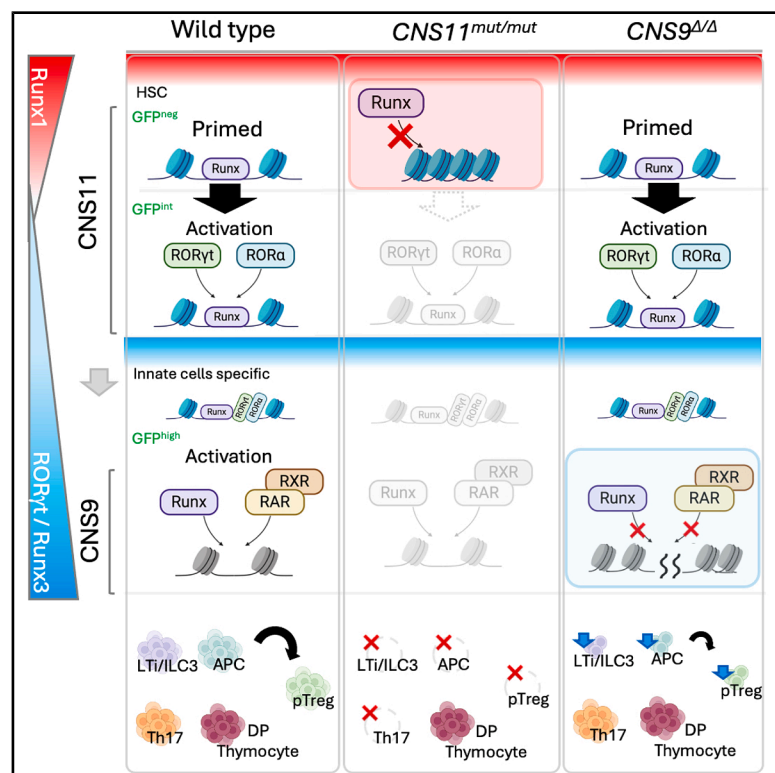


A hierarchical *Rorc*(γ t) *cis*-regulatory cascade orchestrates differentiation of ROR γ t⁺ innate immune cells

Graphical abstract



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In brief

Transcription factor ROR γ t is a central regulator of type 3 immunity, essential for host defense and tissue homeostasis. Fukui et al. identify a hierarchical *CNS11*-*CNS9* enhancer cascade that governs ROR γ t expression across innate lymphocytes and antigen-presenting cells. Disruption of this transcriptional regulation perturbs lymphoid organ development and gut immune balance.

Highlights

- Sequential regulation by *CNS11* and *CNS9* governs ROR γ t expression in innate immune cells
- *CNS11* priming in HSCs/MPPs is required for ROR γ t induction in peripheral immune cells
- *CNS9*-driven ROR γ t enhancement is primarily required for ROR γ t⁺ APC differentiation
- *CNS9* is essential for control of type 2 immunity via pTreg cell induction by ROR γ t⁺ APCs



Article

A hierarchical *Rorc*(γ t) *cis*-regulatory cascade orchestrates differentiation of ROR γ t⁺ innate immune cells

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SUMMARY

Beyond their well-established roles in type 3 immunity, ROR γ t⁺ innate immune cells are also essential for secondary lymphoid organ (SLO) formation and gut homeostasis. However, the transcriptional mechanisms governing ROR γ t expression in these cells, including group 3 innate lymphoid cells (ILC3s), lymphoid tissue inducer (LTi) cells, and antigen-presenting cells (APCs), remain largely unresolved. Here, we identified two key *cis*-regulatory elements within conserved non-coding sequences (CNS) 9 and 11 in the *Rorc* locus, which were sequentially utilized during differentiation. Initially, Runx-binding sites in CNS11 established chromatin accessibility as early as the hematopoietic stem cell (HSC) stage. Disruption of this chromatin priming prevented subsequent transcriptional activation, thereby abolishing the initial induction of ROR γ t in these cells. At later stages, CNS9 played a critical role, particularly in the development of ROR γ t⁺ APCs, contributing to colonic peripheral Treg (pTreg) cell induction. This hierarchical transcriptional regulation was essential for SLO formation and postnatal type 3 immunity and for restraining excessive intestinal type 2 immune responses through pTreg cell induction.

INTRODUCTION

The nuclear hormone receptor RAR-related orphan receptor γ t (ROR γ t), first identified in thymocytes,¹ is a central regulator of type 3 immunity,^{2–4} orchestrating antimicrobial defense, tissue repair, and barrier homeostasis. ROR γ t is expressed in group 3 innate lymphoid cells (ILC3s), γ T17 cells, Th17 cells,^{3–10} and more recently described antigen-presenting cell (APC) populations^{11–13} that induce colonic ROR γ t⁺ regulatory T (Treg) cells,^{13–16} highlighting broader roles beyond classical lymphoid cells. ROR γ t is also essential for lymphoid tissue inducer (LTi) cells,^{6,17} which drive lymph node (LN) and Peyer's patch (PP) organogenesis through lymphotoxin expression.¹⁸ Fetal LTi cells arise from the fetal liver integrin (Int.) $\alpha_4\beta_7$ -positive precursors^{8,19} shortly after Id2 induction,^{20–22} representing a pivotal evolutionary step in adaptive immunity. However, the molecular

mechanisms initiating ROR γ t expression during LTi specification remain incompletely defined.

