proxy for late-G1 (the stage with the most prominent structural changes). We reasoned that if STAG2 displacement of STAG1 were a major regulatory mechanism, acute STAG2 depletion should trigger a detectable increase in chromatin-associated STAG1. However, immunoblotting showed that while STAG2 was efficiently depleted, STAG1 levels on the chromatin remained unchanged (new Extended Data Fig. 6g, h). This data indicate that STAG2 does not competitively displace STAG1 from the chromatin.

(2) Quantitative STAG1 ChIP-seq in late-G1 phase cells

To identify potential localized changes in occupancy that global chromatin fractionation might miss, we performed calibrated anti-STAG1 ChIP-seq in late-G1 sorted cells following acute STAG2 depletion. Consistent with our chromatin fractionation data, we found no significant genome-wide increase in STAG1 chromatin occupancy upon the loss of STAG2 (new Extended Data Fig. 6e, f). Importantly, these findings were fully reproduced in asynchronous populations. Notably, the asynchronous-population analyses were independently validated using two distinct anti-STAG1 antibodies (ab4455 and ab317301), further strengthening the robustness of this conclusion (new Extended Data Fig. 6a, b). This dual validation across both sorted and asynchronous cells reinforces our conclusion that STAG1-cohesin does not quantitatively replace STAG2-cohesin on the chromatin.

(3) Preliminary Micro-HiChIP analysis in asynchronous cells

Finally, to directly interrogate the potential changes in the structural role of STAG1 without STAG2, we performed a preliminary STAG1 Micro-HiChIP experiment in asynchronous cells before and after STAG2 depletion. This technique selectively identifies chromatin contacts mediated by STAG1-cohesin. Consistent with the results described above, acute STAG2 depletion did not lead to a detectable increase in STAG1 occupancy at loop anchors (Rebuttal Fig. 1a). However, the contact data revealed a striking trend: the emergence of larger loops specifically associated with STAG1-cohesin following STAG2 depletion (Rebuttal Fig. 1b, c). This suggests that while the physical binding of STAG1 remains stable, its extrusion dynamics may be altered (and perhaps unrestrained) once STAG2 is removed. Because these data are currently preliminary and represent a distinct mechanistic direction, we intend to further validate and present them in a future study dedicated to the functional interplay between these paralogs.



Rebuttal Fig. 1 *Stag2^{mΔID}* cells anti-STAG1 Micro-Hi-CHIP

a, Heat maps of STAG1 Micro-HiChIP signals centered on structural loop anchors in asynchronous *Stag2^{mΔID}* cells with or without 5-Ph-IAA treatment. b, Representative STAG1 Micro-HiChIP contact map showing the emergence of larger STAG1-associated loops upon STAG2 depletion. c, Boxplots showing the $\log_2$ fold change in STAG1-associated loop strength after STAG2 depletion across the indicated loop-size bins.

Together, these independent biochemical and genomic analyses provide no evidence for a competitive displacement mechanism. Instead, they suggest that STAG2 may normally restrain STAG1-mediated loop extrusion through a mechanism other than direct binding competition. We have therefore removed the "competitive displacement" claim from the manuscript and have updated the discussion to reflect this new model of functional interference rather than physical displacement (discussion: line:609–626 of PDF file).

3. Loop extrusion without Stag subunit

At least in vitro, loop extrusion requires the Stag subunits in addition to NIPBL. The presence of a small number of loops in the absence of Stags in vivo is a surprising result. However, it is difficult to demonstrate that the depletion of the Stags is complete, particularly with the western blots shown in Extended Figure 1. Even in the ChIPs shown in that Figure, there are Stag2 signals in the Stag2-AID+auxin conditions.

Please show Stag1 and Stag2 blots for the chromatin fractionation in Figure 5d (this is in fact part of the request made in point 1) Moreover, the authors should immunoprecipitate SMC1 in extracts prepared from the same sorted cells used for the Hi-C (double Stag1AID; Stag2 AID and Smc3AID) and check for the presence of Stags in the immunoprecipitates. Likewise, it would be important to demonstrate the presence of cohesin and CTCF, but no Stag1/2, at the borders of (at least some of) these loops that remain after removal of the two Stag proteins.

RESPONSE: We thank the reviewer for this insightful comment.

To address the reviewer's concern regarding whether cohesin loading was mediated by residual STAG proteins, we performed additional biochemical experiments, ChIP-seq, and functional Hi-C analyses.

(1) Biochemical evidence of STAG-independent cohesin loading.

- To address the completeness of depletion and the composition of the remaining cohesin pool, we performed detailed chromatin fractionation in the *Stag1^{mΔID} Stag2^{mΔID}* cells. Following 5-Ph-IAA treatment, STAG1 and STAG2 proteins become biochemically undetectable in the

chromatin fraction. Crucially, however, RAD21 and SMC1 remain associated with chromatin at approximately 50% of their initial levels (new Fig.5f, g).

While it is impossible to claim absolute "zero" protein levels of STAG1 and STAG2, the fact that STAG proteins are undetectable while ~50% of the cohesin core subunits remain bound to the chromatin demonstrates a non-linear relationship. If cohesin loading were strictly 1-to-1 dependent on STAG proteins, core ring occupancy would decrease proportionally with STAG loss. Our data hence strongly suggest that STAG proteins are not critically required for the initial association of the cohesin ring with chromatin.

- In response to the reviewer's suggestion, we sought to perform anti-SMC1 co-immunoprecipitation to characterize the molecular composition of the cohesin complexes after STAG protein depletion.

While the reviewer suggested using sorted cells, the high salt and detergent conditions required for high-purity co-IP are technically incompatible with the formaldehyde-fixed samples used for sorting. We therefore performed these experiments in asynchronous populations, which, as established in our previous responses, serve as a proxy for the functional state of late-G1 cells.

Upon 5-Ph-IAA treatment, STAG1 and STAG2 were undetectable in the SMC1 immunoprecipitates (new Extended Data Fig. 12c, d), indicating that the remaining cohesin complex on chromatin does NOT contain STAG proteins. Furthermore, RAD21 remains associated with SMC1 without significant reduction, suggesting that the cohesin trimer holocomplex (SMC1, SMC3, RAD21) remains intact and potentially functional (new Extended Data Fig. 12c, d).

