nature portfolio

Peer Review File

SATB1 lowers the T cell receptor signaling threshold for thymocyte positive selection via super-enhancer reprogramming

Corresponding Author: Professor Bingtao Hao

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)
Xue et al. demonstrated that double-positive (DP) thymocytes require SATB1 for proper super-enhancer formation and chromatin looping during positive selection in response to weak TCR signaling. SATB1 expression peaks specifically at the DP stage during positive selection, and SATB1 facilitates super enhancers that increase loop intensity as CD69- DPs transition to CD69+ DPs by directly occupying these regions together with CTCF and cohesin. This SATB1-mediated chromatin regulation lowers the TCR signaling threshold required for positive selection and prevents the inappropriate egress of autoreactive DP cells to the periphery. Consistently, deletion of SATB1 impairs positive selection-associated gene expression programs, resulting in defective CD4 and CD8 single-positive (SP) development. Finally, the authors showed that SATB1 promotes enhancer activity by forming nuclear condensates.

Although the role of SATB1 in regulating the DP super enhancer and chromatin looping has been extensively studied previously, the functional link to TCR threshold and mechanistic insights from nuclear condensates are novel. However, some points need to be addressed to fully support the main conclusions of the manuscript.

Major points:
One of the key findings of this manuscript is that SATB1 lowers the TCR signal threshold; thus, SATB1 loss results in positive selection only for cells that receive strong TCR signals. Although these results were inferred using gene expression data, whether SATB1 actually lowers signal threshold needs to be experimentally supported. Also, it would be helpful to know whether SATB1 is needed to interpret TCR "strength" vs "duration " properly. Finally, if the authors can dissect SATB1 functions in genes involved in different TCR waves (c.f., Steier et al., PMID: 37580604), this would improve the manuscript.

The authors argue that SATB1 is substantially redistributed in CD69- vs. CD69+ thymocytes. However, ~1,600 differentially bound sites (among ~20,000 peaks) seem modest. Are these a small number of peaks enriched for SATB1 functional target genes (genes responding to SATB1 loss during positive selection), and most of the unchanging binding sites are not functional? If not, how does SATB1 execute distinct functions in CD69- vs. CD69+ cells?

Results from ACC-seq seem subtle (e.g., Fig 8h and i). Also, additional evidence is needed to support the claim that SATB1-mediated nuclear condensates regulate positive selection genes. For instance, are diminished Fix-Hex differences in SATB1-deficient cells enriched near CD5 and other TCR signaling genes?

Minor points:
Vav-Cre was a major tool used to delete SATB1, but the results may be confounded by SATB1 deletion at earlier developmental stages.

Fig 1 b-d: It will be helpful to adjust the x-axis to quantitatively represent the GFP levels.

Fig S1e-f: CD4 SP and CD8 SP show higher GFP levels than those of DP. If this result is true, this contradicts previous statements that SATB1 is most highly expressed in DP and its levels decrease in SPs (c.f., lines 56-57).

Line 158: CD69+ DP showed increased SATB1 peaks (Fig 1, Fig S2) and SATB1 expression is also increased during positive selection. Why do SATB1-associated loops decrease in CD69+ DP cells?

Fig 5k: representative flow cytometry plots from spleen show unusual CD4 int CD8 int populations in both WT and KO. Are these properly gated on live singlets?

Are SATB1-regulated loops also developmentally dynamic loops (changing between CD69- and CD69+ DPs, related to Fig 7, S10)?

Fig S10b, the changes in the bottom right panel seem very modest. Adjusting the axis scale may help recognize differences.

Some figures were not called in the main text (Fig 5C, Fig 7D).

Figures 8d, h, and i need statistics.

Reviewer #2

(Remarks to the Author)
Positive selection is one of the most critical tests that a developing T cell must pass. The authors note ”the mechanisms by which thymocytes translate weak and transient TCR signals into lineage-defining transcriptional programs [during positive selection] remain unclear”. This study examines the role in positive selection of SATB1 (“a nuclear architectural protein that organizes higher-order chromatin and coordinates gene expression programs in T cells”)
The paper is in many ways a technological tour-de-force, defining a role for SATB1 to “promote the expression of positive selection-associated genes by enhancing super-enhancer (SE) activation”. There is no doubt that this data will be interesting and inspiring to many workers in this field. The limitation to much of the work is that many of the experiments produce correlative data (which of course is important and informative but more is needed to support the conclusions that are drawn), and the data generated from the KO mice, while promising, do not exclude relatively limited effects (see below). The paper could be markedly improved: these are some specific suggestions:
1. methods
a. what is the genetic back ground of the mice? Are the strains all inbred?
b. Clarify-“Then evey antibodys add 1μl to 106 cells “.
c. Does not appear Fc block used for FACS– false positives can result
d. What was done to eliminate doublets? (FACS)
e. Clarify- “The sorted cells through staining with antibodies targeting CD4, CD8a and CD69 antibody. “
f. Clarify “493. The CD69+DP cells sorted by FACS,PN-1000268)”
g. Since CD69 is a critical antigen in this study, the supplemental data should show an FMO control, and a sort purity analysis (FACS)
h. correct typos: “613 incubation with a secondary antibody conjuction with Aleax 488 fluorescence”
i. was a Viability dye used? Without this, false positives are often increased (FACS)
j. “CUT&Tag experiments were performed as described before with minimal changes” – provide a citation.
k. Clarify – “689 ATAC-seq library preparation ..690 Initially, approximately 5 × 104 cell pellets” (do they mean cell pellets that each contain 5 × 104 cells?)
2. Results
a. Figure 1 eGFP, eGFP expression in CD69- DP and CD69+ DP; there is more GFP in CD69+. Please show FSC vs GFP or show cells of the same FSC gate to ensure that the result is not driven by the fact that larger cells may have more GFP protein without reflecting increased transcription of the locus,
b. Clarify “187 Multiple studies have shown that super-enhancer regulates the expression of cellular characteristic genes”.
c. Figure S7 c,d, are the displayed cells gated first for DP? Does not say so..
d. Since the SATB1 KO uses a regulated Cre, has the team evaluated efficiency of deletion in the DP subsets 1-4? There would be a strong potential for selection and competition by non-deleted cells.
e. Is TCRalpha rearrangement normal in the SATB1 KO cells? Its stated that RAG gene expression depends on SATB1. Decreased TCRab+ cells would impair positive selection. Is the TCRbeta antibody used specific for full TCRab complexes? Without addressing this, the model proposed for the centrality of SATB1 in regulating a large number of genes in DP cells is not well supported.
f. the scRNA seq could be so informative but why on sorted CD69+ DP thymocytes? why not on the entire DP ?
g. its very important to consider the ways in which the KO may distort the correct identification of DP cells as SATB1 regulates both CD4 and CD8, or may include aberrant cell populations due to developmental impedance - consider comparison to Li et al https://doi.org/10.1186/s13073-021-00861-7 and using the flow analysis scheme they employ

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Most of my previous comments have been adequately addressed or clarified by the authors, and I believe the manuscript

has improved substantially. I thank the authors for their careful responses and revisions.

I have only a few minor points that still require attention:
• Figures 1e,f and Supplementary Figures S1d,f: Statistical analyses are missing.
• Line 306 (Figure 6h): The text refers to "CD69- DP cells," whereas both Figure 6h and its legend are labeled "CD69+ DP cells." Please clarify and ensure consistency.
• Methods lines 617-618: The reference and the name of the software used are missing.
• Methods lines 771-772: The reported concentration of 1 M $CaCl_2$ appears to be unphysiologically high. Please verify whether this value is correct.

Reviewer #2

(Remarks to the Author)
This is a thorough revision of an important study. The work advances this field significantly by adding in-depth understanding of the role of SATB1 in T cell development. The authors address both reviewers comments very well - the paper is much improved with the addition of new data and re-examination of some of the conclusions (or data interpretation). This is an outstanding body of work and needs no further revision

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Major points:
One of the key findings of this manuscript is that SATB1 lowers the TCR signal threshold; thus, SATB1 loss results in positive selection only for cells that receive strong TCR signals. Although these results were inferred using gene expression data, whether SATB1 actually lowers signal threshold needs to be experimentally supported. Also, it would be helpful to know whether SATB1 is needed to interpret TCR "strength" vs "duration " properly. Finally, if the authors can dissect SATB1 functions in genes involved in different TCR waves (c.f., Steier et al., PMID: 37580604), this would improve the manuscript.

Response:

We thank the reviewer for these insightful and constructive comments.

To directly evaluate whether SATB1 affects TCR signaling beyond transcriptional readouts, we performed calcium flux assay in both $CD69^-$ DP and $CD69^+$ DP thymocytes upon CD3 stimulation. Satb1-deficient DP thymocytes showed reduced Ca^{2+} mobilization compared with WT cells, with a relatively modest reduction in CD69- DP cells and a more pronounced impairment in $CD69^+$ DP cells (**Figure 6k-l**). In particular, the response after extracellular Ca^{2+} addition was markedly reduced in Satb1-deficient $CD69^+$ DP cells, whereas ionomycin induced robust Ca^{2+} responses in both WT and Satb1-deficient cells. These results indicate that SATB1 is required for optimal TCR-induced Ca^{2+} signaling rather than for general cellular Ca^{2+} mobilization capacity.

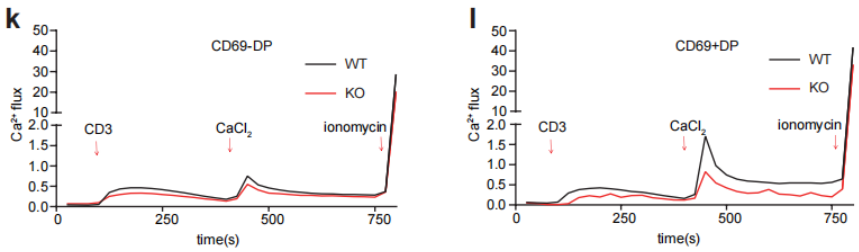


Figure 6k and 6l

We agree that a definitive measurement of signaling threshold would require dose-response stimulation using graded TCR ligands or antibody concentrations. Therefore, we have moderated the wording in the revised manuscript and now state that SATB1 supports TCR responsiveness during positive selection.

Furthermore, we performed pseudotime trajectory analysis on single-cell RNA-seq data to examine the temporal dynamics of TCR-responsive genes. The expression dynamics of Cd8a, Cd5, Runx3 and other key genes revealed two sequential TCR signaling waves during CD8 lineage commitment, which is highly consistent with the model reported in Steier et al. We further profiled the expression of a panel of genes associated with TCR signaling and T cell lineage specification, systematically dissecting the temporal dynamics of TCR signal transduction during the developmental transition of $CD69^+$ DP cells. These analyses have been incorporated into

Figure 5g-h and Supplementary Figure S10 of the new version.

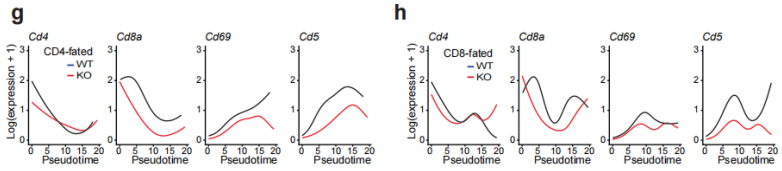


Figure 5g-h

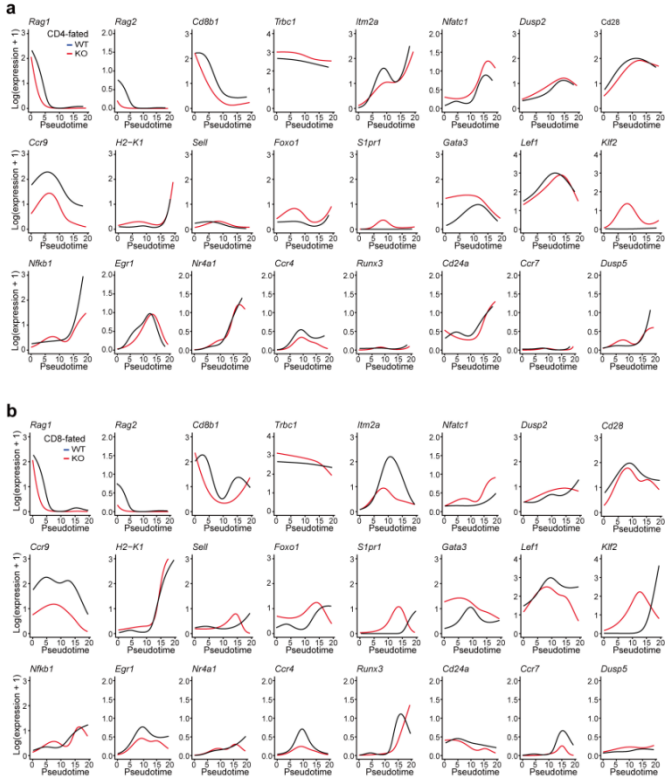


Figure S10 Expression of positive selection related genes in CD69$^+$ DP cells

(a) Smoothed expression profiles of positive selection related genes along pseudotime in CD4-fated trajectories from wild-type and Satb1-deficient thymocytes.