Postnatally, ROR γ t continues to govern innate and adaptive lymphocyte function to contain extracellular microbes and prevent commensal dissemination.^{7,23} Dysregulated ROR γ t activity contributes to autoimmunity,^{2,24,25} yet the transcriptional control of ROR γ t in innate immune cells remains less understood than in Th17 cells.^{26,27}

In this study, we aimed to elucidate the transcriptional regulation of *Rorc*(γ t) across different cell types and developmental stages, with a particular focus on innate immune cells, including LTi cells, ILC3s, and ROR γ t⁺ APCs. Through epigenetic analyses and the generation of genetically engineered mouse models, we identified two essential *cis*-regulatory elements (CREs) within the conserved non-coding sequences (CNS) 9 and 11 at the *Rorc* locus. These CREs are sequentially utilized during the differentiation of ROR γ t⁺



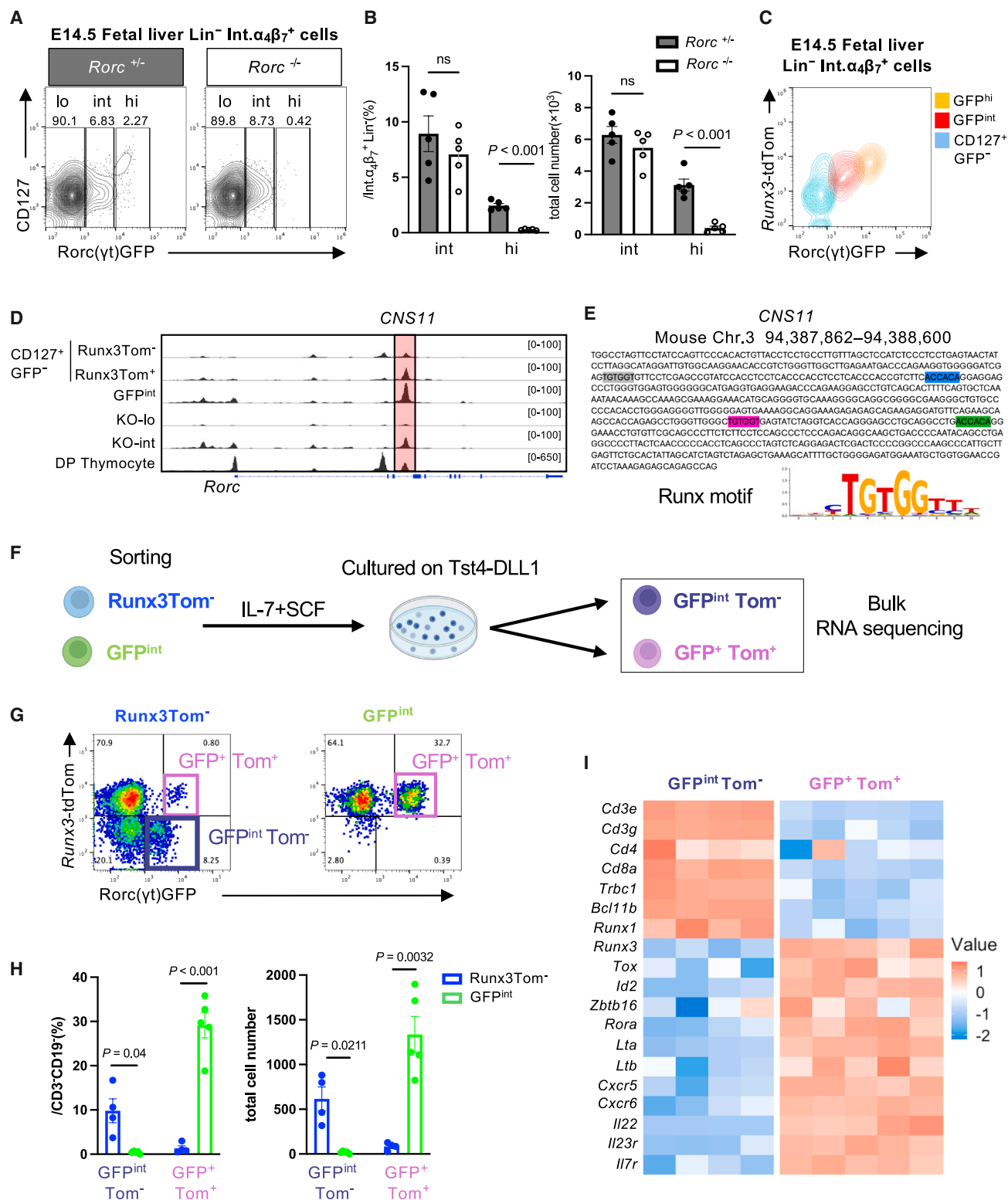


Figure 1. Development of ROR γ t⁺ cells in the fetal liver

(A and B) Flow cytometry of fetal liver (FL) Lin[−]CD3e[−]B220[−]Ter119[−]ST2[−]and NKp46[−]Int.α4β7⁺ cells from E14.5 *Rorc*^{+/−} and *Rorc*^{−/−} mice carrying *Rorc*(γt)*Gfp* (A). Frequencies and numbers of GFP^{int} and GFP^{hi} subsets among Lin[−]Int.α4β7⁺ cells (*n* = 5 per group) (B).