(2) Cohesin binding profiles upon STAG protein depletion.

We performed calibrated ChIP-seq for CTCF and RAD21 in late-G1 *Stag1^{mAID} Stag2^{mAID}* cells. We found that despite ~50% of cohesin remaining on chromatin globally, Most cohesin peaks at CTCF sites were lost (Extended Data Fig. 12a, b). This is highly consistent with the established role of STAG proteins as essential adaptors for CTCF-mediated cohesin arrest.

We speculate that the residual loop signals noted by the reviewer likely originate from trace amounts of STAG proteins that remain below the threshold of Western blot detection. However, this does not contradict our primary biochemical observation: that a substantial pool of cohesin can associate with chromatin independently of the STAG.

(3) P_S curve analysis as a global assessment of cohesin extrusion activity upon STAG loss.

To further examine whether chromatin-bound cohesin can actively extrude loops in the absence of STAG proteins, we assessed the contact probability decay curve P_S , which provide a more reliable global reflection of extrusion activity than discrete puncta (loop signals) that depend on stable CTCF anchor points. In our original manuscript, we compared STAG1/2 co-depleted cells to an SMC3-depletion line (a true negative control for cohesin presence) and found that STAG-depleted cells maintain significantly more short-range contacts, signifying extrusion activity.

In the revised manuscript, we further compare our data to NIPBL-depleted late-G1 samples (PMID: 39975341), which have previously been shown to retain partial extrusion activity of cohesin. Crucially, we found that the P_S curve of STAG1/2 co-depleted cells display stronger extrusion

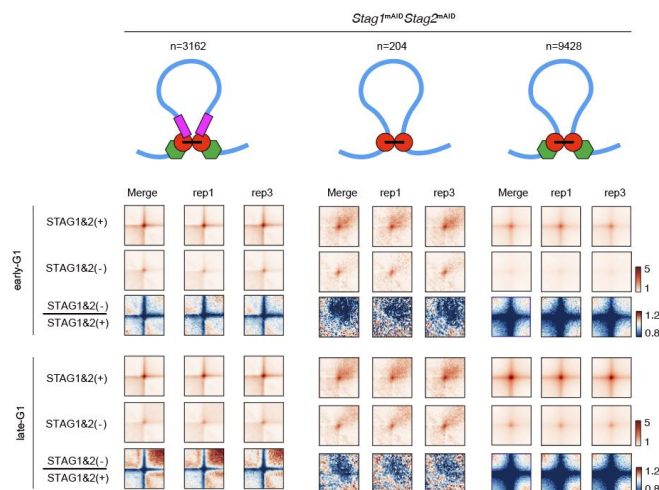
signatures than those of NIPBL-depleted cells (new Extended Data Fig. 13i). This finding definitively confirms that STAG1/2 co-depleted samples retain measurable loop extrusion activity.

In summary, through these orthogonal biochemical and functional analyses, we are confident that a substantial fraction of cohesin remains chromatin-associated as an intact core trimer upon STAG1 and 2 loss. While we acknowledge that trace residual STAG proteins may facilitate a minor background of traditional, CTCF-anchored loop formation, our data collectively support the conclusion that the core cohesin ring possesses a basal, "intrinsic" capacity for chromatin association and loop-extrusion in the absence of STAG proteins(discussion: line:656–671 of PDF file).

4. What happens with non-structural cohesin loops? These would include loops that have cohesin, CTCF, and CRE-element on both sites of the loop, but also other loops that may have cohesin but no CTCF.

RESPONSE: In response to the reviewer's query, we extended our analysis to two additional classes of non-structural loops: (1) those where both anchors are co-occupied by CTCF/cohesin and overlap candidate cis-regulatory elements (cCREs), and (2) those where both anchors are RAD21-positive but CTCF-negative.

Aggregate Peak Analysis (APA) shows that both categories exhibit a reduction in contact strength upon STAG depletion; however, the magnitude of this effect is significantly weaker than that observed for the structural loops (Rebuttal Fig. 2). This observation is expected, as these alternative loop classes are likely less dependent on the specific CTCF-cohesin anchoring mechanisms. While these results are consistent with our broader model, we have opted not to include them in the revised manuscript to maintain focus on the primary architectural framework (loop extrusion). This ensures the central mechanistic conclusions regarding STAG-dependent organization remain clear, particularly as the CTCF-negative class may contain transient or spurious loops. We provide this analysis in the rebuttal for the reviewer's reference.



Rebuttal Fig. 2 Non-structural cohesin-associated loops are modestly affected by STAG1&STAG2 depletion.

Additional issues:

5. The quality of the ChIPs shown in Extended Fig 1 is rather variable, with low enrichment for Stag1 in Stag1^{MAID} clone, for instance. It would be better to show also a common subunit (Rad21 or SMC1/3). Please indicate number of called peaks in each condition, whether they colocalize with CTCF or not. Previous reports have shown that cohesin positions without CTCF are enriched in

Stag2.

RESPONSE: We thank the reviewer for this important comment. To address this concern, we repeated the ChIP-seq experiments and included spike-in controls for calibration. In addition, we have added data for the common cohesin subunit RAD21, and we now report the number of called peaks in each condition as well as the number of peaks colocalizing with CTCF. These results are now presented in the revised manuscript (new Extended Data Fig. 1f-j).

6. I am confused with the saddle plots presented throughout the manuscript

(1) The values for late G1 in Stag1(+) and Stag2(+) control conditions are quite different (4.56 and 5.24, respectively), when they should be the same.

(2) The two replicates of the Stag1-AID cell lines show changes in opposite directions (4.12 to 3.96 in rep1; 5.80 to 5.94 in rep2 (Extended Figure 3d)

(3) The Smc3(+) has a much lower score than the Stag1(+) or Stag2(+): 2.44
What is the reason for these differences?

RESPONSE: We thank the reviewer for this meticulous observation.