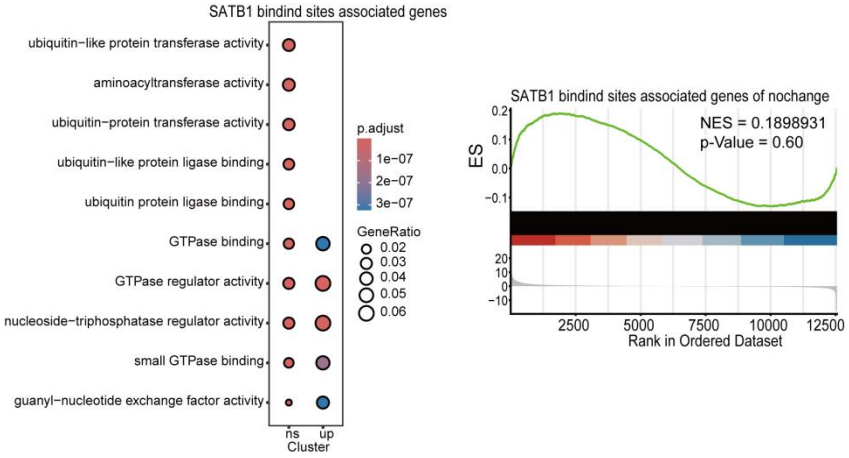
(b) Smoothed expression profiles of positive selection related genes along pseudotime in CD8-fated trajectories from wild-type and Satb1-deficient thymocytes.

The authors argue that SATB1 is substantially redistributed in CD69- vs. CD69+ thymocytes. However, ~1,600 differentially bound sites (among ~20,000 peaks) seem modest. Are these a small number of peaks enriched for SATB1 functional target genes (genes responding to SATB1 loss during positive selection), and most of the unchanging binding sites are not functional? If not, how does SATB1 execute distinct functions in CD69- vs. CD69+ cells?

Response:

We thank the reviewer for this thoughtful and insightful comment. We agree that the number of differentially bound SATB1 sites is modest relative to the total number of SATB1 peaks, and we have therefore performed additional analyses to distinguish constitutive binding from functionally dynamic binding during positive selection.

Genes associated with constitutive SATB1 peaks showed limited transcriptional changes upon Satb1 deletion in CD69^{+} DP cells, suggesting that many unchanged SATB1-binding events are not detectably coupled to gene-expression changes under this developmental condition. In contrast, as shown in Figure 6e, genes within SATB1-mediated loops were significantly downregulated in Satb1-deficient CD69^{+} DP cells, indicating that loop-associated SATB1 binding better identifies functional regulatory interactions than simple nearest-gene annotation. Because many SATB1 binding sites are located at distal enhancers (Figure S3a), nearest-gene assignment may underestimate or misassign their regulatory targets. We therefore interpret the approximately 1,600 dynamic SATB1 sites as a functionally enriched subset that is preferentially linked to enhancer–promoter communication and positive-selection gene activation. We have revised the manuscript to clarify this point and to avoid implying that all SATB1-binding sites are equally functional during the CD69^{-} to CD69^{+} DP transition.



Results from ACC-seq seem subtle (e.g., Fig 8h and i). Also, additional evidence is needed to support the claim that SATB1-mediated nuclear condensates regulate positive selection genes. For instance, are diminished Fix-Hex differences in SATB1-deficient cells enriched near CD5 and other TCR signaling genes?

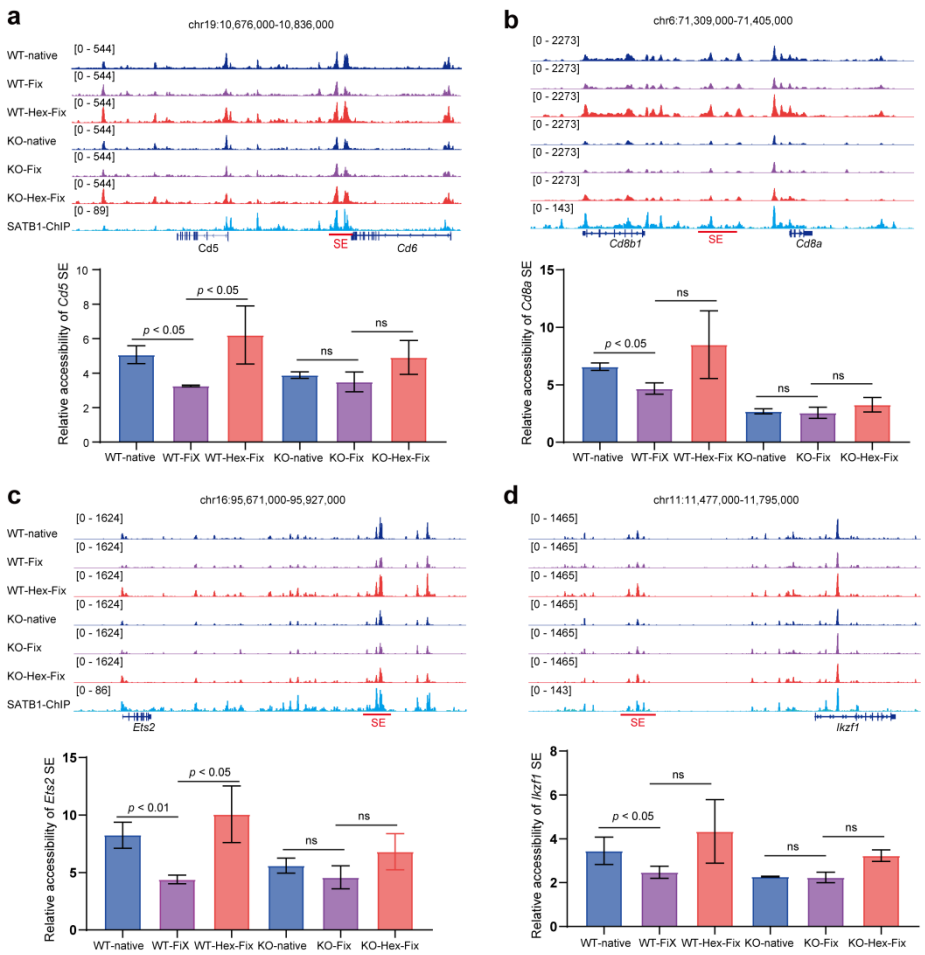
Response:

We thank the reviewer for this constructive comment. To strengthen the connection between SATB1-associated condensate-sensitive chromatin accessibility and positive-selection genes, we expanded the ACC-seq analysis to additional TCR signaling and positive-selection loci, including Cd5, Cd8a, Ets2 and Ikzf1. These newly added loci showed reduced Fix-Hex differences at super-enhancer (SE) regions in Satb1-deficient CD69^{+}DP cells, similar to the pattern observed at the Ets1 locus.

We have revised the text to describe Fix–Hex differences as an operational readout of condensate-sensitive chromatin accessibility, rather than as definitive proof of condensate dependency. The Cd5 locus is now included in Figure 8h, and additional loci including Ets1 are presented in Supplementary Figure S15/S16. Together, these analyses support the conclusion that SATB1 contributes to condensate-sensitive accessibility at super-enhancers associated with positive-selection genes.

We also acknowledge that 1,6-hexanediol can have condensate-independent effects. Therefore, we have moderated the wording in the revised manuscript and now state that SATB1-dependent SEs

display condensate-sensitive accessibility, which is consistent with a condensate-associated mechanism of enhancer regulation.



Minor points:

Vav-Cre was a major tool used to delete SATB1, but the results may be confounded by SATB1 deletion at earlier developmental stages.

Response:

We thank the reviewer for this important concern regarding potential developmental confounding effects with Vav-Cre.

We agree that Vav-Cre-mediated deletion could potentially introduce effects from earlier hematopoietic stages. To address this concern, we analyzed Satb1 deletion using CD4-Cre, which mediates deletion at the DP stage. Satb1$^{fl/fl}$ CD4-Cre mice showed comparable positive-selection defects to those observed in Satb1$^{fl/fl}$ Vav-Cre mice, including reduced CD69^{+}/CD5^{+} DP populations and impaired SP development (Figure S7). These findings support a DP-stage requirement for SATB1 during positive selection and indicate that the major phenotype is not solely inherited from earlier developmental defects.

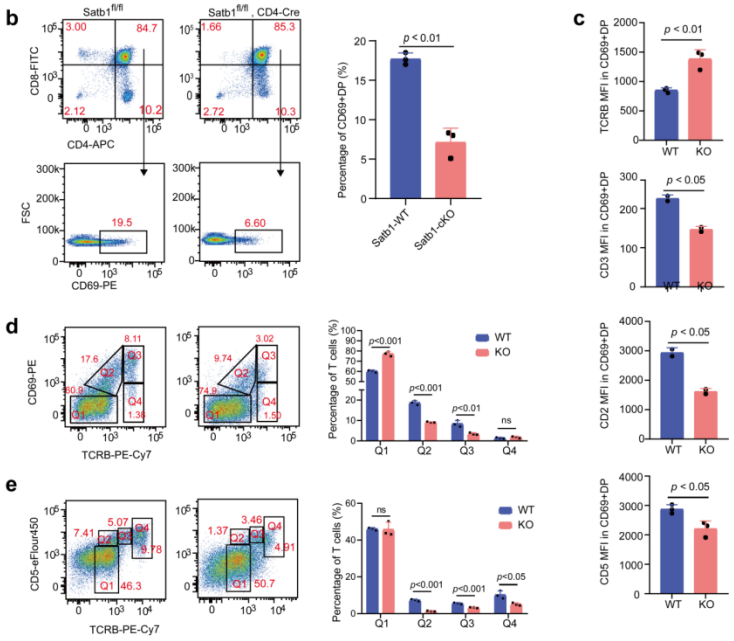


Figure S7b-e Conditional deletion of Satb1 in DP thymocytes impairs positive selection

(b) Flow cytometry analysis of thymocytes from Satb1fl/fl (WT) and Satb1fl/fl; CD4-Cre (cKO) mice. Representative plots show CD4 and CD8 expression (top) and CD69 expression within DP thymocytes (bottom). Right, quantification of the frequency of CD69^{+} DP cells. Data are shown as mean ± s.d.; P values were determined by two-sided Student's t test.

(c) Quantification of mean fluorescence intensity (MFI) of TCRβ, CD3, CD2, and CD5 on CD69^{+} DP thymocytes from WT and Satb1 cKO mice. Data are shown as mean ± s.d.; P values were determined by two-sided Student's t test.

(d) Flow cytometry analysis of thymocytes stratified by TCRβ and CD69 expression, defining four developmental stages (Q1-Q4) corresponding to sequential phases of positive selection. Representative plots (left) and quantification of population frequencies (right) are shown for WT and Satb1 cKO mice.

(e) Flow cytometry analysis of thymocytes stratified by TCRβ and CD5 expression, defining four developmental stages (Q1-Q4). Representative plots (left) and quantification of population frequencies (right) are shown for WT and Satb1 cKO mice.

Fig 1 b-d: It will be helpful to adjust the x-axis to quantitatively represent the GFP levels.

Response:

We thank the reviewer for this suggestion.

We have revised the x-axis labels in Figure 1b–d to quantitatively represent GFP fluorescence intensity. We also replaced the label "SATB1$^{-/eGFP}$" with "GFP" in the histogram axis for clarity.