(C) Flow cytometry of CD127⁺GFP[−], GFP^{int}, and GFP^{hi} subsets in E14.5 FL Lin[−]Int.αβ⁺ cells from *Rorc*(*yt*)*Gfp*; *Runx3*^{Tom/+} mice.

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innate immune cells. Initially, Runx-binding sites in *CNS11* establish early chromatin accessibility, priming the region as early as the hematopoietic stem cell (HSC) stage—an essential step in initiating ROR γ t expression. Subsequently, the primed *CNS11* undergoes progressive activation through the cooperative action of Runx with other transcription factors, including the ROR family, thereby amplifying ROR γ t expression. Once ROR γ t is induced, its expression is further reinforced in a runt-related transcription factor and core-binding factor subunit β complex (Runx/CBF β)- and retinoic acid receptor α (RARA)/retinoid X receptor γ (RXRG)-dependent manner via *CNS9*, which plays a critical role in the development of ROR γ t⁺ APCs and contributes to colonic peripheral Treg (pTreg) induction. This hierarchical *Rorc*(γ t) transcriptional program is indispensable for secondary lymphoid organ (SLO) formation, postnatal type 3 immunity, and the suppression of type 2 immune responses via pTreg induction.

RESULTS

Stepwise development of LT_i precursors in the fetal liver

To understand the *Rorc*(γ t) regulation in LT_i cell development, we first examined fetal liver Lin[−]Int.α₄β₇⁺ populations,¹⁹ using *Rorc*(γ t)*Gfp* bacterial artificial chromosome (BAC) transgenic (Tg) mice.¹⁵ Based on the GFP intensity, fetal liver Lin[−]Int.α₄β₇⁺ cells were classified into three populations: GFP^{lo}, GFP^{int}, and GFP^{hi} cells, which correspond to distinct developmental stages of LT_i cells⁹ (Figure 1A). In various cell types, master regulatory factors can control their own expression.²⁸ To examine whether ROR γ t regulates its own expression, we utilized *Rorc*^{−/−} mice (Figure S1A) carrying the *Rorc*(γ t)*Gfp* transgene and monitored *Rorc*(γ t) promoter activity in fetal liver Lin[−]Int.α₄β₇⁺ cells.

In *Rorc*^{−/−}; *Rorc*(γ t)*Gfp* mice, a population with intermediate GFP intensity, referred to hereafter as knockout (KO)-GFP^{int} (KO-int), was identified (Figure 1A). The frequency and number of these GFP-intermediate cells among the Lin[−]Int.α₄β₇⁺ fraction were comparable between *Rorc*^{+/-} and *Rorc*^{−/−} mice. In contrast, GFP^{hi} cells were scarcely detected in *Rorc*^{−/−} mice (Figures 1A and 1B). Assay for transposase-accessible chromatin using sequencing (ATAC-seq) and RNA sequencing (RNA-seq) analyses revealed that KO-int cells and GFP^{int} cells shared similar epigenomic and transcriptomic features (Figures S1B and S1C). Together, these results suggest a sequential, two-step regulation of *Rorc*(γ t) during LT_i differentiation: an intermediate expression state (GFP^{int}) followed by a ROR γ t-dependent transition to a high-expression state (GFP^{hi}).

GFP^{int} populations with activated *CNS11* are precursors committed to the LT_i lineage

Next, we aimed to elucidate the transcriptional network of GFP^{int} cells, the first population in which ROR γ t promoter activation is

observed. ATAC-seq footprinting analysis revealed a genome-wide increase in the ROR and the Runx family transcription factor binding in GFP^{int} cells compared with the heterogeneous GFP^{lo} population (Figure S1D). These findings suggest that, alongside the ROR family, the Runx family plays a key regulatory role in GFP^{int} cells.

The Runx transcription factor complexes are composed of a DNA-binding Runx protein and its non-DNA-binding partner, CBF β . A previous study demonstrated a reduction in the number of LT_i cells and in ROR γ t expression levels within LT_i cells in the fetal intestine of mice lacking the predominant transcript variants of Runx1 and CBF β during the fetal stage.²⁹ However, given that Runx1 or CBF β deficiency in hematopoietic cells affects common lymphoid progenitor (CLP) differentiation,^{30,31} which includes LT_i progenitors, it remains unclear whether the Runx family is directly involved in ROR γ t expression in LT_i cells.

To address this issue, we first examined the dynamics of *Runx1* and *Runx3* transcript levels during LT_i cell development, particularly from the CD127⁺GFP[−] fraction—which is enriched for LT_i progenitors—to fully differentiated LT_i cells. Genome-wide analysis confirmed that each population accurately represented its respective differentiation stage (Figures S1C and S1E). In the fetal liver, *Runx1* transcript levels were relatively higher than those of *Runx3* at the CD127⁺GFP[−] stage but gradually decreased during differentiation, whereas *Runx3* transcript levels progressively increased (Figure S1F). Further analysis of *Runx3* expression in fetal liver populations of *Runx3*-tdTomato mice³² carrying *Rorc*(γ t)*Gfp* revealed a strong positive correlation between *Runx3* and *Rorc*(γ t) expression (Figure 1C). Based on these findings, we hypothesized that Runx3 expression marks distinct stages of LT_i cell differentiation.