(3) Baseline variability: Absolute saddle scores are influenced by clone-to-clone variability and batch effects, and therefore are not intended for direct quantitative comparison across different engineered cell lines/clones (e.g., *Stag1*^{mAID}, *Stag2*^{mAID}, and *Smc3*^{mAID} backgrounds). Furthermore, because these experiments involve complex synchronization protocols, subtle batch-to-batch variabilities in nocodazole release kinetics and cell cycle progression can influence baseline compartment strength. Our main conclusions are based on matched comparisons between untreated and auxin-treated conditions within the same clone/replicate.

(2) Discrepancy between *Stag1*^{mAID} clones.

We agree that the two replicates show minor fluctuations in opposite directions. In our view, this inconsistency demonstrates that acute STAG1 depletion has a negligible effect on compartment strength. Because the biological impact is so minimal, the observed fluctuations between conditions are at the noise level and are susceptible to minor batch-to-batch experimental variability.

(3) Baseline compartment score in *Smc3*^{mAID} cells.

We apologize for this error. This was the result of a manual error during data processing. We have now corrected this number in the revised manuscript (new Extended Data Fig. 13d). The corrected values are now consistent with the range observed in our other cell lines. We thank the reviewer for bringing this to our attention.

C. Additional suggestions.

7. For the analyses of contact probability, using the derivative of $P(s)$ provides a better way to assess the changes related to contacts in the range of either cohesin loops or compartments.

RESPONSE: We thank the reviewer for this insightful suggestion. we have revised the manuscript to include slope analysis of the P_s curves, as suggested (new Fig. 5h, i; new Extended Data Fig. 13g-i).

8. Figure 5g, the Y axis should be “protein levels” instead of “expression”

RESPONSE: We thank the reviewer for pointing this out. We have corrected the Y-axis label in Figure 5g (new Fig. 5g) to "protein level" as suggested.

Citations

9-Line 437 "This result mildly contrasts with prior reports of transcriptional alterations upon Stag1 loss, a discrepancy that may reflect cell type specific differences or technical variations^{34,36}". Ref 34 (Rao 2017) examines total cohesin loss; ref 36 (Holzman 2019) is a proteomic study that quantifies cohesin and regulators in HeLa cells. Thus, they are not "reports of transcriptional alterations upon Stag1 loss". Instead, maybe cite, Remeseiro 2012 (PMID: 22415368), Cuadrado 2019 (PMID: 31216471) or Pezic 2023 (PMID: 37802073).

RESPONSE: We thank the reviewer for bringing up these studies. We have updated the citations to include the following references that are more appropriate for discussing STAG1 depletion and its transcriptional impact:

- Remeseiro 2012 (PMID: 22415368)
- Cuadrado 2019 (PMID: 31216471)
- Pezic 2023 (PMID: 37802073)

These changes are reflected in the revised manuscript (Result: lines 575–577 of PDF file).

10-Line 487 "Cohesin loading is widely attributed to Nipbl, which exhibits high DNA-binding affinity ref 8,45-48. It is therefore surprising that depletion of Stag proteins extensively reduces cohesin loading and loop formation"

Previous reports have shown in vivo and in vitro the importance of Stag subunits for cohesin loading, for instance:

-in vivo: Alonso Gil 2023 (PMID: 36898992) shows presence of Stag1 and Stag2 cohesins on chromatin after strong downregulation of NIPBL;

- in vitro: Murayama 2014 (PMID: 24291789) shows the requirement of Scc3/Stag for DNA entrapment; the cited ref 8 shows that Stag subunit is required for in vitro loop formation, in addition to NIPBL; ref. 46 and 47 also suggest an important role for Stag1 subunit is binding DNA.

RESPONSE: We thank the reviewer for providing these helpful references and comments.

We have thoroughly discussed the potential implications of our results in light of previous studies. (discussion: lines 646–655 of PDF file).

Reviewer #3:

The manuscript by Wang et al. reveals distinct and compensatory roles for STAG1 and STAG2 across early- and late-G1. The authors show that STAG2 plays an important role in forming short loops—including enhancer–promoter (E-P) loops—and in suppressing large loops by competing with STAG1, whereas STAG1 depletion has more modest effects including attenuation of large loops during post-mitotic genome refolding in the G1E-ER4 cell line. The authors further suggest that STAG2-mediated loop extrusion is constrained by CTCF-bound barriers rather than by genomic travel distance. Finally, co-depletion of STAG1 and STAG2 results in a synergistic loss of virtually all structural loops and a more severe disruption of transcription than either single deletion.

While some results are simply consistent with prior observations, this study also reports novel and interesting findings, including a potential mechanism for STAG2-dependent short-loop formation (CTCF barrier vs. travel-distance limitation) and the possibility of cohesin-mediated loop extrusion without STAG subunits—both of which may require further experimental validation. In addition, the balance/abundance between STAG1 and STAG2 in the G1E-ER4 cell line is not discussed and may be an important confounding factor. By addressing the concerns outlined below, this manuscript could be reconsidered for publication. Overall, this read to me like an interesting and important contribution to the field.

RESPONSE: We thank the reviewer for their thoughtful and constructive feedback. We appreciate the positive assessment of our work and acknowledge the important points raised.

Regarding the balance and abundance of STAG1 and STAG2 in the G1E-ER4 cell line, we have now included additional experimental data to address this concern, providing quantitative measurements of STAG1/STAG2 levels. We also clarified the possible influence of this balance on our findings in the revised manuscript.

Major points

1. Line 120: Fig. 1a shows STAG1 tagged with mAID–GFP and STAG2 with mAID–mCherry, but this is not clearly stated in the main text. Please state this explicitly, as large tags can affect protein stability. In addition, please provide immunoblots for STAG1 and STAG2 (using isoform-specific antibodies) alongside the parental cell line to confirm that tagging does not markedly reduce protein levels; STAG proteins are prone to downregulation after tagging.

RESPONSE: We thank the reviewer for this comments.

We have now explicitly stated the tag configuration of all relevant cell lines in main text and methods section of the revised manuscript, including that STAG1 was tagged with mAID–GFP and STAG2 was tagged with mAID–mCherry (Results: lines 125–128 of PDF file; Methods: lines 740–745 of PDF file).

