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A role for cohesin in T cell receptor rearrangement and thymocyte differentiation

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Cohesin enables post-replicative DNA repair and chromosome segregation by holding sister chromatids together from the time of DNA replication in S-phase until mitosis[1]. There is growing evidence that cohesin also forms long range chromosomal cis-interactions[2–4] and may regulate gene expression[2–10] in association with CTCF (ref. 8, 9), mediator[4] or tissue-specific transcription factors[10]. Human cohesinopathies like Cornelia de Lange Syndrome are thought to result from impaired non-canonical cohesin functions[7], but a clear distinction between cohesin's cell division-related and cell division-independent functions as exemplified in Drosophila[11–13] has not been demonstrated in vertebrate systems. To address this, we deleted the cohesin locus *Rad21* in mouse thymocytes at a time in development when these cells stop cycling and rearrange their T cell receptor alpha locus (*Tcra*). *Rad21* deficient thymocytes had a normal lifespan and retained the ability to differentiate, albeit with reduced efficiency. Loss of *Rad21* led to a defective chromatin architecture at the *Tcra*

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Author contributions. VS and MM conceived the study with critical input from DGS, LA, AGF, MSK and KM. VS, BH, KTK, TL, HMB, KEB, GT, KH and MM conducted experiments, KTK, DJA, KM, GT and DGS designed and generated novel materials, TC, AT and HM analysed data, VS and MM wrote the paper and all authors discussed the results and commented on the manuscript.

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locus, where cohesin binding sites flank the TEA promoter and the Eα enhancer, and demarcate *Tcra* from interspersed *Tcrd* elements and neighbouring housekeeping genes. Cohesin was required for long-range promoter-enhancer interactions, *Tcra* transcription, H3K4me3 histone modifications that recruit the recombination machinery[14,15] and *Tcra* rearrangement. Provision of pre-rearranged T cell receptor transgenes largely rescued thymocyte differentiation, demonstrating that among thousands of potential target genes across the genome[4,8–10] defective *Tcra* rearrangement was limiting for the differentiation of cohesin-deficient thymocytes. These findings firmly establish a cell division-independent role for cohesin in *Tcra* locus rearrangement and provide a comprehensive account of the mechanisms by which cohesin enables cellular differentiation in a well-characterised mammalian system.

The somatic rearrangement of lymphocyte receptor loci is central to adaptive immunity[16]. Gene segments distributed over millions of base pairs of genomic DNA are transcribed, brought into proximity with each other, and recombined in a cell lineage- and developmental stage-specific fashion[17–19]. In developing thymocytes, proliferation and differentiation are tightly linked and the activity of Rag (recombinase activating gene) proteins is restricted to the G1 phase of the cell cycle[20]. Early thymocytes at the $CD4^-$ $CD8^-$ double negative (DN) stages 1 and 2 proliferate in response to cytokines and briefly arrest at the DN3 stage, where they rearrange the T cell receptor (TCR) beta locus (Fig. 1a). Pre-T cell receptor signals drive a phase of proliferation that extends to the early $CD4^+$ $CD8^+$ double positive (DP) stage. Shortly after the acquisition of CD4 and CD8, DP thymocytes lose the expression of the transferrin receptor CD71 (ref. 21) and become small, non-proliferating $CD71^-$ DP cells (Fig. 1a), which represent the great majority of thymocytes. During their life span of 3 to 4 days, DP thymocytes undergo multiple rounds of TCR alpha (*Tcra*) rearrangement[16,18,19]. Successful TCR expression and engagement selects a minority (3–5%) of DP thymocytes for differentiation via a $CD4^+$ $CD8^{lo}$ intermediate stage towards long-lived CD4 or CD8 SP cells, again with minimal proliferation[16,22] (Fig. 1a).