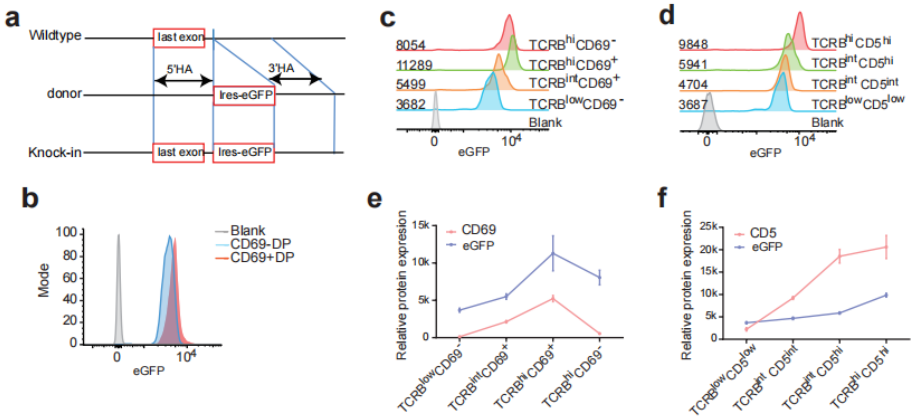


Figure 1b–d

Fig S1e-f: CD4 SP and CD8 SP show higher GFP levels than those of DP. If this result is true, this contradicts previous statements that SATB1 is most highly expressed in DP and its levels decrease in SPs (c.f., lines 56-57).

Response:

We thank the reviewer for highlighting this point. We have revised the text to distinguish Satb1 mRNA expression, Satb1-IRES-eGFP reporter signal, and endogenous SATB1 protein abundance. The previous statement (lines 56–57) referred to Satb1 mRNA levels, which have been reported to peak in DP thymocytes and decrease in SP cells. In our Satb1-IRES-eGFP reporter mice, however, GFP fluorescence is higher in SP thymocytes than in DP thymocytes. Because IRES-eGFP fluorescence may reflect Satb1 locus activity, reporter translation, and/or GFP protein stability, it should not be interpreted as a direct measurement of endogenous SATB1 protein abundance. We have revised the manuscript accordingly and now describe these data as Satb1 reporter activity rather than SATB1 protein expression.

Line 158: CD69+ DP showed increased SATB1 peaks (Fig 1, Fig S2) and SATB1 expression is also increased during positive selection. Why do SATB1-associated loops decrease in CD69+ DP cells?

Response:

We thank the reviewer for this insightful question.

First, total chromatin loops decrease globally from CD69$^-$DP (~40,000) to CD69$^+$DP (~33,000), which contributes to the reduction in absolute number of SATB1-associated loops. Second, Figure 2d displays the proportion of SATB1-associated loops relative to total loops, not absolute counts. The reduced proportion of SATB1 loops in CD69$^+$DP cells suggests that gained SATB1 binding peaks are concentrated in specific genomic regions, rather than being broadly distributed across all loop anchors. It suggests that SATB1 is redistributed toward a restricted set of functional enhancer-associated interactions during the CD69$^-$ to CD69$^+$ DP transition. We have clarified this point in the revised manuscript.

Fig 5k: representative flow cytometry plots from spleen show unusual CD4 int CD8 int populations in both WT and KO. Are these properly gated on live singlets?

Response:

We thank the reviewer for this important comment. We agree that the CD4^{int}CD8^{int} population in

spleen should be carefully distinguished from doublets or other flow-cytometric artifacts. We therefore re-examined the gating strategy. All spleen samples were first gated on lymphocytes by FSC-A/SSC-A, followed by sequential singlet gating using FSC-A/FSC-H and SSC-A/SSC-H before CD4/CD8 analysis. Thus, the $CD4^{int}CD8^{int}$ population is unlikely to be caused by doublet contamination. We have added the complete gating strategy to Figure S8a and clarified this in the Methods.

After this stringent singlet gating, the $CD4^{int}CD8^{int}$ population remained detectable. A small population was present in WT spleen, and its frequency was increased in Satb1-deficient mice. Thus, this population is unlikely to reflect doublet contamination. Rather, we interpret it as a peripheral CD4/CD8 double-positive or DP-like T-cell population. This interpretation is consistent with previous reports that SATB1 is required for maintaining thymocyte/T-cell identity and that Satb1 deficiency can result in abnormal CD4/CD8 lineage features and increased peripheral DP-like T-cell populations.

We have revised the manuscript to describe these cells more cautiously as peripheral $CD4^{int}CD8^{int}$ or CD4/CD8 double-positive T cells, rather than conventional thymic DP cells, and have added the gating strategy to support this conclusion.

Are SATB1-regulated loops also developmentally dynamic loops (changing between CD69- and CD69+ DPs, related to Fig 7, S10)?

Response:

We thank the reviewer for this important question. We compared SATB1-regulated loops in Satb1-deficient $CD69^+$ DP cells with loops that change during the normal $CD69^-$ to $CD69^+$ DP transition in WT cells. Using stringent loop-calling criteria, only approximately 30% of loops were stably maintained during this transition. Importantly, a substantial fraction of loops altered by Satb1 deletion in $CD69^+$ DP cells did not simply overlap with developmentally dynamic loops observed in WT cells. These results suggest that SATB1 loss induces specific regulatory-loop defects in $CD69^+$ DP cells, rather than merely reflecting a shift along the normal developmental trajectory. We have clarified this point in the revised text.

Fig S10b, the changes in the bottom right panel seem very modest. Adjusting the axis scale may help recognize differences.

Response:

We thank the reviewer for this helpful suggestion.

The axis scale in Figure S11b (Fig S10b in the old version) has been adjusted to better visualize the differences, as recommended.

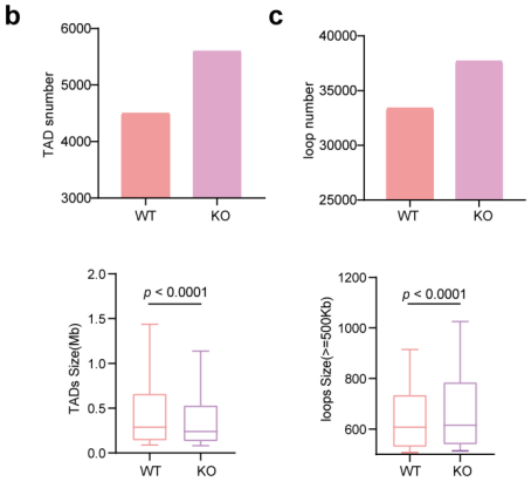


Figure S11b-c

Some figures were not called in the main text (Fig 5C, Fig 7D).

Response:

We thank the reviewer for this comment.

Figure 5C is now cited in the main text at line 248. Figure 7D is already cited at line 313, as indicated.

Figures 8d, h, and i need statistics.

Response:

We thank the reviewer for this comment.

Statistical analyses have been added to Figures 8d, h, and i as requested.

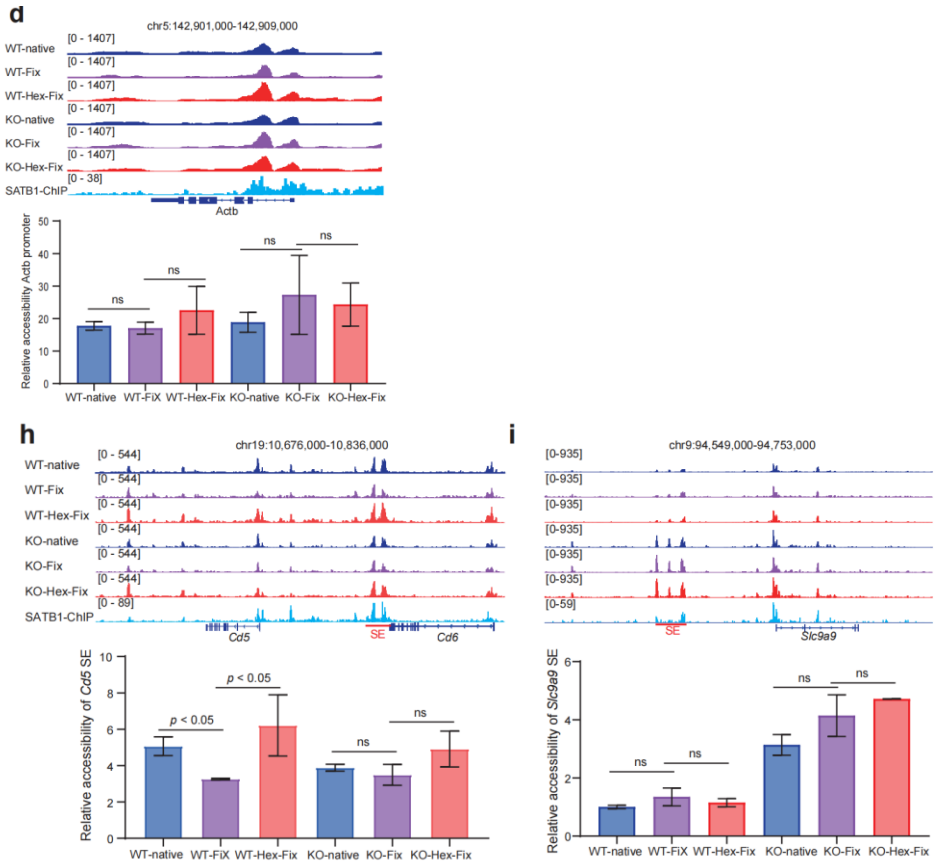


Figure 8d, 8h-i

Reviewer #2 (Remarks to the Author):

The paper is in many ways a technological tour-de-force, defining a role for SATB1 to "promote the expression of positive selection-associated genes by enhancing super-enhancer (SE) activation". There is no doubt that this data will be interesting and inspiring to many workers in this field. The limitation to much of the work is that many of the experiments produce correlative data (which of course is important and informative but more is needed to support the conclusions that are drawn), and the data generated from the KO mice, while promising, do not exclude relatively limited effects (see below). The paper could be markedly improved: these are some specific suggestions:

1. methods

a. what is the genetic back ground of the mice? Are the strains all inbred?

Response:

All mouse strains used in this study were maintained on a C57BL/6 background. Age- and sex-matched littermate controls were used for all experiments unless otherwise indicated. We have added this information to the Methods section.

b. Clarify-"Then evey antibodys add 1μl to 106 cells ".

Response:

We thank the reviewer for pointing out this unclear sentence. We have revised the Methods to state that cells were stained with fluorochrome-conjugated antibodies at empirically titrated concentrations for 30 min on ice. This replaces the ambiguous statement regarding "1 μl antibody per 10^6 cells."

c. Does not appear Fc block used for FACS– false positives can result

Response:

We appreciate the reviewer's concern regarding potential non-specific antibody binding. Fc block was not used in the original thymocyte staining experiments. Because the key analyses were performed primarily on thymocytes and gated T-lineage populations, Fc receptor-mediated background is expected to be limited. In addition, all antibodies were directly conjugated monoclonal antibodies and were used at empirically titrated concentrations. We have clarified this point in the Methods.

For peripheral lymphoid tissues, cells were first gated on $CD3^+$ T cells before CD4/CD8 analysis, which further minimizes potential contamination from Fc receptor-expressing non-T cells. We have also added CD69 FMO controls and post-sort purity analyses to strengthen the reliability of the flow-cytometric gating.

d. What was done to eliminate doublets? (FACS)

Response:

Doublets and aggregates were excluded by sequential gating using FSC-A/FSC-H and SSC-A/SSC-H parameters before downstream analysis. The complete gating strategy has been added to Figure S8a.