To address the concern regarding protein stability, we performed immunoblotting to compare the levels of endogenous STAG1 and STAG2 in our tagged lines against the parental cell line. Analysis across three independent biological replicates confirmed that mAID-tagging did not significantly reduce the total protein levels of either STAG1 or STAG2 (new Extended Data Fig. 1a-d; new Extended Data Fig. 11a, b). These results demonstrate that the large tags do not compromise protein stability, ensuring that our model system maintains physiological levels of the cohesin subunits prior to depletion.

2. Line 123-125: It is better to include data from the parental cell line to demonstrate that these tagging do not compromise cell proliferation.

RESPONSE: We thank the reviewer for this helpful suggestion. We have now included proliferation data of the parental cell line in the revised manuscript (new Extended Data Fig. 1e; new Extended Data Fig. 11e).

Our results show that the *Stag2*^{mAID} and *Stag1*^{mAID}*Stag2*^{mAID} cell lines proliferate at rates comparable to the parental cells. We did observe that the *Stag1*^{mAID} cell line exhibits a slightly slower growth rate relative to the parental line. However, this baseline difference does not affect our conclusions or the interpretation of our degradation experiments. All functional and genomic comparisons for STAG1 are performed within the *Stag1*^{mAID} background (comparing 5-Ph-IAA-treated to untreated conditions), ensuring that any observed phenotypes are a direct result of acute protein depletion rather than basal growth kinetics. We have updated the manuscript to explicitly note the slower growth characteristics of the *Stag1*^{mAID} line to ensure full transparency for the reader. (Results: lines 131–134 of PDF file)

3. Line 125-127: It is unclear whether spike-in normalization was used. Spike-in controls are necessary to discuss this statement. Please also discuss whether depletion of one STAG subunit leads to compensatory increases in the other, as this is important for interpreting downstream results.

RESPONSE: We thank the reviewer for raising these important points. We address the concerns as follows:

(1) Spike-in normalization:

To ensure quantitative accuracy in our ChIP-seq experiments, we performed calibrated ChIP-seq with spike-in controls. We have now updated the manuscript to include these spike-in-normalized results. Notably, the findings from are entirely consistent with our original CPM-based ChIP-seq data, further confirming the robustness of our conclusions regarding STAG chromatin occupancy (new Extended Data Fig. 1i, j; new Extended Data Fig. 3f, g; new Extended Data Fig. 6a).

(2) Compensatory effects of STAG subunit depletion:

To investigate potential cross-talk or compensation between the two paralogs, we analyzed the chromatin binding of the remaining STAG subunit following the acute depletion of the other. Using spike-in-normalized ChIP-seq, we found that:

- Depletion of STAG1 did not result in a compensatory increase or alteration in STAG2 binding (new Extended Data Fig. 3f, g).
- Depletion of STAG2 did not alter STAG1 binding levels or distribution (new Extended Data Fig. 6a, b, e, f).

This lack of compensation was consistently observed in both asynchronous and late-G1 cell populations. These results demonstrate that the acute loss of one STAG subunit does not trigger a reciprocal change in the chromatin association of the other

4. Line 128-132: Although the synchronization approach has been validated previously, please include representative FACS plots demonstrating effective cell-cycle control and provide appropriate citations^{1,2}.

RESPONSE: We thank the reviewer for the suggestion. We have included representative FACS plots for the cell lines used in this study, confirming effective and consistent enrichment of early- and late-G1 populations (new Extended Data Fig. 1k; new Extended Data Fig. 11f). We have also added the requested citations in the revised manuscript (Methods: lines 822–824 of PDF file).

5. Line 151-158: While cohesin abundance influences compartmentalization, the more relevant citations for loop-extrusion-dependent effects should be 3–5. If you intend to argue for differential loop extrusion rather than altered cohesin abundance after depletion, the authors should include RAD21 (or SMC1/3) western blots to show that total cohesin levels are comparable between conditions; otherwise, differential abundance cannot be excluded.

RESPONSE: We thank the reviewer for highlighting the importance of distinguishing between cohesin abundance and extrusion processivity. We have addressed this by performing chromatin fractionation to quantify the core cohesin machinery remaining on the genome after STAG2 depletion. Our results demonstrate that chromatin-associated levels of RAD21 and SMC1 remain unaffected by STAG2 loss (new Extended Data Fig. 6g, h), confirming that total cohesin occupancy is not significantly reduced. Consequently, the observed weakening of chromatin compartments cannot be attributed to change in cohesin level; rather, it likely arises from the enhanced processivity of STAG1-cohesin in the absence of STAG2. To avoid potential confusion, we have removed the phrase “longer residence time” from the revised text and now state more cautiously that the remaining cohesin may compensate, at least in part, for STAG2 function, while compartmentalization is only slightly reduced.

6. Line 179: The purpose of Fig. 5d is unclear. If the intended message is that larger loops are more affected by STAG1 loss, consider showing differential loop strength as box plots stratified by genomic distance quantiles.

RESPONSE: We thank the reviewer for this suggestion. After re-evaluating the data, we found that acute STAG1 depletion causes only a very slight reduction in structural loop strength. We therefore chose to replot these data as density plots of loop strength in early- and late-G1 cells, which more clearly illustrate the main conclusion that STAG1 loss has only a marginal reduction in structural loop (new Extended Data Fig. 3b).

7. Line 187-188: This is previously shown in Hela cells⁶ and prior work shows that STAG1/STAG2 ratios are cell-type specific^{5,7}. It is better to quantify STAG1- and STAG2-cohesin in G1E-ER4 cells (e.g., by mass spectrometry). This is an important prerequisite for interpreting variant-specific effects throughout the manuscript.