To address the role of cohesin in *Tcra* rearrangement and thymocyte differentiation we combined a conditional allele encoding the cohesin subunit Rad21 (*Rad21*^{lox}, fig. 1b, Supplementary Fig. 1a) with a Cre transgene under the control of *Cd4* regulatory elements (CD4Cre), which becomes active at the transition from the $CD4^-$ $CD8^-$ DN to the DP stage[23]. Pilot experiments with YFP reporters showed CD4Cre-dependent accumulation of YFP after the $CD71^+$ proliferative stage in non-dividing DP thymocytes (Supplementary fig. 1b). Proliferating CD4Cre *Rad21*$^{lox/lox}$ $CD71^+$ DP cells showed partial locus deletion but retained >50% *Rad21* mRNA and protein (Fig. 1c). *Rad21* genomic deletion was essentially complete (>97%) and *Rad21* RNA and protein levels were substantially reduced in non-dividing DP thymocytes (Fig. 1c). Hence, cohesin was selectively depleted from non-dividing thymocytes. Importantly, CD4Cre *Rad21*$^{lox/lox}$ DP thymocyte numbers were normal (Fig. 1d). Intermediate $CD4^+8^{lo}$ and mature CD4 SP and CD8 SP thymocytes accumulated slowly in CD4Cre *Rad21*$^{lox/lox}$ mice (Supplementary fig. 2a) but were present in normal numbers by 6 weeks of age (Fig. 1d).

Bromodeoxyuridine (BrdU) incorporation into replicating DNA can identify proliferating thymocyte populations and track their differentiation[22]. Pulse-chase experiments labeled

proliferating ($CD71^+$) DP but not non-proliferating $CD4^+8^{lo}$, CD4 SP or CD8 SP thymocytes[22] (Fig. 1e, 2 hour time point). During the subsequent chase period, BrdU-labeled DP cells differentiated to become $CD4^+8^{lo}$ and eventually CD4 or CD8 SP (Fig. 1e, left panel). This sequence of differentiation was preserved in CD4Cre *Rad21*$^{lox/lox}$ thymocytes, but the proportion of DP thymocytes that became CD4 SP or CD8 SP was reduced (Fig. 1e, right panel, and supplementary fig. 2b).

In continuous BrdU labeling experiments, the percentage of $BrdU^+$ cells indicates population turnover[22]. Importantly, cohesin-deficient and control DP thymocytes labeled with similar kinetics (Fig. 1f). Consistent with the pulse-labeling data (Fig. 1e), the accumulation of $CD4^+8^{lo}$, and CD4 SP subsets was reduced in CD4Cre *Rad21*$^{lox/lox}$ mice (Supplementary fig. 2c). Hence, cohesin depletion impaired the differentiation of DP thymocytes, but not their survival.

Unlike many other differentiated cell types, mature thymocytes can be induced to re-enter the cell cycle. *In vitro* activated CD4Cre *Rad21*$^{lox/lox}$ CD4 SP thymocytes showed abnormal mitotic figures with multiple spindles, chromosome segregation defects (Fig. 1g, supplementary fig. 3), and poor survival (Fig. 1g). CD4Cre-mediated deletion of *Rad21* therefore generates thymocytes that die when forced to divide, yet have a normal lifespan as non-dividing cells *in vivo*. This affords the interrogation of cohesin functions in interphase, independent of essential cohesin functions during cell division.

Rad21 chromatin immunoprecipitation and sequencing (ChIP-seq) mapped cohesin to key positions within the *Tcra* locus in DP thymocytes (Fig. 2a). Cohesin was abundant at the locus control region[24], which separates the *Tcra* enhancer, Eα, from the neighbouring *Dad1* housekeeping gene[25,26]. Other prominent cohesin sites separated the *Tcra* TEA promoter from the *Tcrd* enhancer, Eδ, which controls *Tcrd* gene segments that are interspersed within the *Tcra* locus but follow a distinct developmental stage-specific programme[18]. Cohesin colocalisation with the insulator protein CTCF (ref. 8, 9) is found at the *Dad1* site 10kb downstream of Eα, while at the Jα49 promoter cohesion associates with its loading protein Nipbl and mediator subunits[4] more than with CTCF (Supplementary fig. 4). Interestingly, the major *Tcra* regulatory elements Eα and TEA bound copious amounts of cohesin, Nipbl, and mediator as well as CTCF (Supplementary fig. 4).