To test this, we further subdivided the CD127⁺GFP[−] fraction (Figure 1C) into two populations: CD127⁺Runx3Tom[−] and CD127⁺Runx3Tom⁺, hereafter referred to as Runx3Tom[−] and Runx3Tom⁺, respectively (Figure S1G). Their enrichment for ILC-related transcription factor binding—including TCF7^{31,33} and GATA3³⁴—further supported the idea that Runx3Tom⁺ cells are more differentiated than Runx3Tom[−] cells (Figure S1H).

CREs play a crucial role in controlling lineage-specific gene expression in helper T cells.³⁵ In Th17 cells, there are 11 conserved non-coding sequences (*CNS1–CNS11*) within the *Rorc* locus.²⁶ ATAC-seq analysis identified an open chromatin region corresponding to *CNS11*^{26,27,36} (Chr3:94,387,862–94,388,600), which becomes accessible at the Runx3Tom[−] stage, when ROR γ t is not yet expressed, and is progressively activated during differentiation into the GFP^{int} stage, along with the upregulation of Runx3 (Figure 1D). Four Runx-binding sites within *CNS11* were identified in the JASPAR database (Figure 1E), suggesting their involvement in LT_i cell differentiation. In contrast, DP thymocytes exhibited a relatively low

(D) ATAC-seq at the *Rorc* locus, in E14.5 FL Lin[−]Int.α₄β₇⁺ subsets from *Rorc*^{+/-} and *Rorc*^{−/−} mice (KO-lo and KO-int) carrying *Rorc*(γ t)*Gfp*, and in P1 DP thymocytes.

(E) Mouse genome sequence of the *Rorc* *CNS11* locus with core Runx motifs highlighted.

(F–H) *In vitro* culture of sorted GFP^{int} and Runx3Tom[−] FL cells on Tst4-DLL1 stromal cells with IL-7/SCF (F). Flow cytometry of GFP^{int}Tom[−] (purple) and GFP⁺Tom⁺ (magenta) cells in CD45.2⁺CD3ε[−]CD19[−] populations from Runx3Tom[−] and GFP^{int} subsets after culture (G). Frequencies and numbers (Runx3Tom[−]: *n* = 4, GFP^{int}: *n* = 5) (H).

(I) Bulk RNA-seq of GFP^{int} Tom[−] (*n* = 4) and GFP⁺Tom⁺ (*n* = 5) cells.

Data are shown as mean ± SEM; representative of ≥2 (C and D) or pooled from ≥3 (B, H, and I) experiments. Two-tailed *t* test (ns, *p* > 0.05).