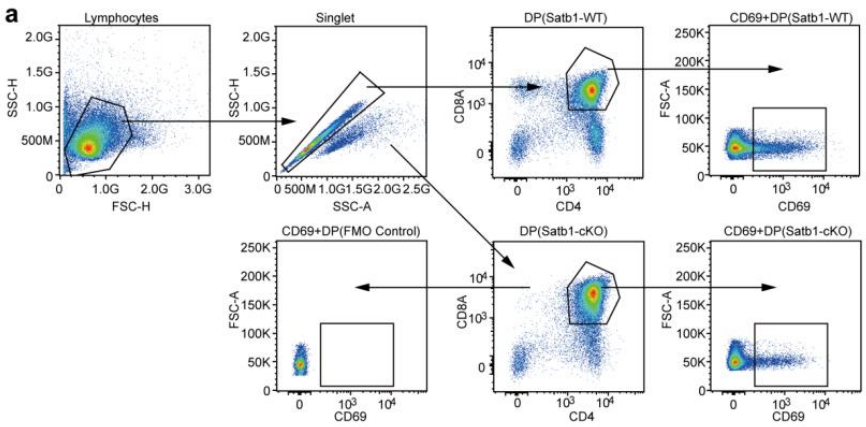


Figure S8a

e. Clarify- "The sorted cells through staining with antibodies targeting CD4, CD8a and CD69 antibody. "

f. Clarify "493. The CD69+DP cells sorted by FACS,PN-1000268)"

Response:

We have revised the Methods to state: "CD69$^+$ DP thymocytes were sorted on a BD FACSAria III after staining with antibodies against CD4, CD8α and CD69." The incorrect catalog number and unclear wording have been removed.

g. Since CD69 is a critical antigen in this study, the supplemental data should show an FMO control, and a sort purity analysis (FACS)

Response:

We thank the reviewer for this important suggestion. Because CD69 is central to our study, we added CD69 FMO controls to define the CD69$^+$ gate. We also performed post-sort purity analysis by reanalyzing a fraction of sorted CD69$^+$ DP cells. Representative FMO controls and post-sort purity plots have been added to Figure S8a. The Methods and figure legends have been revised accordingly.

h. correct typos: "613 incubation with a secondary antibody conjuction with Aleax 488 fluorescence"

Response:

We have corrected this sentence to: "Cells were incubated with an Alexa Fluor 649-conjugated secondary antibody." The spelling errors have been corrected in the Methods.

i. was a Viability dye used? Without this, false positives are often increased (FACS)

Response:

We appreciate the reviewer's concern. Viability dye was not used in the original experiments, and we have revised the Methods to avoid implying otherwise. Dead cells and debris were minimized by using freshly prepared single-cell suspensions and were reduced during analysis by FSC/SSC lymphocyte gating. We acknowledge that viability dye would further improve exclusion of dead cells, and we will incorporate viability dye in future flow-cytometry experiments. Importantly, the key conclusions are supported by independent readouts, including surface-marker analysis, sorted-

cell transcriptomics, and Ca^{2+} flux assays.

j. "CUT&Tag experiments were performed as described before with minimal changes" – provide a citation.

Response:

We have added the appropriate citation for the CUT&Tag protocol and revised the sentence to: "CUT&Tag experiments were performed with minor modifications as previously described (Kaya-Okur et al., 2019)."

k. Clarify – "689 ATAC-seq library preparation ..690 Initially, approximately 5 × 104 cell pellets" (do they mean cell pellets that each contain 5 × 104 cells?)

Response:

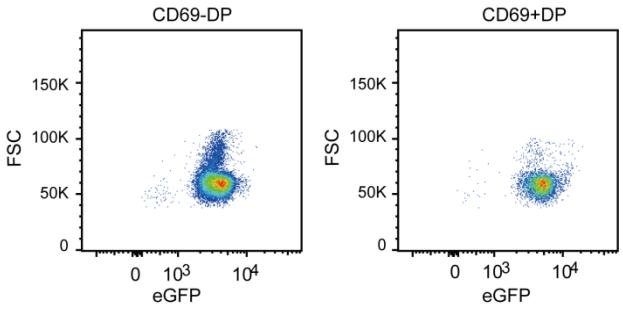
We thank the reviewer for pointing out this ambiguity. We have revised the Methods to state that approximately 5×10^4 cells were used for each ATAC-seq library.

2. Results

a. Figure 1 eGFP, eGFP expression in CD69- DP and CD69+ DP; there is more GFP in CD69+. Please show FSC vs GFP or show cells of the same FSC gate to ensure that the result is not driven by the fact that larger cells may have more GFP protein without reflecting increased transcription of the locus,

Response:

We appreciate the reviewer's careful concern about potential cell size/granularity effects on GFP mean fluorescence intensity (MFI). To exclude that the higher GFP signal in $CD69^+$ DP cells simply reflects larger cell size, we analyzed eGFP expression within the same FSC gate. Even when comparing size-matched cells, $CD69^+$DP cells still exhibited significantly higher eGFP expression than $CD69^-$ DP cells. These results indicate that the increased GFP signal is not simply driven by larger cell size and supports increased Satb1 reporter activity during positive selection. We have added this analysis to the revised figure.



b. Clarify "187 Multiple studies have shown that super-enhancer regulates the expression of cellular characteristic genes".

Response:

We have clarified and revised the sentence for accuracy and grammar: "Multiple studies have demonstrated that super-enhancers regulate the expression of cell-type-specific signature genes."

c. Figure S7 c,d, are the displayed cells gated first for DP? Does not say so..

Response:

We thank the reviewer for pointing this out. The cells shown in Figure S7c,d were analyzed from total thymocytes rather than pre-gated DP cells, because the purpose of this panel was to compare the overall distribution of thymocyte developmental populations. We have clarified the gating strategy in the figure legend and Methods. The analysis follows established flow-cytometric strategies for assessing thymic positive and negative selection.

d. Since the SATB1 KO uses a regulated Cre, has the team evaluated efficiency of deletion in the DP subsets 1-4? There would be a strong potential for selection and competition by non-deleted cells.

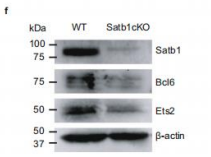
Response:

We thank the reviewer for raising this important point. We agree that, when using a Cre-mediated conditional knockout system, incomplete deletion could potentially generate a competitive advantage for non-deleted cells and complicate the interpretation of developmental phenotypes.

We have previously evaluated the efficiency of SATB1 deletion in thymocytes in our previous study (Feng et al. 2022 Nature Communications) using the same conditional knockout strategy. In that study, SATB1 protein was efficiently depleted in the vast majority of DP thymocytes, indicating that the system achieves robust deletion at the DP stage.

Importantly, the developmental and transcriptional phenotypes observed in Satb1 cKO mice were not restricted to a minor subset of cells but were consistently detected across the DP compartment and downstream thymocyte populations. This argues against the possibility that the main phenotype was driven by expansion or selection of a small population of non-deleted escape cells. In addition, the persistence of positive-selection defects in the OT-I TCR transgenic background further supports the conclusion that the phenotype reflects a cell-intrinsic requirement for SATB1 rather than selective outgrowth of undeleted cells.

To address the reviewer's concern more explicitly, we have now clarified in the revised manuscript that SATB1 deletion efficiency in DP thymocytes had been validated in our previous study. We also added a statement noting that the broad reduction of SATB1 protein in sorted DP thymocytes makes it unlikely that non-deleted cells account for the observed defects.



e. Is TCRalpha rearrangement normal in the SATB1 KO cells? Its stated that RAG gene expression depends on SATB1. Decreased TCRab+ cells would impair positive selection. Is the TCRbeta antibody used specific for full TCRab complexes? Without addressing this, the model proposed for the centrality of SATB1 in regulating a large number of genes in DP cells is not well supported.

Response:

We thank the reviewer for this important comment. We agree that defects in TCRα rearrangement and/or reduced surface TCRαβ expression could contribute to impaired positive selection and therefore need to be carefully considered.

SATB1-dependent regulation of TCR rearrangement has been established in our previous studies. In our previous study (Hao et al. 2015 J Exp Med), we reported that SATB1 deficiency affected Rag1/Rag2 expression and impaired Tcra rearrangement. In a subsequent study (Feng et al. 2021 Genome), we further characterized how SATB1 shapes the adult thymic TCR repertoire, with a particularly strong effect on Jα usage during TCRα rearrangement. Thus, we agree with the reviewer that TCRα rearrangement is not completely normal in Satb1-deficient thymocytes.

To address this issue directly in the cell population analyzed in the present study, we have now added new Tcra 5′RACE sequencing data from $CD69^+$ DP thymocytes. Consistent with our previous findings, Satb1 deficiency altered Vα–Jα usage in $CD69^+$ DP cells, with relatively increased usage of proximal Jα segments and reduced usage of distal Jα segments. We have added these data as a heatmap of Vα–Jα combinations in $CD69^+$ DP thymocytes from WT and Satb1 cKO mice in the revised manuscript as **Figure S7f**. These results confirm that SATB1-dependent changes in TCRα repertoire formation are also present in the positively selecting $CD69^+$ DP compartment.

Regarding the TCRβ staining, the antibody used detects surface TCRβ. In DP thymocytes, stable surface TCRβ staining largely reflects expression of the assembled αβ TCR complex, because TCRβ requires pairing with TCRα and association with the CD3 complex for efficient surface expression. Therefore, reduced $TCR\beta^+$ or $TCR\alpha\beta^+$ DP cells can, at least in part, reflect impaired TCRα rearrangement or reduced αβ TCR surface expression. We have clarified this point in the revised text and have avoided interpreting reduced TCRβ staining as evidence for a TCRβ-specific defect alone.

Importantly, however, the positive-selection phenotype cannot be explained solely by altered endogenous TCRα rearrangement. Positive-selection defects persisted in OT-I TCR transgenic mice, in which a prearranged TCR specificity bypasses much of the variability associated with endogenous TCRα repertoire formation. This result indicates that, in addition to its role in TCRα rearrangement and repertoire formation, SATB1 also regulates rearrangement-independent processes required for DP thymocyte responsiveness during positive selection.

We have therefore revised the manuscript to make this interpretation explicit: SATB1 contributes to positive selection through at least two connected mechanisms. First, it supports normal TCRα rearrangement and repertoire formation. Second, it regulates a broader DP transcriptional and chromatin program that controls TCR responsiveness and positive-selection competence independently of endogenous TCRα repertoire variability. This revised interpretation better reflects both our previous work and the new $CD69^+$ DP Tcra 5′RACE data added in Figure S7f.

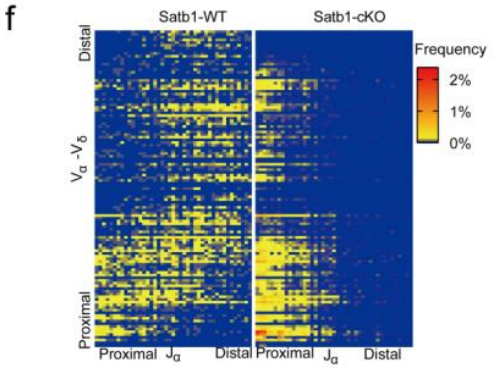


Figure S7f Heatmap of Vα–Jα combination in CD69^{+} DP thymocytes from WT and *Satb1* cKO mice.

f. the scRNA seq could be so informative but why on sorted CD69+ DP thymocytes? why not on the entire DP ?

Response:

We appreciate the reviewer's important question. We chose to perform scRNA-seq on sorted CD69^{+} DP thymocytes because this population represents the key compartment undergoing positive selection and is markedly reduced in Satb1-deficient mice. In Satb1 cKO mice, CD69^{+} DP cells represent only approximately 5% of total DP thymocytes. Therefore, scRNA-seq of unsorted total DP cells would be dominated by pre-selection CD69^{-} DP cells and would under-sample the rare but functionally critical CD69^{+} DP population. By sorting CD69^{+} DP cells, we were able to resolve the heterogeneity and transcriptional trajectories within the positively selecting compartment. We have clarified this rationale in the revised manuscript and included the sorting strategy in Figure S8a.

g. its very important to consider the ways in which the KO may distort the correct identification of DP cells as SATB1 regulates both CD4 and CD8, or may include aberrant cell populations due to developmental impedance - consider comparison to Li et al https://doi.org/10.1186/s13073-021-00861-7 and using the flow analysis scheme they employ

Response:

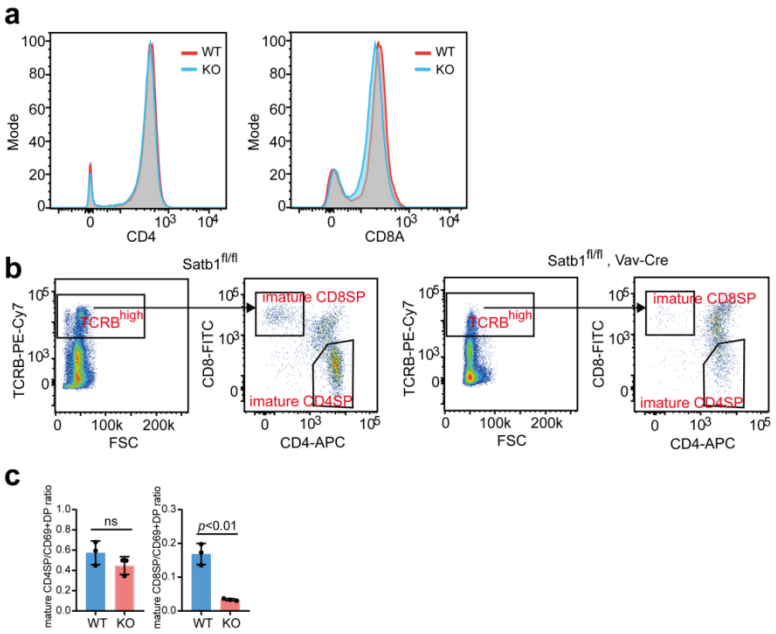
We greatly appreciate the reviewer's valuable comment and the suggestion to consider the gating strategy used by Li et al. (2021). We agree that Satb1 deficiency could distort CD4/CD8 expression and thereby complicate the identification of DP and SP populations.