RNA-sequencing (RNA-seq) indicated that *Tcra* constant region (Cα) transcripts were considerably more abundant than transcripts from the neighbouring *Dad1* gene and the *Tcrd* constant region (Cδ) in control DP thymocytes. In cohesin-depleted small DP thymocytes, Cδ and *Dad1* transcripts were elevated at the expense of Cα transcripts (Fig. 2b) as confirmed by real time RT-PCR (Supplementary fig. 5a). Moreover, transcription across the *Tcra* joining elements, Jα, was skewed: control DP thymocytes preferentially transcribed distal (3') Jα elements, while cohesin-depleted DP thymocytes preferentially transcribed proximal (5') Jα elements (Fig. 2b).

Transcription of lymphocyte receptor loci facilitates rearrangement[27] in part via the trimethylation of histone H3 at lysine 4 (H3K4me3). H3K4me3 recruits Rag2 protein[14,15,28], which together with Rag1 forms the recombinase complex[18]. ChIP showed

reduced H3K4me3 deposition (Fig. 2c) and Rag binding (Fig. 2d,e) at distal Jα elements in CD4Cre *Rad21*$^{lox/lox}$ DP thymocytes. Hence, cohesin deficiency affected *Tcra* transcription, H3K4me3 histone modifications and the recruitment of Rag recombinases.

Primary *Tcra* rearrangements involve proximal (5') Jα elements and occur in early CD71^{+} DP thymocytes, while secondary rearrangements involve progressively more distal (3') Jα elements in non-dividing DP thymocytes[18] (Supplementary fig. 1b). CD71^{+} DP CD4Cre *Rad21*$^{lox/lox}$ thymocytes had near-normal Rad21 protein levels (Fig. 1c) and Cδ, Cα and *Dad1* transcription (Supplementary fig. 5b), and primary rearrangements of proximal Jα elements were present at normal levels (Fig. 3a, b). In contrast, secondary *Tcra* rearrangements were substantially impaired in non-dividing CD4Cre *Rad21*$^{lox/lox}$ DP thymocytes (Fig. 3a, b), which were depleted of cohesin (Fig. 1c). The usage of the distal Jα22 element, for example, was reduced on average by 86% (Fig. 3b, middle), reflecting a progressive under-representation of 3' Jα segments (Fig. 3b, right). This was confirmed by analysis of additional Vα gene families (Supplementary fig. 6a) and by RT-PCR-based copy number analysis of mature *Tcra* transcripts (Supplementary fig. 6b). Therefore, primary rearrangements occurred before the depletion of cohesin, while reduced cohesin expression impaired secondary rearrangements in non-dividing DP thymocytes. Normal BrdU labeling kinetics (Fig. 1f) exclude decreased lifespan as an explanation for aberrant *Tcra* rearrangement[29]. Consistent with the defective recruitment of Rag proteins to the *Tcra* locus (Fig2d, e), double strand breaks were reduced in cohesin-deficient thymocytes (Fig. 3c). This identifies Rag cleavage, rather than double strand break repair as the limiting step for *Tcra* rearrangements in cohesin-deficient thymocytes.

Since *Tcra* rearrangement changes the positioning of regulatory elements[18], altered *Tcra* transcription (Fig. 2b) could either be a direct consequence of cohesin depletion, or result indirectly from defective rearrangement. To distinguish between these possibilities we compared intronic *Tcra* transcript copy numbers in the absence of rearrangement in *Rag1*-deficient CD4Cre *Rad21*$^{lox/wt}$ and CD4Cre *Rad21*$^{lox/lox}$ DP thymocytes. Cohesin depletion reduced the transcription (Fig. 4a) and H3K4me3 (Supplementary fig. 7). at Jα independently of *Tcra* rearrangement.