RESPONSE: We entirely agree that establishing the endogenous ratio of STAG1 to STAG2 in G1E-ER4 cells is essential for interpreting our results. To provide a rigorous quantification, we employed two independent approaches:

- (1) Quantitative mass spectrometry analysis: As suggested by the reviewer, we performed quantitative mass spectrometry to determine the relative levels of the two STAG paralog. By comparing the signal intensities of paralog-specific peptide across three biological replicates, we estimate that the ratio of STAG1 and STAG2 is approximately 1:5 (new Extended Data Fig. 4h, i; Results: lines 229–232 of PDF file).
- (2) Western blot quantification: To further substantiate our findings in MS, we adopted CRISPR/Cas9 genome editing and generated endogenous FLAG-P2A-HALO knock-in lines for STAG1 and STAG2, respectively. We first confirmed that FLAG tagging did not significantly alter the protein levels of STAG1 and STAG2 (new Extended Data Fig. 4a-e). Using a single anti-FLAG antibody to ensure equal detection sensitivity to STAG paralogs, we quantified their relative abundance via Western blot. This approach independently confirmed a STAG1:STAG2 ratio of approximately 1:5 (new Extended Data Fig. 4f, g; Results: lines 222–227 of PDF file).

- (3) These results confirm the relative abundance of STAG1 and STAG2 in G1E-ER4 cells and are consistent with previous quantitative proteomic measurements of cohesin subunits (PMID: 31204999). We have incorporated these data into the revised manuscript, with additional details in the Methods section.

8. Line 209-215: This idea could further be supported by western blots comparing STAG1 and STAG2 levels in early- vs. late-G1 to investigate changes in their relative abundance.

RESPONSE: We thank the reviewer for the suggestion. We agree that resolving the temporal stoichiometry of STAG1 and STAG2 is of high interest. However, direct biochemical quantification of sorted cells is technically prohibitive in our system, as the formaldehyde fixation required for cell-cycle sorting precludes high-quality protein extraction and accurate immunoblotting.

Instead, we investigated whether STAG1 is truly displaced by STAG2 on chromatin using chromatin fractionation before and after STAG2 loss. For this analysis, we used asynchronous populations. Since the majority of cells in an asynchronous culture have progressed past early-G1, their cohesin is considered "fully loaded". Therefore, asynchronous cells can be viewed as a proxy of late-g1 phase, where we observe the most dramatic structural differences. Our chromatin fractionation data from these populations (new Extended Data Fig. 6g, h) shows that the acute depletion of STAG2 did not lead to an increase in STAG1 chromatin occupancy. Consistent with this, calibrated STAG1 ChIP-seq in asynchronous *Stag2^{mAID}* cells using two independent anti-STAG1 antibodies (new Extended Data Fig. 6a, b), as well as in late-G1 sorted cells (new Extended Data Fig. 6e), likewise showed no detectable increase in STAG1 chromatin occupancy upon STAG2 loss. This result indicates that STAG2 does not physically displace STAG1 from the genome.

Combining our findings with previous work (Wutz et al., 2024; PMC11716112) showing that STAG2 associates with chromatin with slower kinetics than STAG1 after mitosis, we suggest that STAG2 progressively accumulates to become the dominant paralog by late-G1. Rather than physical displacement, STAG2 likely functions to suppress STAG1-mediated large loop formation through alternative unknown mechanisms. We have updated the manuscript text (discussion: lines 590–626 of PDF file) to reflect this refined model.

9. Line 217-218: This section is potentially interesting but difficult to follow and should be rewritten for clarity. The hypothesis could be tested more directly by conditional CTCF depletion (e.g., using a dTAG system)⁸.

RESPONSE: We thank the reviewer for this helpful suggestion. We agree that this section in the original manuscript was not sufficiently clear, and we have therefore rewritten it substantially for clarity in the revised version (Results: lines 321–377 of PDF file). To more directly test the role of CTCF in constraining STAG2-mediated loop extrusion, we performed STAG2-directed Micro-HiChIP in a conditional *Ctcf^{mAID}* depletion background (new Extended Data Fig. 9a). This allowed us to specifically track the behavior of STAG2-associated chromatin contacts upon acute removal of the CTCF barrier.

Our findings from this experiment provide robust support for our hypothesis:

- Upon CTCF depletion, canonical STAG2-associated loops were markedly reduced or lost, confirming that CTCF is required to stabilize STAG2-cohesin at specific anchors (new Extended Data Fig. 9a-c).
- Critically, we observed a significant increase in long-range contacts that span across the original CTCF anchor sites. This indicates that without the CTCF roadblock, STAG2-

associated cohesin is no longer arrested and instead continues to extrude past these previous boundaries to form more distal contacts (new Extended Data Fig. 9b-d).

- Analysis of CRE-centered compiled virtual 4C profiles showed that, upon CTCF depletion, contacts in regions proximal to CREs were reduced, whereas contacts at more distal regions were increased (new Extended Data Fig. 9d, e). This pattern further supports the idea that, without CTCF, STAG2-associated cohesin can travel farther from CRE-associated loading regions and engage more distant chromatin contacts.

These results provide direct evidence that STAG2-cohesin is susceptible to CTCF-mediated arrest, and that its inability to form larger loops in wild-type cells is a consequence of CTCF-imposed roadblocks rather than an inherent lack of processivity. We have rewritten this section for clarity and incorporated the Micro-HiChIP data into the revised manuscript (new Extended Data Fig. 9 and Results: lines 363–377 of PDF file).

10. Line 281-286: This is very interesting point. Although many studies associate STAG2 with (super)enhancers, to this reviewer's knowledge there is no direct evidence of preferential loading at these regions. It may be better to consider adding NIPBL ChIP-seq with and without STAG2 to test this. If comparing early vs. late G1, does NIPBL loading shift from euchromatin (early G1) to heterochromatin (late G1)?

RESPONSE: We thank the reviewer for this constructive comment. To address this, we performed calibrated NIPBL ChIP-seq in early- vs. late-G1 cells.

We prioritized the temporal comparison (early vs. late) as we reasoned it would more directly reveal the shifting landscape of cohesin loading throughout G1. We did not perform NIPBL ChIP-seq following STAG2 depletion, as NIPBL acts upstream to recruit cohesin to chromatin; therefore, STAG2 loss is not expected to alter the fundamental binding pattern of the loader itself.