To address this concern, we re-examined CD4 and CD8 surface expression in Satb1-deficient thymocytes. Although Cd4 and Cd8a transcripts were reduced in our transcriptomic data, surface CD4 and CD8 protein levels were only modestly affected, supporting the reliable identification of CD4^{+}CD8^{+} DP cells by flow cytometry.

More importantly, we realized that directly defining CD4^{+}CD8^{-} and CD4^{-}CD8^{+} cells as CD4SP and CD8SP populations in the original analysis could include immature single-positive cells and aberrant intermediates. We therefore revised the gating strategy by first selecting TCRβ^{hi} thymocytes and then defining mature CD4SP and CD8SP cells as TCRβ^{hi}CD4^{+}CD8^{-} and

TCRβ^{hi}CD4^{-}CD8^{+} cells, respectively. We reanalyzed the developmental ratios of mature CD4SP/CD69^{+} DP and mature CD8SP/CD69^{+} DP populations and updated Figure 4a and the corresponding quantification.

This revised gating strategy minimizes contamination from immature SP cells and developmentally aberrant populations and makes the flow-cytometric analysis more rigorous.



REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Major points:

One of the key findings of this manuscript is that SATB1 lowers the TCR signal threshold; thus, SATB1 loss results in positive selection only for cells that receive strong TCR signals. Although these results were inferred using gene expression data, whether SATB1 actually lowers signal threshold needs to be experimentally supported. Also, it would be helpful to know whether SATB1 is needed to interpret TCR "strength" vs "duration " properly. Finally, if the authors can dissect SATB1 functions in genes involved in different TCR waves (c.f., Steier et al., PMID: 37580604), this would improve the manuscript.

Response:

We thank the reviewer for these insightful and constructive comments.

To directly evaluate whether SATB1 affects TCR signaling beyond transcriptional readouts, we performed calcium flux assay in both $CD69^-$ DP and $CD69^+$ DP thymocytes upon CD3 stimulation. Satb1-deficient DP thymocytes showed reduced Ca^{2+} mobilization compared with WT cells, with a relatively modest reduction in CD69- DP cells and a more pronounced impairment in $CD69^+$ DP cells (**Figure 6k-l**). In particular, the response after extracellular Ca^{2+} addition was markedly reduced in Satb1-deficient $CD69^+$ DP cells, whereas ionomycin induced robust Ca^{2+} responses in both WT and Satb1-deficient cells. These results indicate that SATB1 is required for optimal TCR-induced Ca^{2+} signaling rather than for general cellular Ca^{2+} mobilization capacity.

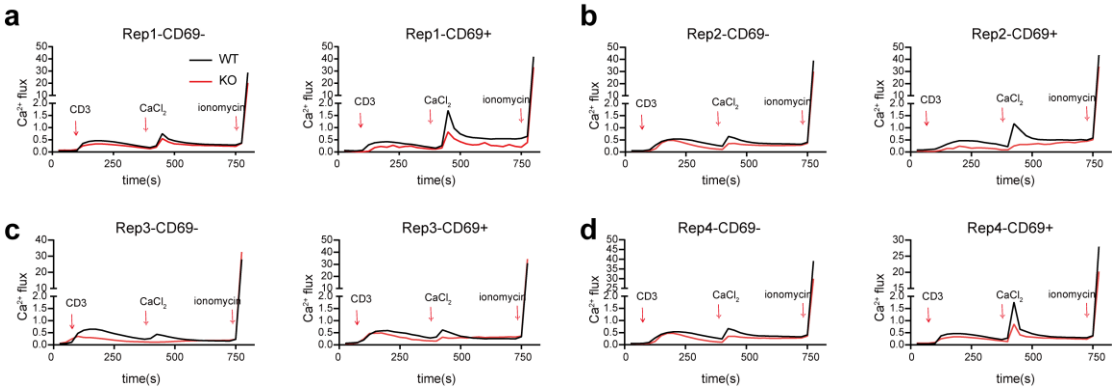


Figure 6k-l and S11

We agree that a definitive measurement of signaling threshold would require dose-response stimulation using graded TCR ligands or antibody concentrations. Therefore, we have moderated the wording in the revised manuscript and now state that SATB1 supports TCR responsiveness during positive selection.

Furthermore, we performed pseudotime trajectory analysis on single-cell RNA-seq data to examine the temporal dynamics of TCR-responsive genes. The expression dynamics of Cd8a, Cd5, Runx3 and other key genes revealed two sequential TCR signaling waves during CD8 lineage commitment, which is highly consistent with the model reported in Steier et al. We further

profiled the expression of a panel of genes associated with TCR signaling and T cell lineage specification, systematically dissecting the temporal dynamics of TCR signal transduction during the developmental transition of CD69^{+} DP cells. These analyses have been incorporated into Figure 5g-h and Supplementary Figure S10 of the new version.

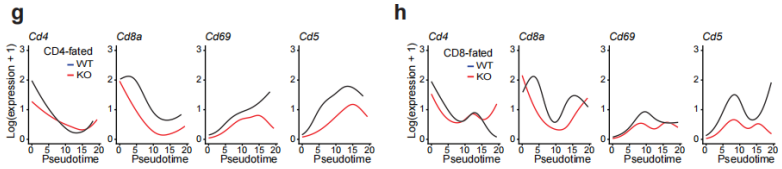


Figure 5g-h

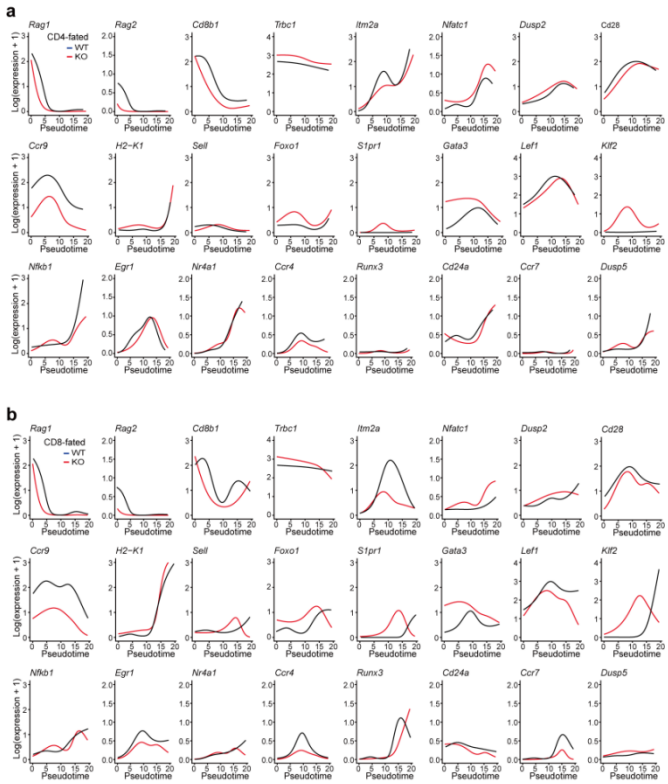


Figure S10 Expression of positive selection related genes in CD69^{+} DP cells

(a) Smoothed expression profiles of positive selection related genes along pseudotime in CD4-fated trajectories from wild-type and Satb1-deficient thymocytes.

(b) Smoothed expression profiles of positive selection related genes along pseudotime in CD8-fated trajectories from wild-type and Satb1-deficient thymocytes.

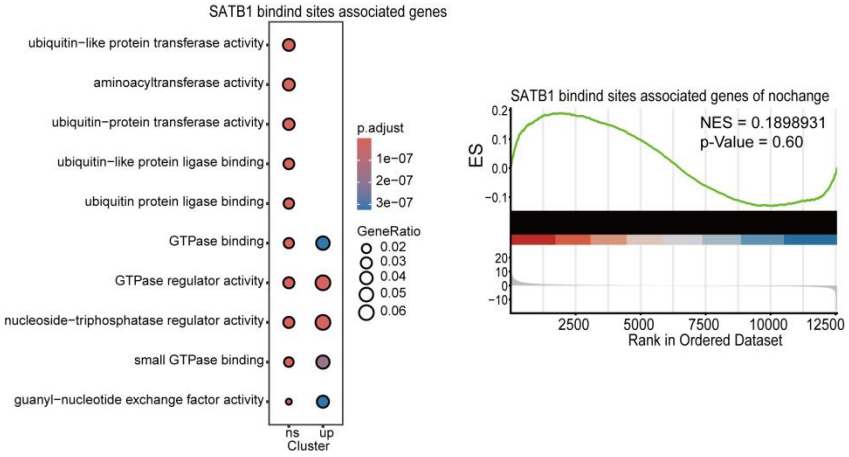
The authors argue that SATB1 is substantially redistributed in CD69^{-} vs. CD69^{+} thymocytes. However, ~1,600 differentially bound sites (among ~20,000 peaks) seem modest. Are these a small number of peaks enriched for SATB1 functional target genes (genes responding to SATB1 loss during positive selection), and most of the unchanging binding sites are not functional? If not, how does SATB1 execute distinct functions in CD69^{-} vs. CD69^{+} cells?

Response:

We thank the reviewer for this thoughtful and insightful comment. We agree that the number of

differentially bound SATB1 sites is modest relative to the total number of SATB1 peaks, and we have therefore performed additional analyses to distinguish constitutive binding from functionally dynamic binding during positive selection.

Genes associated with constitutive SATB1 peaks showed limited transcriptional changes upon Satb1 deletion in CD69^{+} DP cells, suggesting that many unchanged SATB1-binding events are not detectably coupled to gene-expression changes under this developmental condition. In contrast, as shown in Figure 6e, genes within SATB1-mediated loops were significantly downregulated in Satb1-deficient CD69^{+} DP cells, indicating that loop-associated SATB1 binding better identifies functional regulatory interactions than simple nearest-gene annotation. Because many SATB1 binding sites are located at distal enhancers (Figure S3a), nearest-gene assignment may underestimate or misassign their regulatory targets. We therefore interpret the approximately 1,600 dynamic SATB1 sites as a functionally enriched subset that is preferentially linked to enhancer–promoter communication and positive-selection gene activation. We have revised the manuscript to clarify this point and to avoid implying that all SATB1-binding sites are equally functional during the CD69^{-} to CD69^{+} DP transition.



Results from ACC-seq seem subtle (e.g., Fig 8h and i). Also, additional evidence is needed to support the claim that SATB1-mediated nuclear condensates regulate positive selection genes. For instance, are diminished Fix-Hex differences in SATB1-deficient cells enriched near CD5 and other TCR signaling genes?