To explore how cohesin affects *Tcra* transcription we analysed long-range interactions between the TEA promoter and Eα, which are separated by approximately 80 kb of genomic DNA and together regulate the transcription of *Tcra*. In chromosome conformation capture (3C) assays[30], Eα interacted strongly with TEA in DP thymocytes (Fig. 4b) but cohesin depletion reduced these interactions to the level found in pre-B cells, where *Tcra* is not detectably transcribed (Fig. 4b). Hence, the extent of *Tcra* enhancer-promoter interactions was cell type-specific, correlated with *Tcra* transcription, and was cohesin-dependent. A role for cohesin in additional enhancer-promoter interactions during sequential *Tcra* rearrangements is suggested by Eα contacts with promoters between Jα49 and Jα37 (Fig. 4b), which can drive Jα transcription in the absence of TEA[18], and by the cohesin binding to numerous Vα promoters (Fig. 2a). Cohesin depletion also affected Eα interactions with the neighbouring *Dad1* cohesin site, and therefore the topology of the *Tcra* locus control region[24], which has CTCF-dependent transcriptional insulator function[25,26] (Fig. 4b). As

cohesin mediates CTCF-dependent transcriptional insulation[8,9], increased *Dad1* expression at the expense of Cα (Fig. 2b) may indicate impaired insulator function.

To test whether aberrant *Tcra* rearrangement caused inefficient differentiation we equipped cohesin-deficient thymocytes with transgenes encoding rearranged TCRs. Compared to endogenously rearranged TCRs (Fig. 4c, top), the expression of MHC class II (Fig. 4c, middle) or MHC class I restricted TCRs (Fig. 4c, bottom) markedly improved the generation of CD4Cre *Rad21*$^{lox/lox}$ CD4^{+}8^{lo} and SP thymocytes.

In summary, cohesin shapes the chromatin architecture of the *Tcra* locus by mediating cell type-specific long-range interactions between enhancer and promoter elements that control transcription, H3K4me3 deposition, Rag recombinase recruitment, and ultimately *Tcra* rearrangement. These defects compromise thymocyte differentiation by limiting the number and diversity of sequential *Tcra* rearrangements. Hence, cohesin contributes to cellular differentiation in a well-characterised mammalian system.

Methods summary

The conditional *Rad21* allele was generated by inserting *LoxP* sites into introns 4 and 6 (Supplementary fig. 1a). Methods used for RT- and genomic PCR (ref. 2), flow cytometry[2], 3C analysis[2], ChIP for cohesin[2], histone modifications[30] and Rag proteins[30] have been described. See supplementary methods for other mouse strains, BrdU labeling and detection, ChIP-seq and RNA-seq protocols, ligation-mediated PCR, confocal microscopy, Tcra rearrangement assays and copy number measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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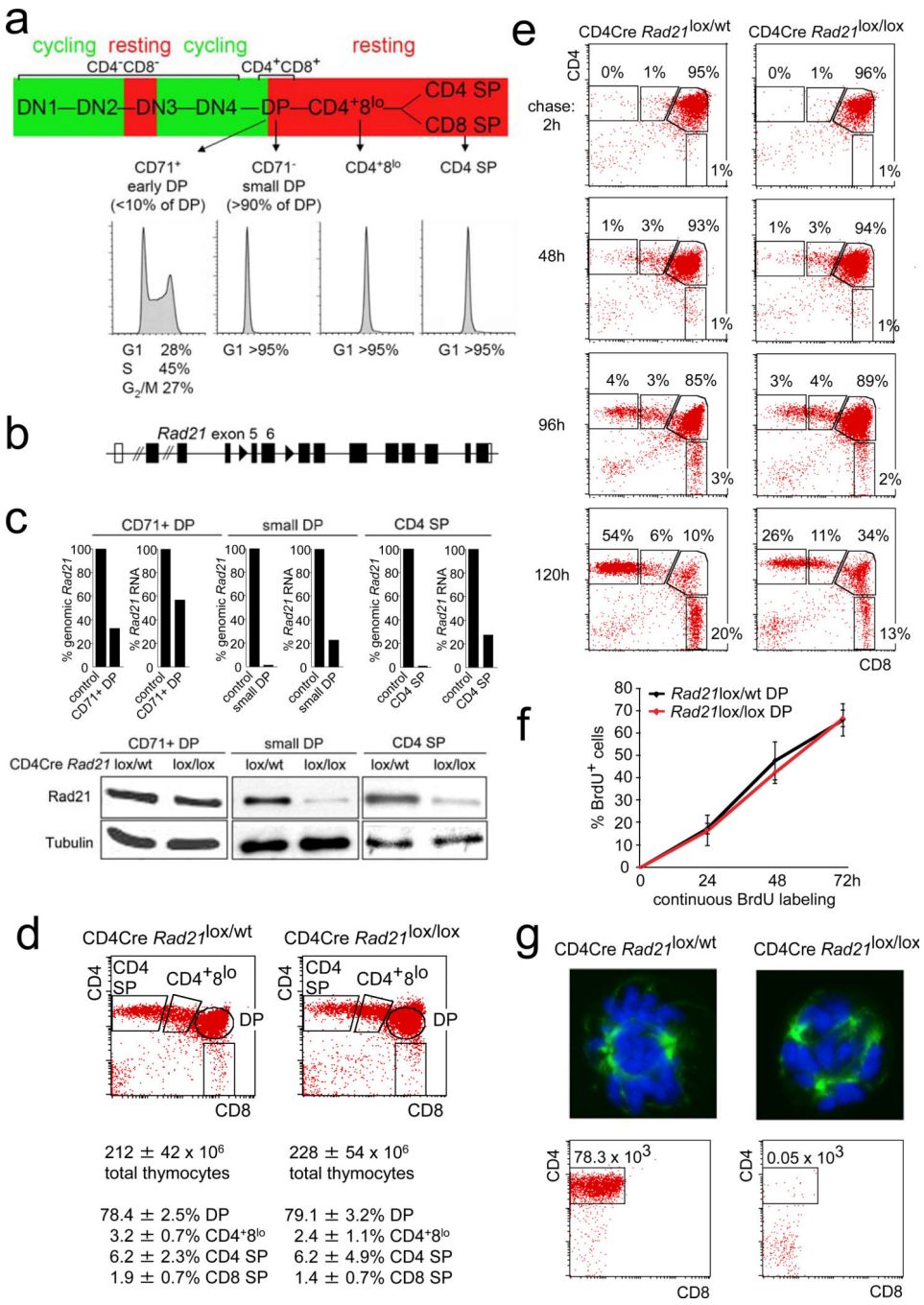