Our results show that NIPBL is highly enriched at candidate cis-regulatory elements (CREs) throughout G1 (new Extended Data Fig. 10c, d), consistent with several recent findings (PMID: 39975341) suggesting that enhancers serve as high-density "hubs" for cohesin loading despite occupying a small fraction of the genome. Notably, as cells proceed from early- to late-G1 phase, we also observed a dramatic increase in NIPBL occupancy at regions extending outside enhancers (new Extended Data Fig. 10d). These data suggest that while euchromatic loading is established early, cohesin loading at heterochromatin becomes more robust as G1 progresses.

We have incorporated these calibrated NIPBL occupancy data into the manuscript, providing a mechanistic basis for the temporal shift in STAG2-cohesin localization.

11. Line 297-299: the same with comment 1.

RESPONSE: We thank the reviewer for this comment. We have clarified in the revised manuscript that in the double-edited cell line, STAG1 is tagged with mAID–GFP and STAG2 with mAID–mCherry (Results: lines 431–434 of PDF file; Methods: lines 765-768 of PDF file).

Additionally, we performed Western blot analysis to assess STAG1 and STAG2 protein levels in the double-edited cell lines and compared them to the parental cell line. This was done across three independent biological replicates, and quantification of the band intensities showed that AID-tagging did not significantly reduce protein levels for either STAG1 or STAG2 (new Extended Data Fig. 11a, b).

12. Line 348-49: This is also novel and intriguing finding. Ideally, it would be tested by an in vitro

loop-extrusion assay or an ATPase assay while it is a lot of work.

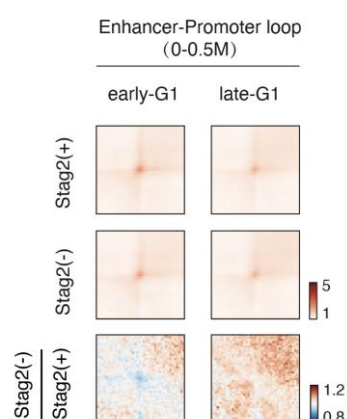
RESPONSE: We thank the reviewer for highlighting the novelty of this finding. While we agree that a full biochemical characterization of STAG-less cohesin, including in vitro loop extrusion and ATPase assays, would be an exciting next step, we believe it is outside the scope of the current study, which focuses on the distinct in vivo architectural roles of STAG1 and STAG2.

That said, we performed a biochemical experiment to assess the integrity of the cohesin complex in the absence of both STAG subunits. Using SMC1 co-immunoprecipitation (co-IP), we found that the core cohesin trimer (SMC1, SMC3, and RAD21) remains intact and associated following the loss of both STAG1 and STAG2 (new Extended Data Fig. 12c, d). This finding, combined with our in vivo genomic data, provides strong evidence that the core complex remains stable and may retain the capacity to associate with chromatin and mediate structural contacts. We have added a discussion of these results to the revised manuscript (discussion: lines 646–671 of PDF file).

13. Line 376-378: How many of the affected loops are putative E-P loops?

RESPONSE: We thank the reviewer for this important question. In our initial analysis, we focused primarily on structural loops (typically anchored by CTCF/cohesin but not CREs) to establish the architectural framework of the G1 genome. To address the reviewer's query, we have now specifically analyzed putative Enhancer-Promoter (E-P) loops following STAG2 depletion.

To address this, we specifically examined 2,988 putative E-P loops, defined as contacts between two cis-regulatory elements (CREs) where at most one anchor is occupied by CTCF/cohesin, and the genomic distance is under 500 kb to avoid confounding with A-type mini-compartments. We found that these E-P loops were weakened upon STAG2 depletion, showing a trend that mirrors our structural loop findings: a distinct reduction in early-G1 that becomes slightly diminished by late-G1. These results support the model that structural loops orchestrated by STAG2 serve as a scaffold that facilitates the formation or stability of more transient E-P contacts (Rebuttal Fig. 3). As this analysis is somewhat peripheral to the main mechanistic focus of the present study, we provide it here in the rebuttal for the reviewer's reference rather than incorporating it into the revised manuscript.



Rebuttal Fig. 3 APA analysis of putative enhancer-promoter loops upon STAG2 depletion.

14. Line 381-384: What are the sets of 'non-sig'? In this type of analysis, the background sets must be selected carefully. It might be suitable to select the genes in which the expression levels are similar.

RESPONSE: We thank the reviewer for this suggestion. To ensure a robust comparison, we have re-performed the analysis using a background set of non-significant genes that were randomly selected to match the expression profile of the down-regulated genes. Our conclusions remained consistent using this expression-matched control group, and we have updated the revised manuscript accordingly (new Fig. 6g).

15. Line 404-406: How is “association with E-P” defined in this analysis? Please specify the criteria.

RESPONSE: Here, “association with E-P” was defined using the ABC model. Because enhancer–promoter interactions play an important role in transcriptional initiation and maintenance (PMID:35691341), we used the ABC framework (PMID: 31784727) to link distal active regulatory elements to gene promoters. Promoters were defined as ± 500 bp around TSSs, and candidate enhancers were identified from distal ATAC-seq- and H3K27ac-marked regions. For each element–promoter pair within 5 Mb, we calculated an ABC score based on element activity and Hi-C-derived contact frequency/probability, normalized across all candidate elements linked to that promoter. Genes were classified as E–P-associated if their promoters had at least one distal enhancer connection with an ABC score > 0.025 . Therefore, the percentages shown in lines 404–406 indicate the fraction of genes in each group with at least one high-confidence ABC-supported enhancer–promoter interaction. Details of the analytical workflow and parameter settings are also provided in the revised Methods section.

$$\text{ABC score} = \frac{\text{Activity} * \text{Contact} * \text{Probability}}{\sum_{\text{gene}} \text{Activity} * \text{Contact} * \text{Probability}}$$

Discussion: Please revise the Discussion section after addressing the points above.

RESPONSE: We have now revised the Discussion section accordingly after addressing the points above, and the updated text now provides a clearer and more balanced interpretation of our findings.

Minor points

1. Line 74, 303, etc: “SMC3” is capitalized whereas “Rad21” appears mixed-case. Similar inconsistencies occur elsewhere. Please use consistent nomenclature: proteins in all capitals (e.g., SMC3, RAD21) and genes in italicized lowercase with an initial capital (e.g., Stag1, Stag2, Rad21) where appropriate.