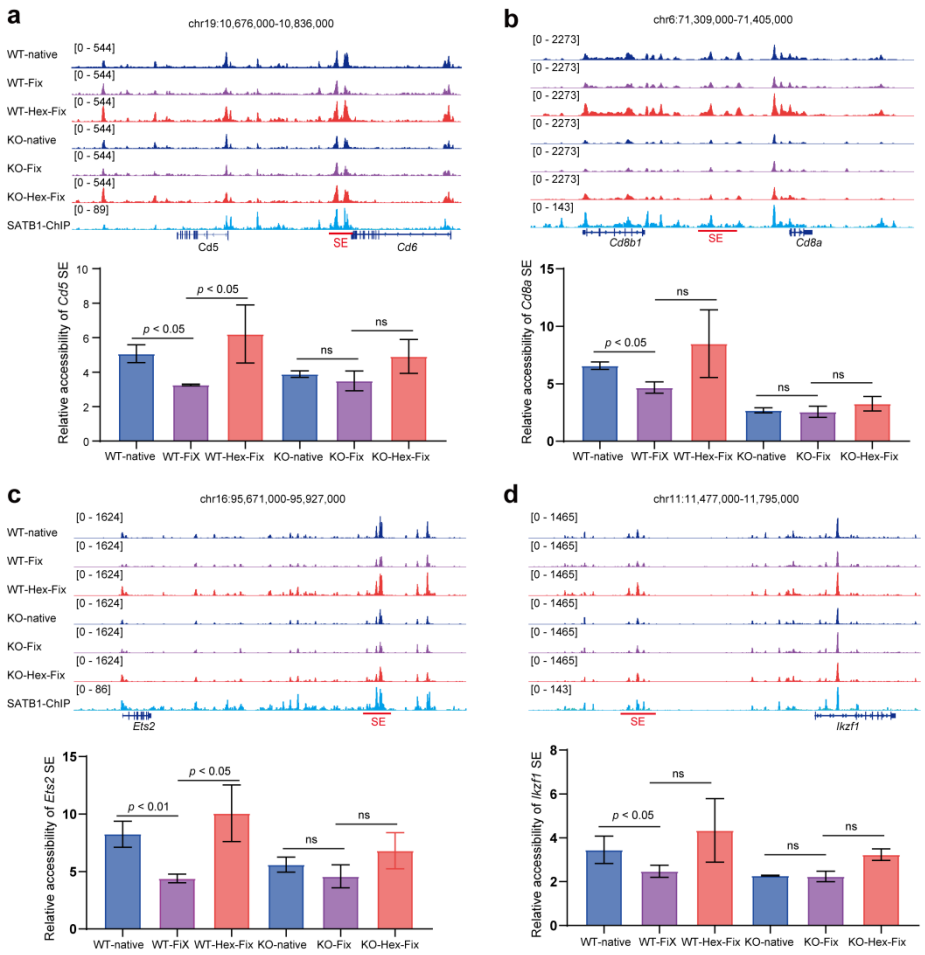
Response:

We thank the reviewer for this constructive comment. To strengthen the connection between SATB1-associated condensate-sensitive chromatin accessibility and positive-selection genes, we expanded the ACC-seq analysis to additional TCR signaling and positive-selection loci, including Cd5, Cd8a, Ets2 and Ikzf1. These newly added loci showed reduced Fix-Hex differences at super-enhancer (SE) regions in Satb1-deficient CD69^{+}DP cells, similar to the pattern observed at the Ets1 locus.

We have revised the text to describe Fix–Hex differences as an operational readout of condensate-sensitive chromatin accessibility, rather than as definitive proof of condensate dependency. The Cd5 locus is now included in Figure 8h, and additional loci including Ets1 are presented in Supplementary Figure S15/S16. Together, these analyses support the conclusion that SATB1 contributes to condensate-sensitive accessibility at super-enhancers associated with positive-

selection genes.

We also acknowledge that 1,6-hexanediol can have condensate-independent effects. Therefore, we have moderated the wording in the revised manuscript and now state that SATB1-dependent SEs display condensate-sensitive accessibility, which is consistent with a condensate-associated mechanism of enhancer regulation.



Minor points:

Vav-Cre was a major tool used to delete SATB1, but the results may be confounded by SATB1 deletion at earlier developmental stages.

Response:

We thank the reviewer for this important concern regarding potential developmental confounding effects with Vav-Cre.

We agree that Vav-Cre-mediated deletion could potentially introduce effects from earlier hematopoietic stages. To address this concern, we analyzed Satb1 deletion using CD4-Cre, which mediates deletion at the DP stage. Satb1$^{fl/fl}$ CD4-Cre mice showed comparable positive-selection defects to those observed in Satb1$^{fl/fl}$ Vav-Cre mice, including reduced CD69^{+}/CD5^{+} DP populations and impaired SP development (Figure S7). These findings support a DP-stage requirement for SATB1 during positive selection and indicate that the major phenotype is not solely inherited from earlier developmental defects.

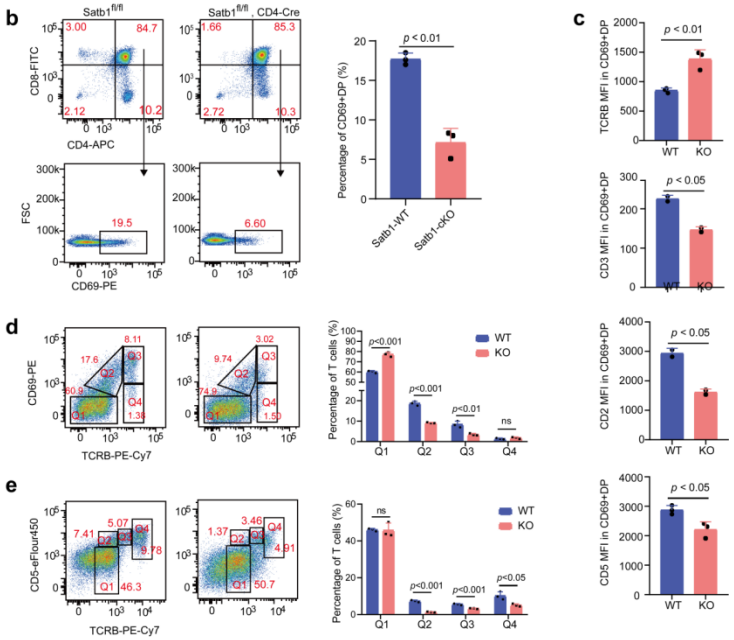


Figure S7b-e Conditional deletion of Satb1 in DP thymocytes impairs positive selection

(b) Flow cytometry analysis of thymocytes from Satb1fl/fl (WT) and Satb1fl/fl; CD4-Cre (cKO) mice. Representative plots show CD4 and CD8 expression (top) and CD69 expression within DP thymocytes (bottom). Right, quantification of the frequency of $CD69^+$ DP cells. Data are shown as mean ± s.d.; P values were determined by two-sided Student's t test.

(c) Quantification of mean fluorescence intensity (MFI) of TCRβ, CD3, CD2, and CD5 on $CD69^+$ DP thymocytes from WT and Satb1 cKO mice. Data are shown as mean ± s.d.; P values were determined by two-sided Student's t test.

(d) Flow cytometry analysis of thymocytes stratified by TCRβ and CD69 expression, defining four developmental stages (Q1-Q4) corresponding to sequential phases of positive selection. Representative plots (left) and quantification of population frequencies (right) are shown for WT and Satb1 cKO mice.

(e) Flow cytometry analysis of thymocytes stratified by TCRβ and CD5 expression, defining four developmental stages (Q1-Q4). Representative plots (left) and quantification of population frequencies (right) are shown for WT and Satb1 cKO mice.

Fig 1 b-d: It will be helpful to adjust the x-axis to quantitatively represent the GFP levels.

Response:

We thank the reviewer for this suggestion.

We have revised the x-axis labels in Figure 1b–d to quantitatively represent GFP fluorescence intensity. We also replaced the label "$SATB1^{-/eGFP}$" with "GFP" in the histogram axis for clarity.

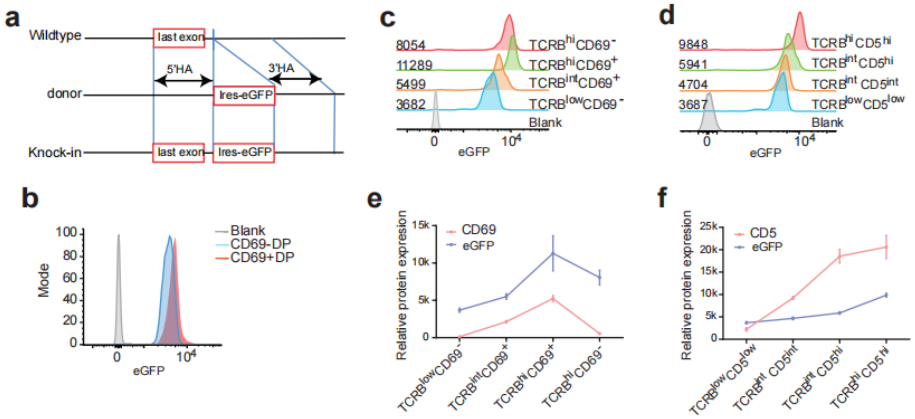


Figure 1b–d

Fig S1e-f: CD4 SP and CD8 SP show higher GFP levels than those of DP. If this result is true, this contradicts previous statements that SATB1 is most highly expressed in DP and its levels decrease in SPs (c.f., lines 56-57).

Response:

We thank the reviewer for highlighting this point. We have revised the text to distinguish Satb1 mRNA expression, Satb1-IRES-eGFP reporter signal, and endogenous SATB1 protein abundance. The previous statement (lines 56–57) referred to Satb1 mRNA levels, which have been reported to peak in DP thymocytes and decrease in SP cells. In our Satb1-IRES-eGFP reporter mice, however, GFP fluorescence is higher in SP thymocytes than in DP thymocytes. Because IRES-eGFP fluorescence may reflect Satb1 locus activity, reporter translation, and/or GFP protein stability, it should not be interpreted as a direct measurement of endogenous SATB1 protein abundance. We have revised the manuscript accordingly and now describe these data as Satb1 reporter activity rather than SATB1 protein expression.

Line 158: $CD69^+$ DP showed increased SATB1 peaks (Fig 1, Fig S2) and SATB1 expression is also increased during positive selection. Why do SATB1-associated loops decrease in $CD69^+$ DP cells?

Response:

We thank the reviewer for this insightful question.

First, total chromatin loops decrease globally from $CD69^-$DP (~40,000) to $CD69^+$DP (~33,000), which contributes to the reduction in absolute number of SATB1-associated loops. Second, Figure 2d displays the proportion of SATB1-associated loops relative to total loops, not absolute counts. The reduced proportion of SATB1 loops in $CD69^+$DP cells suggests that gained SATB1 binding peaks are concentrated in specific genomic regions, rather than being broadly distributed across all loop anchors. It suggests that SATB1 is redistributed toward a restricted set of functional enhancer-associated interactions during the $CD69^-$ to $CD69^+$ DP transition. We have clarified this point in the revised manuscript.

Fig 5k: representative flow cytometry plots from spleen show unusual CD4 int CD8 int populations in both WT and KO. Are these properly gated on live singlets?

Response:

We thank the reviewer for this important comment. We agree that the $CD4^{int}CD8^{int}$ population in

spleen should be carefully distinguished from doublets or other flow-cytometric artifacts. We therefore re-examined the gating strategy. All spleen samples were first gated on lymphocytes by FSC-A/SSC-A, followed by sequential singlet gating using FSC-A/FSC-H and SSC-A/SSC-H before CD4/CD8 analysis. Thus, the $CD4^{int}CD8^{int}$ population is unlikely to be caused by doublet contamination. We have added the complete gating strategy to Figure S8a and clarified this in the Methods.

After this stringent singlet gating, the $CD4^{int}CD8^{int}$ population remained detectable. A small population was present in WT spleen, and its frequency was increased in Satb1-deficient mice. Thus, this population is unlikely to reflect doublet contamination. Rather, we interpret it as a peripheral CD4/CD8 double-positive or DP-like T-cell population. This interpretation is consistent with previous reports that SATB1 is required for maintaining thymocyte/T-cell identity and that Satb1 deficiency can result in abnormal CD4/CD8 lineage features and increased peripheral DP-like T-cell populations.

We have revised the manuscript to describe these cells more cautiously as peripheral $CD4^{int}CD8^{int}$ or CD4/CD8 double-positive T cells, rather than conventional thymic DP cells, and have added the gating strategy to support this conclusion.

Are SATB1-regulated loops also developmentally dynamic loops (changing between CD69- and CD69+ DPs, related to Fig 7, S10)?