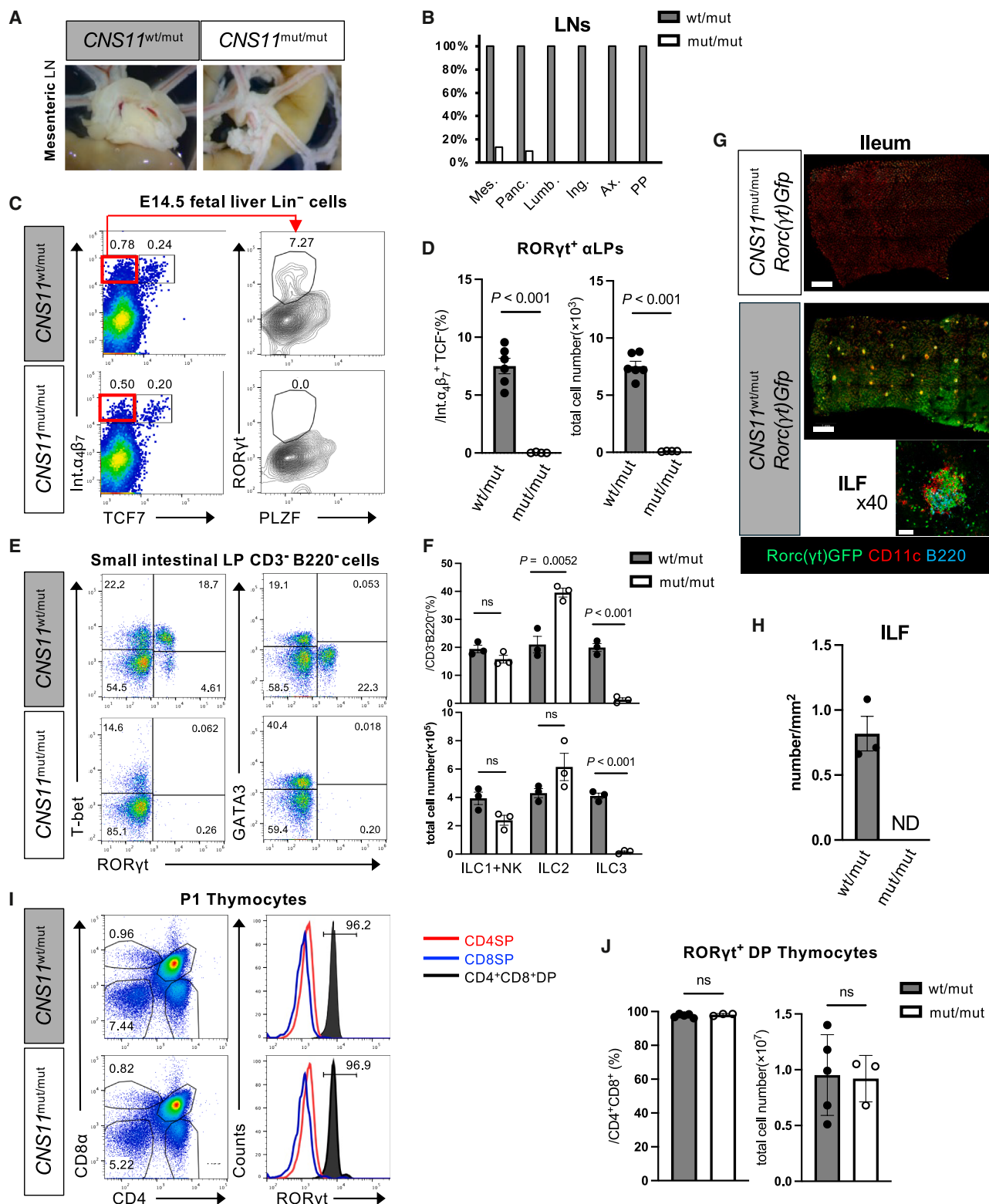


Figure 2. Runx-binding sequences in *Rorc* *CNS11* are essential for fetal LTi and ILC3 development

(A) Mesenteric LNs in 6-week-old *CNS11*^{wt/mut} and *CNS11*^{mut/mut} mice.

(B) LN and PP frequencies ($n = 3$ per group). **Abbreviations:** Mes., mesenteric; Panc., pancreatic; Lumb., lumbar; Ing., inguinal; Ax., axillary; PP, Peyer's patch.

(C and D) Flow cytometry of E14.5 FL Lin⁻ cells from *CNS11*^{wt/mut} and control *CNS11*^{wt/mut} mice (C). Frequencies and numbers of PLZF⁺RORyt⁺ cells among Lin⁻ Int.α4β7⁺TCF7⁺ cells (*CNS11*^{wt/mut}, $n = 4$; *CNS11*^{wt/mut}, $n = 6$) (D).

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CNS11 peak, with a more prominent peak just upstream of *CNS11*. This upstream peak was also observed in *Runx3Tom⁻* cells but gradually diminished as LT_i maturation progressed (Figure 1D).

To examine the relationship between these two peaks and cell lineages, we performed *in vitro* culture experiments using the *Runx3Tom⁻* population, which possesses both the DP thymocyte-related peak and the *CNS11* peak, and the *GFP^{int}* population, which has only the *CNS11* peak (Figures 1D and 1F). In the presence of Tst4-DLL1 stromal cells, which express the Notch ligand DLL-1, *Runx3Tom⁻* cells differentiated into two populations: *GFP^{int}Runx3tdTomato⁻* (*GFP^{int}Tom⁻*) cells and *GFP^{hi}Runx3tdTomato⁺* (*GFP⁺Tom⁺*) cells. Based on their transcriptomic profiles, *GFP^{int}Tom⁻* cells corresponded to CD4⁺CD8α⁺ DP thymocytes, whereas *GFP⁺Tom⁺* cells represented LT_i cells (Figures 1G–1I). Under the same culture conditions, *GFP^{int}* cells exclusively gave rise to *GFP⁺Tom⁺* cells (Figures 1G–1I). These findings suggest that the *GFP^{int}* population, which has activated *CNS11*, largely consists of precursors committed to the LT_i lineage.

Runx-binding sites in *CNS11* are indispensable for LT_i and ILC3 differentiation

Next, to investigate the relevance of four Runx-binding sites within the *CNS11* region in the development of LT_i cells from the *GFP^{lo}* to *GFP^{int}* stage, we generated *Rorc CNS11^{mut/mut}* mice, which carry homozygous mutations in all four Runx-binding sites (Figure S2A). These mice lacked LNs and PPs (Figures 2A and 2B) due to the complete absence of fetal LT_i cells at E14.5 (Figures S2B and S2C). Moreover, in the fetal liver of *CNS11^{mut/mut}* mice, the most immature LT_i precursor cells expressing RORγt³⁷ were also absent (Figures 2C and 2D). These results suggest that the four Runx-binding sites within *CNS11* are indispensable for the initial expression of RORγt during differentiation into the *GFP^{int}* stage.

A previous study demonstrated that Runx3 is required for RORγt induction during ILC3 development.³⁸ Although Runx3-dependent RORγt induction has been attributed to the direct binding of Runx3 to the *Rorc(γt)* promoter region,³⁸ it remains unclear whether the Runx/CBFβ complex utilizes *CNS11* for ILC3 lineage specification.

Adult intestinal ILC3s are heterogeneous and comprise CCR6⁺T-bet⁻ ILC3s and T-bet⁺ ILC3s.^{8,39} Due to their shared gene expression profiles and functional redundancy with fetal LT_i cells, adult intestinal CCR6⁺T-bet⁻ ILC3s are also referred to as LT_i-like cells.^{4,40} Accumulating evidence—including differences in the precursor populations,^{41,42} transcription factor usage,^{33,43} and cell fate trajectories—supports the notion that T-bet⁺ ILC3s and LT_i-like cells represent distinct lineages.^{5,8,39} In the adult small intestine of *Rorc(γt)Gfp* mice, *GFP^{int}* fractions

were observed in both NKp46⁻ and NKp46⁺ ILC3s (Figure S2D). The shared accessibility of *CNS11* in both fetal LT_i cells and intestinal ILC3s suggests a conserved mechanism of *Rorc(γt)* regulation (Figure S2E). Consistently, *CNS11^{mut/mut}* mice lacked all intestinal ILC3s, further supporting this model (Figures 2E and 2F). Moreover, chromatin integration labeling (ChIL)⁴⁴ followed by sequencing (ChIL-seq) on MNK3 cells,⁴⁵ a widely used ILC3 cell line, revealed CBFβ binding to *CNS11* (Figure S2F). Collectively, these findings demonstrate that the Runx/CBFβ complex directly engages *CNS11* to regulate ILC3 development.