RESPONSE: We thank the reviewer for pointing out the inconsistencies. We have corrected this in the revised version.

2. Line 123 (Extended Data Figure 1C): Please indicate the scale for the color map (e.g., CPM, FPKM). More broadly, ensure that all figures specify units and normalization for color scales either in the panel or in the legend.

RESPONSE: We thank the reviewer for the valuable suggestion. We now ensure that all figures in the manuscript clearly specify units and normalization methods to enhance the clarity and readability of the manuscript.

3. Recent work (PMID: 41107486) suggests that some CRE loops are maintained in mitosis, but this was not discussed here. This seems relevant and should be discussed.

RESPONSE: We sincerely thank the reviewer for the valuable suggestion. We have now included a discussion of this study in the revised manuscript and explored its potential relevance to our findings (discussion: lines 679–687 of PDF file).

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Authors have addressed my initial comments with care. I only have two small standing comments below and recommend timely publication.

- Extended figure 4 there seems to be a typo: below the bargraphs quantifying the western blots the labels of clone #34 read S36. Please double check.

RESPONSE:

We thank the reviewer for pointing out this error. It is now corrected in the new Extended Data Fig. 4.

- Extended Figure 6: I am a little puzzled how chromatin binding of cohesin RAD21 and SMC1 can remain unaffected after removal of STAG2, especially given STAG2 is the most abundant STAG paralog. Does it mean STAGs may not be required for chromatin binding of cohesin? Isn't that surprising? Also is the conclusion of Extended Figure 6 that cohesin binding by ChIP-seq is not altered? How can this be reconciled with altered Hi-C? It would be helpful to discuss these two points briefly.

RESPONSE:

Cohesin level upon STAG2 loss:

We share the reviewer's interest in the observation that RAD21 and SMC1 chromatin occupancy remains stable despite the loss of STAG2, the dominant paralog in these cells. Our data suggests that the core cohesin complex possesses an intrinsic, albeit reduced, affinity for chromatin that does not strictly depend on STAG proteins. Indeed, even upon the co-depletion of both STAG1 and STAG2, we observed that approximately 50% of core cohesin subunits remain chromatin-bound (Fig.5f, g; Extended Data Fig. 12e, f). This indicates that while STAG proteins facilitate or stabilize cohesin, they are not absolute requirements for loading. This is an interesting direction for future study. These discussions are incorporated into the MS (Discussion, Lines 654-687 of PDF file).

ChIP-seq profiles vs. Hi-C:

The reviewer raises a critical point regarding how stable ChIP-seq profiles can coexist with significant Hi-C reorganization. We propose that the altered 3D genome architecture upon STAG2 loss stems from a shift in connectivity rather than occupancy.

ChIP-seq is a "stationary" measurement that captures the presence of a protein at a specific locus; it does not capture the partner with which that locus is interacting. Upon STAG2 loss, STAG1-cohesin likely undergoes a genome-wide redistribution of its looping activity (extrusion more extensively). Even with an identical ChIP-seq footprint, the "looping probability" between distant STAG1-bound sites increases because they are no longer restricted by the presence of STAG2-mediated boundaries or competition.

In short, the stable ChIP-seq profiles does not rule out the possibility that the "wires" connecting these anchors have been re-routed over longer distances. We have clarified this distinction in the revised text (Discussion, Lines 624-627 of PDF file).

Reviewer #2 (Remarks to the Author):

The authors have made a thorough revision of the manuscript, addressing many of the reviewers' comments, and the Discussion has also improved. I still have some problems with the section regarding the double depletion of STAG1/2.

- Figure 5f shows a chromatin fractionation experiment using cells depleted from both STAGs in which despite STAG absence, 50% of SMC1-Rad21 (and presumably SMC3) remains on chromatin. The authors refer to this result in their rebuttal saying "Our data hence strongly suggest that STAG proteins are not critically required for the initial association of the cohesin ring with chromatin." However, the experiment has been performed with asynchronously growing cells, and not cells that exit from mitosis in the absence of STAGs, as is the case for the Hi-C in Figure 5. Thus, these (presumably) trimeric complexes that are left on chromatin in the absence of STAGs may correspond to complexes loaded before STAG depletion, which maintain their association with chromatin. This possibility should be mentioned,

RESPONSE:

We appreciate the reviewer's comment.

Regarding the concern about 'legacy' complexes in our asynchronous fractionation experiments, we would like to highlight the kinetics of cohesin turnover. Previous live-cell imaging studies have established that the mean residence time of chromatin-associated cohesin is approximately 20 minutes (Hansen et al., eLife 2017). Given that our STAG depletion lasted 4 hours. It is highly probable that the majority of the cohesin pool underwent multiple cycles of unloading and re-loading during the treatment period. This suggests that the 50% residual SMC1/Rad21 observed likely reflects the equilibrium state of STAG-independent association rather than stable pre-loaded complexes.

Nevertheless, we acknowledge that a subset of particularly stable cohesin complexes may persist. We now add these discussions in the Discussion section and specifically acknowledge the possibility that pre-loaded complexes could contribute to the residual pool (Lines 480-483, 672-679 of PDF file).

Related to that:

The abstract says, referring to the double depletion of STAG1/2: "approximately 50% of cohesin remains chromatin-associated and retains active extrusion capacity".

As it is written, it seems that the cohesin trimer does loop extrusion as well as the tetramer. I suggest the authors to tone down this statement and say instead "approximately 50% of cohesin remains chromatin-associated and retains SOME (or MEASURABLE) extrusion capacity".

RESPONSE: We have revised the wording in the Abstract and toned down the statement accordingly. (Abstract, lines 51-52 of PDF file).

-Regarding amounts of STAGs left after depletion: it is not possible to determine these amounts without having an idea of the dynamic range of the antibody used for immunoblotting (Extended data 1a-d; Extended data 11a-b). It is essential to load decreasing amounts of the control condition to check the limit of detection, i.e., can the antibody detect a reduction of 80, 90 or 99%? Likewise, in the IP shown in new Extended Data Figure 12 c-d: (c) The signal for STAG1 and STAG2 in 0h are already low

(compare with those of Smc1 or Rad21); better signals are needed for quantifications; (d) it is unclear what exactly is being quantified. For STAG1, in 0h condition, relative protein level is 0.4, but relative to what?