Response:

We thank the reviewer for this important question. We compared SATB1-regulated loops in Satb1-deficient $CD69^+$ DP cells with loops that change during the normal $CD69^-$ to $CD69^+$ DP transition in WT cells. Using stringent loop-calling criteria, only approximately 30% of loops were stably maintained during this transition. Importantly, a substantial fraction of loops altered by Satb1 deletion in $CD69^+$ DP cells did not simply overlap with developmentally dynamic loops observed in WT cells. These results suggest that SATB1 loss induces specific regulatory-loop defects in $CD69^+$ DP cells, rather than merely reflecting a shift along the normal developmental trajectory. We have clarified this point in the revised text.

Fig S10b, the changes in the bottom right panel seem very modest. Adjusting the axis scale may help recognize differences.

Response:

We thank the reviewer for this helpful suggestion.

The axis scale in Figure S11b (Fig S10b in the old version) has been adjusted to better visualize the differences, as recommended.

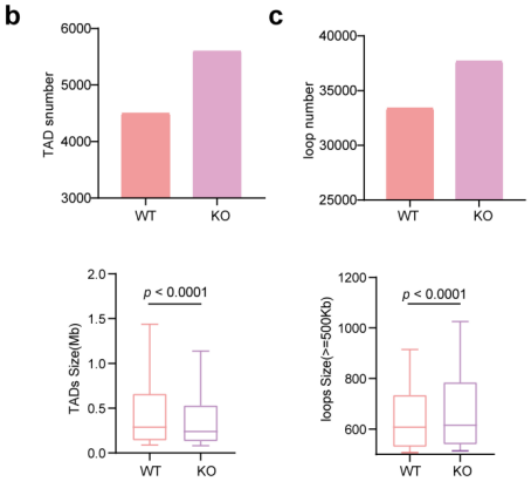


Figure S11b-c

Some figures were not called in the main text (Fig 5C, Fig 7D).

Response:

We thank the reviewer for this comment.

Figure 5C is now cited in the main text at line 248. Figure 7D is already cited at line 313, as indicated.

Figures 8d, h, and i need statistics.

Response:

We thank the reviewer for this comment.

Statistical analyses have been added to Figures 8d, h, and i as requested.

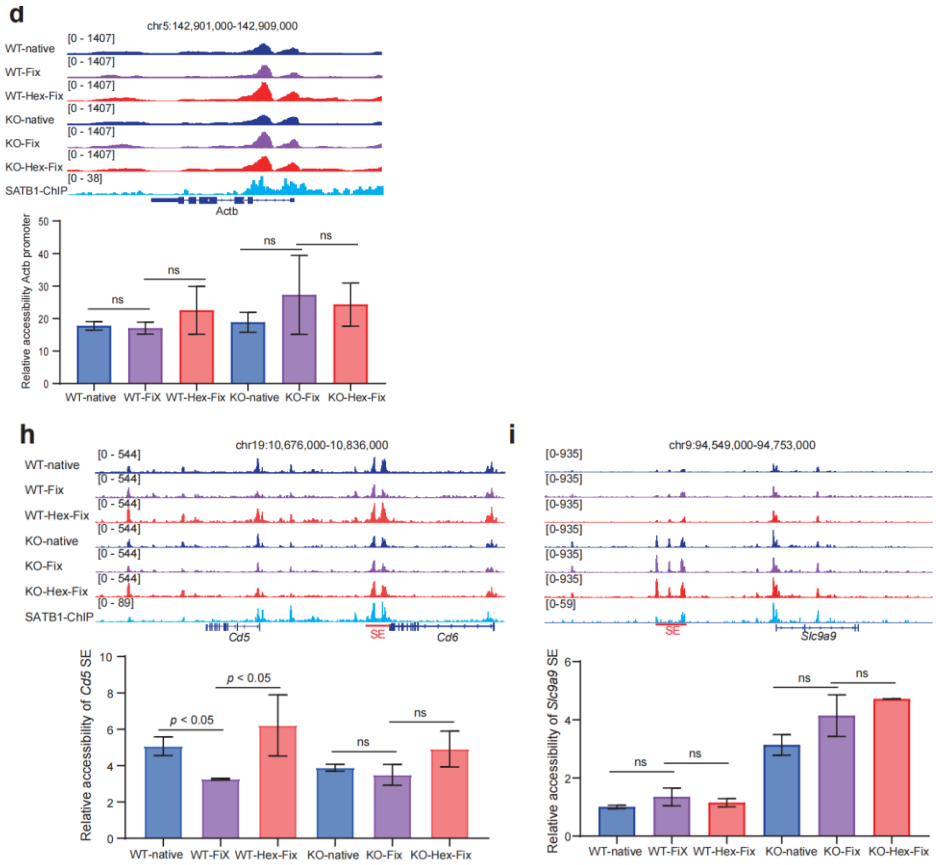


Figure 8d, 8h-i

Reviewer #2 (Remarks to the Author):

The paper is in many ways a technological tour-de-force, defining a role for SATB1 to "promote the expression of positive selection-associated genes by enhancing super-enhancer (SE) activation". There is no doubt that this data will be interesting and inspiring to many workers in this field. The limitation to much of the work is that many of the experiments produce correlative data (which of course is important and informative but more is needed to support the conclusions that are drawn), and the data generated from the KO mice, while promising, do not exclude relatively limited effects (see below). The paper could be markedly improved: these are some specific suggestions:

1. methods

a. what is the genetic back ground of the mice? Are the strains all inbred?

Response:

All mouse strains used in this study were maintained on a C57BL/6 background. Age- and sex-matched littermate controls were used for all experiments unless otherwise indicated. We have added this information to the Methods section.

b. Clarify-"Then evey antibodys add 1μl to 106 cells ".

Response:

We thank the reviewer for pointing out this unclear sentence. We have revised the Methods to state that cells were stained with fluorochrome-conjugated antibodies at empirically titrated concentrations for 30 min on ice. This replaces the ambiguous statement regarding "1 μl antibody per 10^6 cells."

c. Does not appear Fc block used for FACS– false positives can result

Response:

We appreciate the reviewer's concern regarding potential non-specific antibody binding. Fc block was not used in the original thymocyte staining experiments. Because the key analyses were performed primarily on thymocytes and gated T-lineage populations, Fc receptor-mediated background is expected to be limited. In addition, all antibodies were directly conjugated monoclonal antibodies and were used at empirically titrated concentrations. We have clarified this point in the Methods.

For peripheral lymphoid tissues, cells were first gated on $CD3^+$ T cells before CD4/CD8 analysis, which further minimizes potential contamination from Fc receptor-expressing non-T cells. We have also added CD69 FMO controls and post-sort purity analyses to strengthen the reliability of the flow-cytometric gating.

d. What was done to eliminate doublets? (FACS)

Response:

Doublets and aggregates were excluded by sequential gating using FSC-A/FSC-H and SSC-A/SSC-H parameters before downstream analysis. The complete gating strategy has been added to Figure S8a.

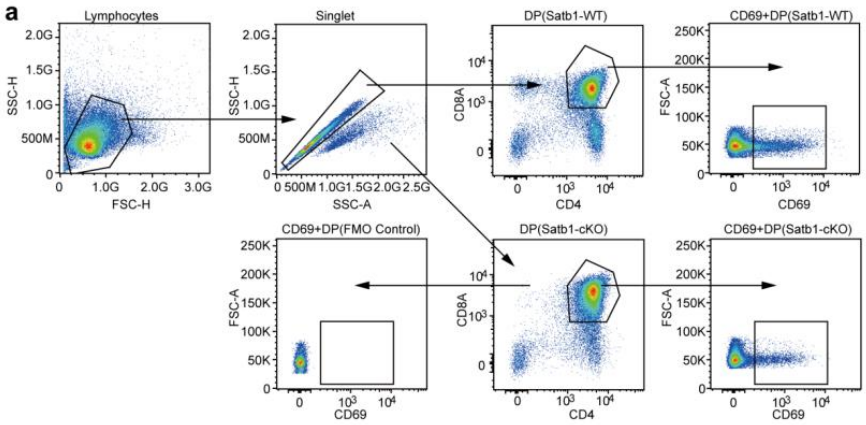


Figure S8a

e. Clarify- "The sorted cells through staining with antibodies targeting CD4, CD8a and CD69 antibody. "

f. Clarify "493. The $CD69^+$ DP cells sorted by FACS,PN-1000268)"

Response:

We have revised the Methods to state: "$CD69^+$ DP thymocytes were sorted on a BD FACSAria III after staining with antibodies against CD4, CD8α and CD69." The incorrect catalog number and unclear wording have been removed.

g. Since CD69 is a critical antigen in this study, the supplemental data should show an FMO control, and a sort purity analysis (FACS)

Response:

We thank the reviewer for this important suggestion. Because CD69 is central to our study, we added CD69 FMO controls to define the $CD69^+$ gate. We also performed post-sort purity analysis by reanalyzing a fraction of sorted $CD69^+$ DP cells. Representative FMO controls and post-sort purity plots have been added to Figure S8a. The Methods and figure legends have been revised accordingly.

h. correct typos: "613 incubation with a secondary antibody conjuction with Aleax 488 fluorescence"

Response:

We have corrected this sentence to: "Cells were incubated with an Alexa Fluor 649-conjugated secondary antibody." The spelling errors have been corrected in the Methods.

i. was a Viability dye used? Without this, false positives are often increased (FACS)

Response:

We appreciate the reviewer's concern. Viability dye was not used in the original experiments, and we have revised the Methods to avoid implying otherwise. Dead cells and debris were minimized by using freshly prepared single-cell suspensions and were reduced during analysis by FSC/SSC lymphocyte gating. We acknowledge that viability dye would further improve exclusion of dead cells, and we will incorporate viability dye in future flow-cytometry experiments. Importantly, the key conclusions are supported by independent readouts, including surface-marker analysis, sorted-

cell transcriptomics, and Ca^{2+} flux assays.

j. "CUT&Tag experiments were performed as described before with minimal changes" – provide a citation.

Response:

We have added the appropriate citation for the CUT&Tag protocol and revised the sentence to: "CUT&Tag experiments were performed with minor modifications as previously described (Kaya-Okur et al., 2019)."

k. Clarify – "689 ATAC-seq library preparation ..690 Initially, approximately 5 × 104 cell pellets" (do they mean cell pellets that each contain 5 × 104 cells?)

Response:

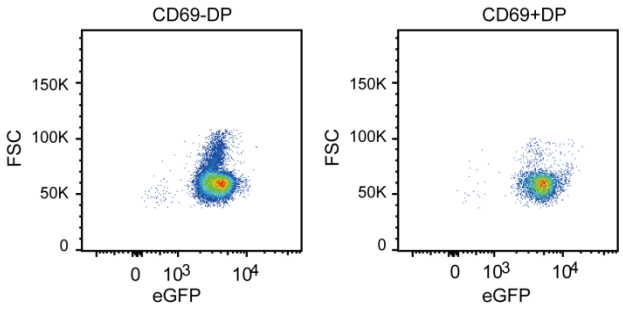
We thank the reviewer for pointing out this ambiguity. We have revised the Methods to state that approximately 5×10^4 cells were used for each ATAC-seq library.

2. Results

a. Figure 1 eGFP, eGFP expression in $CD69^-$ DP and $CD69^+$ DP; there is more GFP in $CD69^+$. Please show FSC vs GFP or show cells of the same FSC gate to ensure that the result is not driven by the fact that larger cells may have more GFP protein without reflecting increased transcription of the locus,

Response:

We appreciate the reviewer's careful concern about potential cell size/granularity effects on GFP mean fluorescence intensity (MFI). To exclude that the higher GFP signal in $CD69^+$ DP cells simply reflects larger cell size, we analyzed eGFP expression within the same FSC gate. Even when comparing size-matched cells, $CD69^+$ DP cells still exhibited significantly higher eGFP expression than $CD69^-$ DP cells. These results indicate that the increased GFP signal is not simply driven by larger cell size and supports increased Satb1 reporter activity during positive selection. We have added this analysis to the revised figure.



b. Clarify "187 Multiple studies have shown that super-enhancer regulates the expression of cellular characteristic genes".

Response:

We have clarified and revised the sentence for accuracy and grammar: "Multiple studies have demonstrated that super-enhancers regulate the expression of cell-type-specific signature genes."

c. Figure S7 c,d, are the displayed cells gated first for DP? Does not say so..