Isolated lymphoid follicles (ILFs) are postnatally formed gut-associated lymphoid tissues (GALTs) distributed throughout the intestinal lamina propria, playing a crucial role in intestinal immunity.⁴⁶ ILFs contain B cells, dendritic cells, and ILC3s, particularly lymphotoxin-expressing LT_i-like cells.^{6,8,46,47} In the ileum of *CNS11^{mut/mut}*, *Rorc(γt)Gfp* mice, which carry *Rorc(γt)Gfp*, ILF formation was completely impaired (Figures 2G and 2H). In contrast, RORγt expression levels in CD4⁺CD8⁺ double-positive (DP) thymocytes of *CNS11^{mut/mut}* mice remained comparable to controls, and their development was unaffected (Figures 2I, 2J, and S2G). These findings suggest that Runx/CBFβ binding to *CNS11* is dispensable for DP thymocyte development but indispensable for the initial expression of RORγt in ILC3s, highlighting its selective role in the early regulation of intestinal ILC3s.

The Runx-binding sites within *CNS11* are essential for the initial establishment of chromatin accessibility

To investigate whether the Runx family directly regulates RORγt expression through Runx-binding sites within *CNS11*, we performed a luciferase reporter assay using HEK-293T cells. Overexpression of either Runx1 or Runx3 did not increase *CNS11*-dependent transcriptional activity (Figure 3A). Consistent with genome-wide analysis (Figure S1D), ROR-binding motifs were confirmed within *CNS11* activated in the *GFP^{int}* population (Figure S3A), prompting us to investigate the ROR family. Using MNK3 cells, chromatin immunoprecipitation sequencing (ChIP-seq) assays further demonstrated the binding of the ROR family members, RORα and RORγt, to *CNS11* (Figure S3B). Either RORα or RORγt alone enhanced *CNS11*-dependent transcriptional activity in a dose-dependent manner, with RORα exhibiting stronger activity than RORγt at lower doses (Figure 3A). Given that IL-17 promoter activity in T cells is regulated through the cooperative action of Runx and ROR family transcription factors,⁴⁸ we examined whether *CNS11* is similarly regulated. As expected, Runx3 enhanced Runx-binding site-dependent *CNS11* reporter activity in the presence of either RORγt or RORα. This enhancement was abolished when the Runx-binding sites within *CNS11* were mutated (Figure 3B), suggesting that *Runx3* and the ROR family synergistically enhance *CNS11*

(E and F) Flow cytometry of small intestinal lamina propria (LP) CD3⁻B220⁻ cells from 8-week-old *CNS11^{mut/mut}* and control *CNS11^{wt/mut}* mice (E). Frequencies and numbers of ILC subsets among CD3⁻B220⁻ cells (*n* = 3 per group) (F).

(G and H) Ileal whole-mount images from 8-week-old *CNS11^{mut/mut}* and control *CNS11^{wt/mut}* mice carrying *Rorc(γt)Gfp*. Confocal microscopy (G top, middle: 10×, and G bottom: 40×) shows ILFs with Rorc(γt)GFP⁺ (green), CD11c⁺ (red), and B220⁺ (blue) cells. Scale bars: 1 mm (10×), 50 μm (40×) (G). Quantification of ILFs defined as aggregates of CD11c⁺ B220⁺ cells. (*n* = 3 per group) (H) (ND; not detected).

(I and J) Flow cytometry of DP, CD4SP, and CD8SP subsets in the thymus from P1 *CNS11^{mut/mut}* and control *CNS11^{wt/mut}* mice (I). Frequencies and numbers of RORγt⁺ DP cells (J) (*CNS11^{wt/mut}*; *n* = 5; *CNS11^{mut/mut}*; *n* = 3).

Data are shown as mean ± SEM; representative of three (G) or pooled from two (D and J) or three (F and H) experiments. Two-tailed *t* test (ns, *p* > 0.05).