RESPONSE:

1. Antibody Detection Range and Protein Quantification

We appreciate the reviewer's suggestion to define the dynamic range of our antibodies. To address this, we performed immunoblotting on serial dilutions of control lysates from the *Stag1^{MAID} Stag2^{MAID}* cell line. This allowed us to establish a standard curve for STAG1, STAG2, RAD21, and SMC1 signals, ensuring our quantifications fall within a linear detection range.

By fitting the signals from 5-Ph-IAA-treated samples into these standard curves, we conclude that:

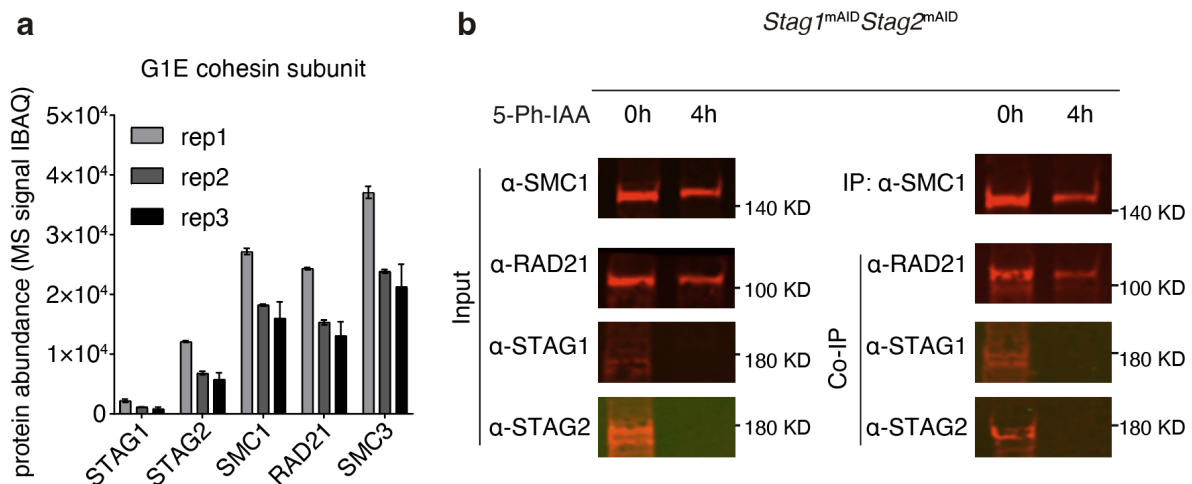
- STAG1 and STAG2: 5-Ph-IAA treatment eliminates >90% of the protein.
- Core Cohesin (RAD21/SMC1): Approximately 40–60% remains chromatin-bound after STAG1/2 co-depletion.

These calibration data and the resulting quantifications have been added to the new Extended Data Fig. 12e, f.

2. CoIP experiments.

Regarding the signal intensity in Extended Data Fig. 12c, we acknowledge that STAG1 and STAG2 signals appear weaker than RAD21 and SMC1. We reason that this discrepancy is not a technical limitation of the blot, but rather a reflection of endogenous protein abundance; our quantitative mass spectrometry data confirms that STAG1 and STAG2 are substantially less abundant than core cohesin subunits (Rebuttal Fig. 1a), with STAG1 being approximately five times less abundant than STAG2. Due to this naturally low abundance, these STAG proteins are particularly challenging to detect as robust, high-intensity bands in immunoprecipitation assays.

To ensure the accuracy of our findings despite these constraints, we repeated the experiment using fluorescent secondary antibodies (Rebuttal Fig. 1b), which provide superior linearity and a broader dynamic range compared to standard HRP-based detection. This fluorescent trial confirmed that while RAD21 and SMC1 were clearly detected in the IPs, STAG1 and STAG2 remained below the threshold of detection (Rebuttal Fig. 1b).



Rebuttal Fig. 1 Lower endogenous abundance of STAG1 and STAG2 and fluorescence-based co-IP validation. **a**, Mass spectrometry-based quantification of cohesin subunits in G1E cells, showing that STAG1 and STAG2 are present at lower abundance than RAD21 and SMC1. Bars represent mean protein abundance from three biological replicates, with technical injections averaged within each biological replicate. **b**, Representative fluorescence-based anti-SMC1 co-IP/immunoblot in the indicated conditions. Fluorescent secondary antibody detection was used to improve signal sensitivity and visualization of weak bands.

3. Normalization:

We apologize for the lack of clarity regarding the normalization in the original Extended Data Fig. 12d, where the value of 0.4 represented an unclearly labeled Co-IP/IP ratio. To improve clarity, we have updated the quantification method by normalizing all protein signals to 1 at the 0h condition, with the 4h condition now expressed as a relative fraction of that baseline (new Extended Data Fig. 12d).

Reviewer #3 (Remarks to the Author):

As I noted in my evaluation of the previous version of this manuscript, I consider this to be an important study that reports several novel and highly interesting findings, including a potential mechanism underlying STAG2-dependent short-loop formation (i.e., a CTCF barrier versus travel-distance limitation) and the possibility that cohesin-mediated loop extrusion can occur even in the absence of STAG subunits.

In the revised manuscript, I was particularly impressed by the extraordinary efforts the authors have made to address my previous concerns. They have responded in a highly rigorous and comprehensive manner through a series of well-designed and informative additional experiments, including calibrated ChIP-seq, Western blotting, and quantitative mass spectrometry to assess STAG variant dosage and the chromatin-associated fraction of the cohesin complex, as well as biochemical experiments evaluating the integrity of the cohesin complex. In addition, the authors have substantially improved the presentation of the manuscript through thoughtful reorganization and clarification of the text.

In my view, all of my major concerns have been fully resolved. The revised manuscript is now highly comprehensive, rigorous, and convincing. I therefore congratulate the authors on this excellent study and their outstanding revision, and I fully support its publication.

RESPONSE: We thank the reviewer.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

RESPONSE: We thank the reviewer.