Figure 1. Genetic cohesin depleted in non-dividing thymocytes

a) Thymocyte differentiation from left to right: $CD4^{-}$ $CD8^{-}$ double negative (DN) stages 1 to 4; $CD4^{+}$ $CD8^{+}$ double positive (DP), $CD4^{+}8^{lo}$; CD4 or CD8 single positive (SP) cells. Proliferation is in green, cell cycle arrest in red. Histograms show DNA content.

b) Conditional *Rad21* allele (see supplementary fig. 1a).

c) Real time genomic PCR of *Rad21* locus deletion, RT-PCR of *Rad21* RNA and western blotting of Rad21 protein.

d) Cell numbers and flow cytometric analysis of thymocyte subsets in 6 week-old CD4Cre *Rad21*$^{lox/lox}$ and CD4Cre *Rad21*$^{lox/lwt}$ mice (mean ± SD, n = 12).
e) Pulse chase analysis of CD4Cre *Rad21*$^{lox/wt}$ and CD4Cre *Rad21*$^{lox/lox}$ thymocytes. Dot blots are gated on BrdU^{+} cells (see supplementary fig. 2b).
f) Continuous BrdU labelling for DP thymocyte turnover. See supplementary Fig. 2c for CD4^{+}8^{lo} and CD4 SP subsets (mean ± SD, n = 3–5 per data point).
g) Top: metaphase spreads of 2 day activated thymocytes stained for alpha-tubulin (green) and DNA (DAPI, blue, see supplementary fig. 3). Bottom: Cells recovered after 5 days.