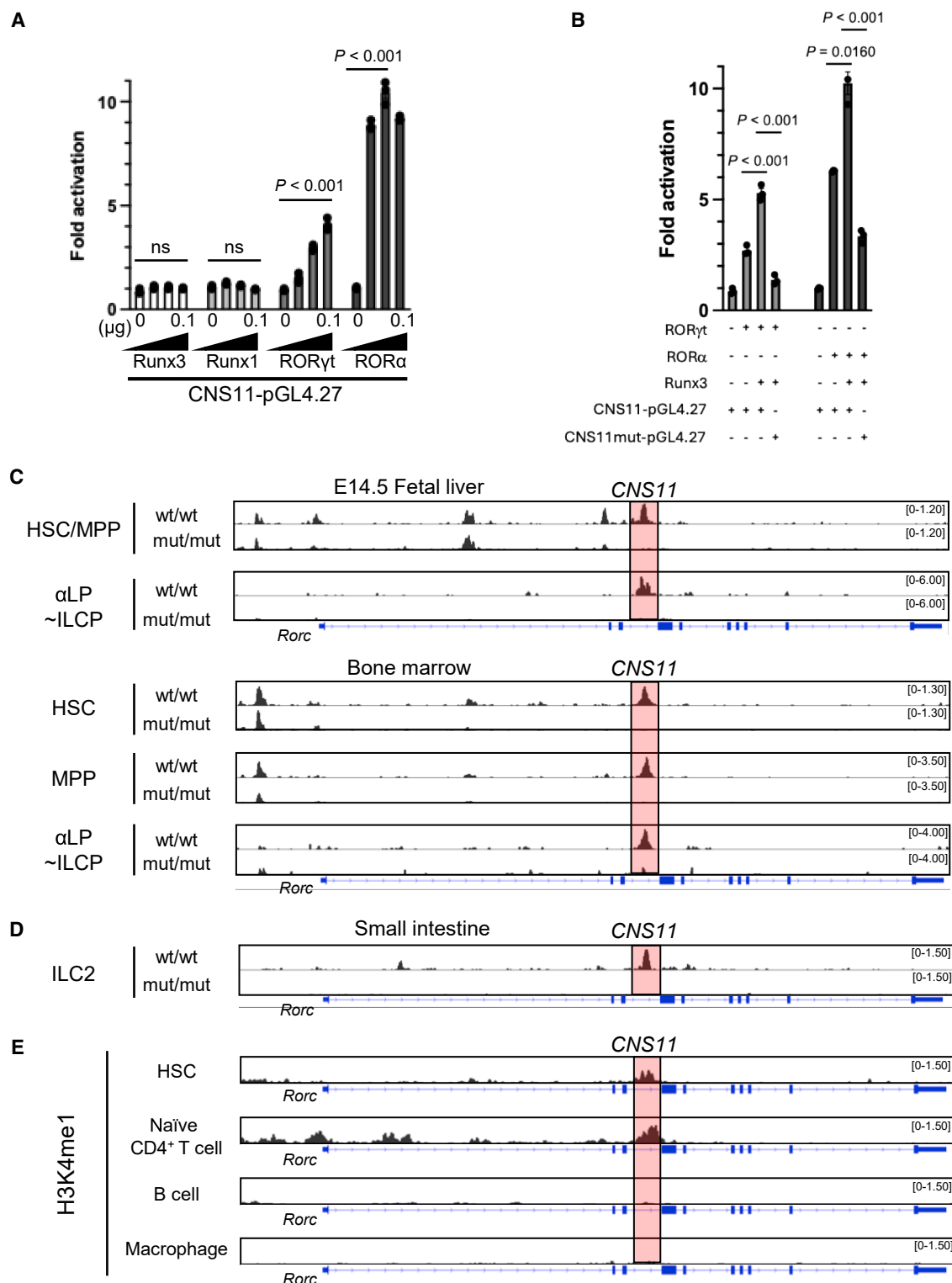


Figure 3. Functional analysis of *Rorc* *CNS11* sequences

(A and B) Luciferase assay in HEK-293T cells transfected with pGL4.27 containing *CNS11* sequences, along with either Runx1-, Runx3-, RORγt-, or RORα-expressing pcDNA3.1(+) vectors or an empty pcDNA3.1(+) vector. The number of vectors carrying each transcription factor ranged from 0.01 to 0.1 μg (A). Luciferase assay in HEK-293T cells transfected with CNS11-pGL4.27 or mutant vector (CNS11mut-pGL4.27) along with a combination of Runx3 (0.05 μg)- and RORγt (0.1 μg)-expressing vectors (left) or Runx3 (0.05 μg)- and RORα (0.01 μg)-expressing vectors (right) (B). Data represent relative luciferase activity normalized to the empty vector.

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activity through Runx-binding sites. However, the ROR family alone could still enhance luciferase activity independently of these Runx-binding sites *in vitro* (Figure S3C). This finding directly contradicts the phenotype of *CNS11*^{mut/mut} mice, in which the initial induction of ROR γ t is completely abolished. This discrepancy raises the possibility that the Runx family exerts additional functions at an earlier stage of development, beyond its synergistic role with the ROR family in activating *CNS11*.

Currently, the Runx family is widely recognized not only for its direct role in regulating target gene expression but also for its crucial function in shaping the chromatin landscape.⁴⁹ In T cells, Runx1 primes the chromatin landscape early in development—even before antigen receptor expression—thereby facilitating the acquisition of lineage-specific effector functions.⁵⁰ In line with this, ROR γ t expression in CD4⁺ T cells was completely absent in *CNS11*^{mut/mut} mice, whereas it was preserved in CD4⁺ T cells from *Cd127*^{Cre/+}; *Cbfb*^{flox/flox} mice,^{51,52} in which CBF β loss occurs after the CLP stage (Figure S3D). Furthermore, adoptive transfer of splenic CD4⁺ T cells into Rag1^{-/-} mice revealed that *CNS11*^{mut/mut} CD4⁺ T cells failed to differentiate into ROR γ t-expressing helper T cells, including Th17 cells and ROR γ t⁺ pTreg cells, whereas wild-type (WT) CD4⁺ T cells efficiently differentiated in the gut (Figure S3E). Collectively, these results demonstrate that CD4⁺ T cells require Runx-binding to *CNS11* in a cell-intrinsic manner, pointing to a potential priming event occurring prior to the CLP stage.