Response:

We thank the reviewer for pointing this out. The cells shown in Figure S7c,d were analyzed from total thymocytes rather than pre-gated DP cells, because the purpose of this panel was to compare the overall distribution of thymocyte developmental populations. We have clarified the gating strategy in the figure legend and Methods. The analysis follows established flow-cytometric strategies for assessing thymic positive and negative selection.

d. Since the SATB1 KO uses a regulated Cre, has the team evaluated efficiency of deletion in the DP subsets 1-4? There would be a strong potential for selection and competition by non-deleted cells.

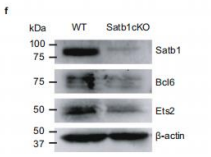
Response:

We thank the reviewer for raising this important point. We agree that, when using a Cre-mediated conditional knockout system, incomplete deletion could potentially generate a competitive advantage for non-deleted cells and complicate the interpretation of developmental phenotypes.

We have previously evaluated the efficiency of SATB1 deletion in thymocytes in our previous study (Feng et al. 2022 Nature Communications) using the same conditional knockout strategy. In that study, SATB1 protein was efficiently depleted in the vast majority of DP thymocytes, indicating that the system achieves robust deletion at the DP stage.

Importantly, the developmental and transcriptional phenotypes observed in Satb1 cKO mice were not restricted to a minor subset of cells but were consistently detected across the DP compartment and downstream thymocyte populations. This argues against the possibility that the main phenotype was driven by expansion or selection of a small population of non-deleted escape cells. In addition, the persistence of positive-selection defects in the OT-I TCR transgenic background further supports the conclusion that the phenotype reflects a cell-intrinsic requirement for SATB1 rather than selective outgrowth of undeleted cells.

To address the reviewer's concern more explicitly, we have now clarified in the revised manuscript that SATB1 deletion efficiency in DP thymocytes had been validated in our previous study. We also added a statement noting that the broad reduction of SATB1 protein in sorted DP thymocytes makes it unlikely that non-deleted cells account for the observed defects.



e. Is TCRalpha rearrangement normal in the SATB1 KO cells? Its stated that RAG gene expression depends on SATB1. Decreased TCRab+ cells would impair positive selection. Is the TCRbeta antibody used specific for full TCRab complexes? Without addressing this, the model proposed for the centrality of SATB1 in regulating a large number of genes in DP cells is not well supported.

Response:

We thank the reviewer for this important comment. We agree that defects in TCRα rearrangement and/or reduced surface TCRαβ expression could contribute to impaired positive selection and therefore need to be carefully considered.

SATB1-dependent regulation of TCR rearrangement has been established in our previous studies. In our previous study (Hao et al. 2015 J Exp Med), we reported that SATB1 deficiency affected Rag1/Rag2 expression and impaired Tcra rearrangement. In a subsequent study (Feng et al. 2021 Genome), we further characterized how SATB1 shapes the adult thymic TCR repertoire, with a particularly strong effect on Jα usage during TCRα rearrangement. Thus, we agree with the reviewer that TCRα rearrangement is not completely normal in Satb1-deficient thymocytes.

To address this issue directly in the cell population analyzed in the present study, we have now added new Tcra 5′RACE sequencing data from $CD69^+$ DP thymocytes. Consistent with our previous findings, Satb1 deficiency altered Vα–Jα usage in $CD69^+$ DP cells, with relatively increased usage of proximal Jα segments and reduced usage of distal Jα segments. We have added these data as a heatmap of Vα–Jα combinations in $CD69^+$ DP thymocytes from WT and Satb1 cKO mice in the revised manuscript as **Figure S7f**. These results confirm that SATB1-dependent changes in TCRα repertoire formation are also present in the positively selecting $CD69^+$ DP compartment.

Regarding the TCRβ staining, the antibody used detects surface TCRβ. In DP thymocytes, stable surface TCRβ staining largely reflects expression of the assembled αβ TCR complex, because TCRβ requires pairing with TCRα and association with the CD3 complex for efficient surface expression. Therefore, reduced $TCR\beta^+$ or $TCR\alpha\beta^+$ DP cells can, at least in part, reflect impaired TCRα rearrangement or reduced αβ TCR surface expression. We have clarified this point in the revised text and have avoided interpreting reduced TCRβ staining as evidence for a TCRβ-specific defect alone.

Importantly, however, the positive-selection phenotype cannot be explained solely by altered endogenous TCRα rearrangement. Positive-selection defects persisted in OT-I TCR transgenic mice, in which a prearranged TCR specificity bypasses much of the variability associated with endogenous TCRα repertoire formation. This result indicates that, in addition to its role in TCRα rearrangement and repertoire formation, SATB1 also regulates rearrangement-independent processes required for DP thymocyte responsiveness during positive selection.

We have therefore revised the manuscript to make this interpretation explicit: SATB1 contributes to positive selection through at least two connected mechanisms. First, it supports normal TCRα rearrangement and repertoire formation. Second, it regulates a broader DP transcriptional and chromatin program that controls TCR responsiveness and positive-selection competence independently of endogenous TCRα repertoire variability. This revised interpretation better reflects both our previous work and the new $CD69^+$ DP Tcra 5′RACE data added in Figure S7f.

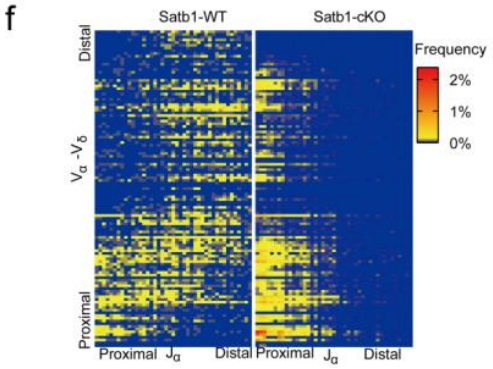


Figure S7f Heatmap of Vα–Jα combination in CD69^{+} DP thymocytes from WT and *Satb1* cKO mice.

f. the scRNA seq could be so informative but why on sorted CD69^{+} DP thymocytes? why not on the entire DP ?

Response:

We appreciate the reviewer's important question. We chose to perform scRNA-seq on sorted CD69^{+} DP thymocytes because this population represents the key compartment undergoing positive selection and is markedly reduced in Satb1-deficient mice. In Satb1 cKO mice, CD69^{+} DP cells represent only approximately 5% of total DP thymocytes. Therefore, scRNA-seq of unsorted total DP cells would be dominated by pre-selection CD69^{-} DP cells and would under-sample the rare but functionally critical CD69^{+} DP population. By sorting CD69^{+} DP cells, we were able to resolve the heterogeneity and transcriptional trajectories within the positively selecting compartment. We have clarified this rationale in the revised manuscript and included the sorting strategy in Figure S8a.

g. its very important to consider the ways in which the KO may distort the correct identification of DP cells as SATB1 regulates both CD4 and CD8, or may include aberrant cell populations due to developmental impedance - consider comparison to Li et al https://doi.org/10.1186/s13073-021-00861-7 and using the flow analysis scheme they employ

Response:

We greatly appreciate the reviewer's valuable comment and the suggestion to consider the gating strategy used by Li et al. (2021). We agree that Satb1 deficiency could distort CD4/CD8 expression and thereby complicate the identification of DP and SP populations.

To address this concern, we re-examined CD4 and CD8 surface expression in Satb1-deficient thymocytes. Although Cd4 and Cd8a transcripts were reduced in our transcriptomic data, surface CD4 and CD8 protein levels were only modestly affected, supporting the reliable identification of CD4^{+}CD8^{+} DP cells by flow cytometry(Figure A).

More importantly, we realized that directly defining CD4^{+}CD8^{-} and CD4^{-}CD8^{+} cells as CD4SP and CD8SP populations in the original analysis could include immature single-positive cells and aberrant intermediates. We therefore revised the gating strategy by first selecting TCRβ^{high} thymocytes and then defining mature CD4SP and CD8SP cells as TCRβ^{high}CD4^{+}CD8^{-} and

TCRβ^{high}CD4^{-}CD8^{+} thymocytes(supplementary Figure 7a), respectively. We reanalyzed the developmental ratios of mature CD4SP/CD69^{+} DP and mature CD8SP/CD69^{+} DP populations and updated Figure 4a and the corresponding quantification(Red box).

This revised gating strategy minimizes contamination from immature SP cells and developmentally aberrant populations and makes the flow-cytometric analysis more rigorous.

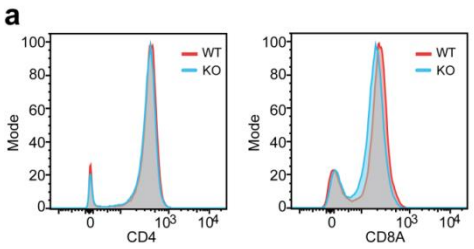


Figure A

Figure 4a(older)

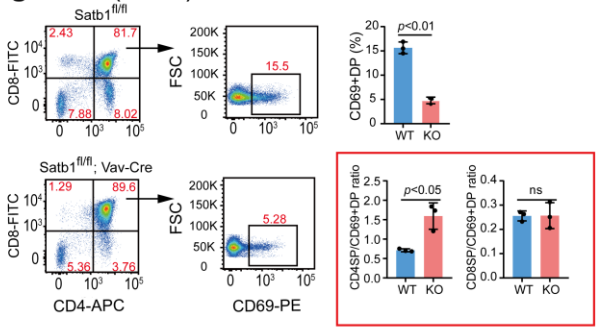
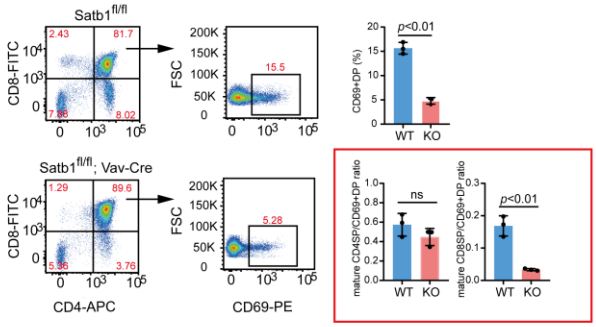
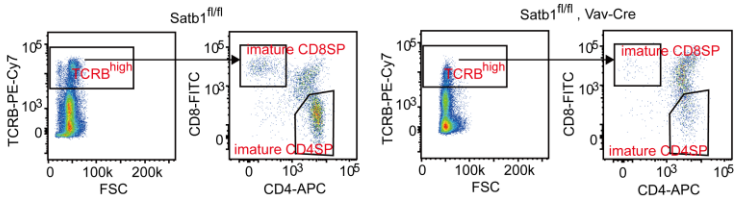


Figure 4a(new)



Supplementary Figure 7a



REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

I have only a few minor points that still require attention:

• Figures 1e,f and Supplementary Figures S1d, f: Statistical analyses are missing.

Response:

We thank the reviewer for this helpful comment. We have now performed statistical analyses for Figures 1e and 1f. Because the four thymocyte populations were measured from the same biologically independent mice, eGFP expression across the indicated populations was analyzed using two-way repeated-measures ANOVA where appropriate. A significant interaction between row factor and column factor was observed, and the p value have been added to the revised figures and figure legends.

For Supplementary Figures S1d and S1f, statistical analyses were also performed, and no significant differences were detected. The statistical tests and sample sizes are now specified in the corresponding figure legend, and non-significant comparisons are indicated as “ns”.

• Line 306 (Figure 6h): The text refers to "CD69- DP cells," whereas both Figure 6h and its legend are labeled "CD69+ DP cells." Please clarify and ensure consistency.

Response:

We appreciate the reviewer’s careful reading. We have corrected “$CD69^-$ DP cells” to “$CD69^+$ DP cells” in the text to ensure consistency with Figure 6h and its legend.

• Methods lines 617-618: The reference and the name of the software used are missing.

Response:

We thank the reviewer for this reminder. In the "Methods" section of the revised manuscript, we have added citations for several software tools, including HiCExplorer (v3.2) and its corresponding references.

•Methods lines 771-772: The reported concentration of 1M $CaCl_2$ appears to be unphysiologically high. Please verify whether this value is correct.

Response:

We thank the reviewer for this careful check. The 1 M $CaCl_2$described in the original text referred to the stock solution concentration and did not represent the final concentration used in the assay. The final working concentration was 1 mM. We have revised the Methods accordingly to report the final working concentration as 1.6 mM.