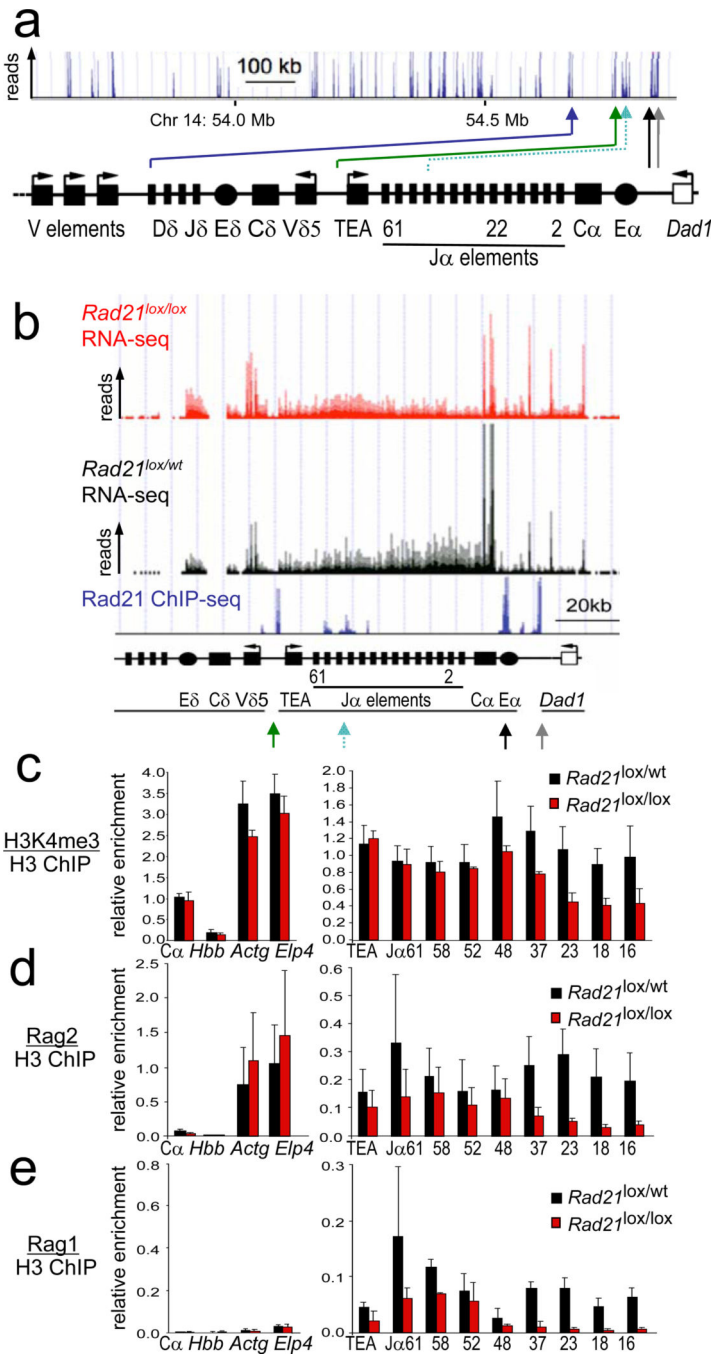

Figure 2. Cohesin affects *Tcra* transcription Rag recombinase recruitment

a) Rad21 ChIP-seq of the 3' part of the *Tcra* locus in DP thymocytes. Arrowheads highlight cohesin sites at the Eα enhancer (black), the *Tcra* locus control region (grey), Jα promoters (turquoise), the TEA promoter (green) and between *Tcrd* elements and V gene segments (blue).

b) RNA-seq of *Tcra* in CD4Cre *Rad21*$^{lox/lox}$ (red) and control *Rad21*$^{lox/wt}$ (black) DP thymocytes. Rad21 ChIP-seq is in blue.

c) ChIP of H3K4me3 relative to total H3 in CD4Cre *Rad21*$^{lox/lox}$ and control DP thymocytes. *Hbb* is a negative and *Actg* and *Elp4* are positive control loci (mean ± SE of 2 independent experiments). p=0.016 for all Jα elements; p=0.38 (NS) for proximal (Jα61–48) and p=0.004 for distal (Jα37–16) Jα elements.

d) ChIP of Rag2 relative to total H3 as in c) (mean ± SE of 3 independent experiments). p=0.0001 for all Jα elements; p=0.054 (NS) for proximal (Jα61–48) and p=0.0001 for distal (Jα37–16) Jα elements.

e) ChIP of Rag1 relative to total H3 as in c) (mean ± SE of 2 independent experiments). p=0.005 for all Jα elements; p=0.17 (NS) for proximal (Jα61–48) and p=0.003 for distal (Jα37–16) Jα elements.

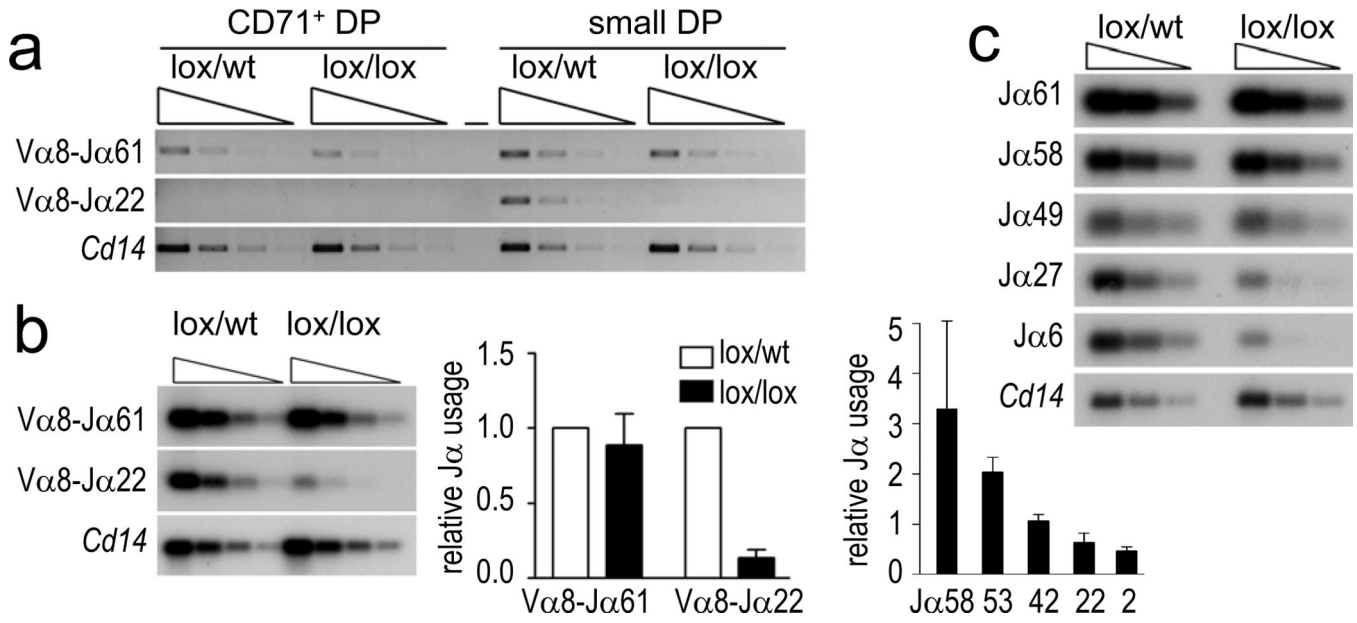

Figure 3. Cohesin affects *Tcra* rearrangement

a) Three-fold dilutions of genomic Vα8-Jα PCR products from CD71^{+} or CD71^{-} DP thymocytes visualised with ethidium bromide. *Cd14* is a genomic control.

b) Genomic PCR products from small DP thymocytes visualised by Southern blotting with Jα-specific probes (left). Usage of the distal Jα22 element was reduced by 86% (middle, mean ± SE, n=3). Southern blotting of Vα8-Jα RT-PCR products from CD4Cre *Rad21*$^{lox/lox}$ normalised to control DP thymocytes (right, mean ± SE, n=3).

c) Double strand breaks in three-fold serially diluted genomic DNA from CD4Cre *Rad21*$^{lox/lox}$ and control DP thymocytes detected by ligation-mediated PCR.

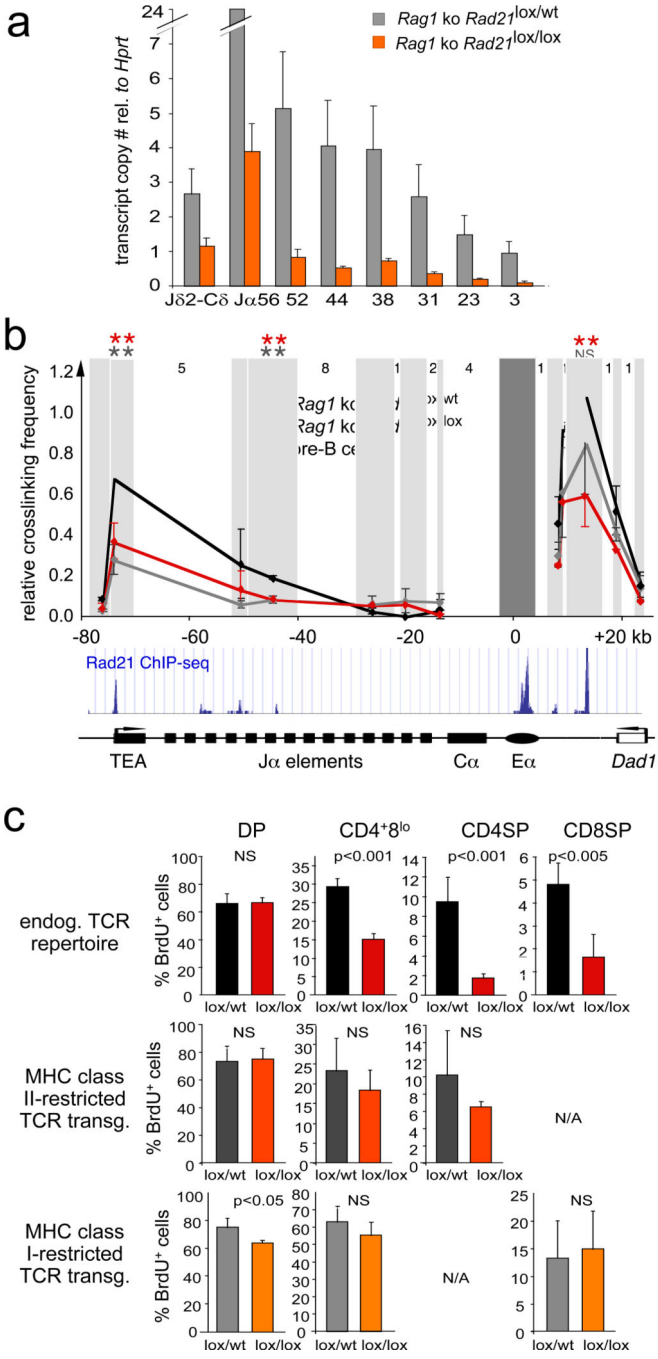

Figure 4. Cohesin mediates long-range interactions between regulatory elements that control *Tcra* transcription

a) Transcript copy number of the unrearranged *Tcra* J region in *Rag1*-deficient CD4Cre *Rad21*$^{lox/lox}$ and *Rad21*$^{lox/wt}$ DP thymocytes (mean ± SE, n=3).

b) 3C analysis of long-range interactions between Eα and *Tcra* restriction fragments (shaded) in *Rag1*-deficient CD4Cre *Rad21*$^{lox/wt}$ (black, mean ± SD, n=3), CD4Cre *Rad21*$^{lox/lox}$ (red, mean ± SD, n=3) DP thymocytes and pre-B cells (grey, mean ± SD, n=3). Intervening HindIII fragment numbers and genomic distances are indicated. Stars: $p < 0.05$

(grey: control thymocytes versus pre-B cells; red: control versus cohesin-depleted thymocytes; NS = not significant).

c) TCR transgenes rescue the differentiation of cohesin-depleted thymocytes. Top: Percentages $BrdU^+$ cells in $CD4^+8^{lo}$, CD4 SP and CD8 SP thymocytes. The differentiation of cohesin-deficient thymocytes is rescued by MHC class II-restricted (middle) and MHC class I-restricted TCR transgenes (bottom), n=3–5 per data point ± SD.