To directly test whether such chromatin priming at the *Rorc* locus is already established during early hematopoiesis, we performed ATAC-seq on various ROR γ t precursor populations, including aLP-ILCP, MPP, and HSC, in both the fetal liver and bone marrow of WT mice. As expected, we found that *CNS11*—the region critical for the initial induction of ROR γ t—already exhibited chromatin accessibility at the HSC/MPP stage. This accessibility was lost in *CNS11*^{mut/mut} mice (Figure 3C). Consistent with this, ChIP-seq analysis of E12.5 fetal liver Lin⁻ cells, relatively enriched for HSCs and multipotent progenitor cells (MPPs),⁵³ revealed clear CBF β binding at *CNS11* in WT cells, whereas this binding was completely absent in *CNS11*^{mut/mut} cells (Figure S3F). To further validate the role of Runx/CBF β in priming chromatin accessibility during early hematopoiesis, we performed ATAC-seq on HSCs/MPPs from the embryonic day (E)14.5 fetal liver of *Vav1*^{Cre/+}; *Cbfb*^{flox/flox} mice, which lack CBF β in early hematopoietic progenitors. HSCs/MPPs from these mice exhibited reduced ATAC peaks within *CNS11* (Figure S3G). Taken together, these findings demonstrate that Runx/CBF β is required for establishing chromatin accessibility at *CNS11* during early hematopoietic differentiation, particularly in HSCs and MPPs.

We further examined *CNS11* accessibility in peripheral cell types that differentiate independently of ROR γ t. *CNS11* was accessible in ILC1,⁵⁴ ILC2,⁵⁵ and naive CD4⁺ T cells⁵⁶—all of which have the potential to express ROR γ t^{9,57,58}—but was inac-

cessible in B cells⁵⁹ and macrophages⁶⁰ (Figure S3H). Consistent with the results from immature precursors, ATAC-seq analysis of the ILC2 subset in *CNS11*^{mut/mut} mice showed that the *CNS11* region was completely closed (Figure 3D). In contrast, in DP thymocytes—which differentiate normally in *CNS11*^{mut/mut} mice—*CNS11* accessibility was obtained, suggesting that its accessibility may be re-established through transcription factors other than Runx (Figure S3I).

Lastly, we examined histone modifications at *CNS11* across various cell types and confirmed that the primed enhancer marker H3K4me1 was enriched in HSCs⁶¹ and naive CD4⁺ T cells⁶² but was not detected in B cells⁶³ or macrophages.⁶⁴ This finding further suggests that *CNS11* exists in a primed state in both HSCs and naive CD4⁺ T cells, preparing them for subsequent activation (Figure 3E).

In summary, the Runx-binding sites within *CNS11* are essential for the initial establishment of chromatin accessibility during early hematopoietic differentiation. Subsequently, these sites function in collaboration with the ROR family to enhance *CNS11* activity.

The fourth Runx-binding site in *CNS11* plays a significant role in the initial formation of *CNS11* chromatin accessibility

To identify the critical Runx-binding motifs in *CNS11* essential for LT α i differentiation and LN formation, we examined genomic conservation across species (Figure S4A). Among the four Runx-binding sites, the third and fourth motifs were completely conserved among placental mammals (Figure 4A). ATAC-seq footprinting analysis of GFP^{int} cells further revealed that the fourth Runx-binding motif exhibited the strongest potential for Runx binding (Figure S4B).

To validate the biological significance of the fourth Runx-binding site, we generated *Rorc* *CNS11*^{4th/4th} mice, which carry homozygous mutations specifically at this site (Figure S4C). These mice exhibited a significantly reduced frequency of peripheral LNs and PPs (Figures 4B and 4C). Consistent with this, *CNS11*^{4th/4th} fetuses almost completely lacked LT α i cells (Figures S4D and S4E). Based on these findings, we concluded that the fourth Runx-binding motif is critical for LT α i differentiation and the formation of LNs and PPs. Next, we analyzed the small intestines of adult *Rorc* *CNS11*^{4th/4th}; *Rorc*(γ)*Gfp* mice. Surprisingly, despite the loss of LNs and PPs, these mice retained ILFs (Figures 4D and 4E). In line with this, T-bet⁻ ILC3s were preserved, whereas T-bet⁺ ILC3s were absent, in *CNS11*^{4th/4th} mice (Figures 4F and 4G).

To investigate the role of the fourth Runx-binding site within *CNS11* in enhancer priming, we performed ATAC-seq on MPPs from the bone marrow and LT α -EGFP⁺ LT α i-like cells from the small intestine of *Rorc* *CNS11*^{4th/4th}; *Lta*^{EGFP} reporter mice (Figure S4F). We found that *CNS11* chromatin accessibility in these cells was significantly reduced (Figure 4H). These

(C and D) ATAC-seq at the *Rorc* locus in E14.5 fetal liver and 8-week-old BM cells (C) and small intestinal ILC2 (D) from *CNS11*^{mut/mut} and control *CNS11*^{wt/wt} mice. (E) ChIP-seq, CUT&Tag, and CUT&RUN analyses of H3K4me1 histone modification at the *Rorc* locus in HSCs, naive CD4⁺ T, B cells, and macrophages. Datasets were obtained from Gene Expression Omnibus (GEO).

Data are shown as mean $\pm$ SEM; representative of two experiments (A–D). Two-tailed *t* test (ns, *p* > 0.05).

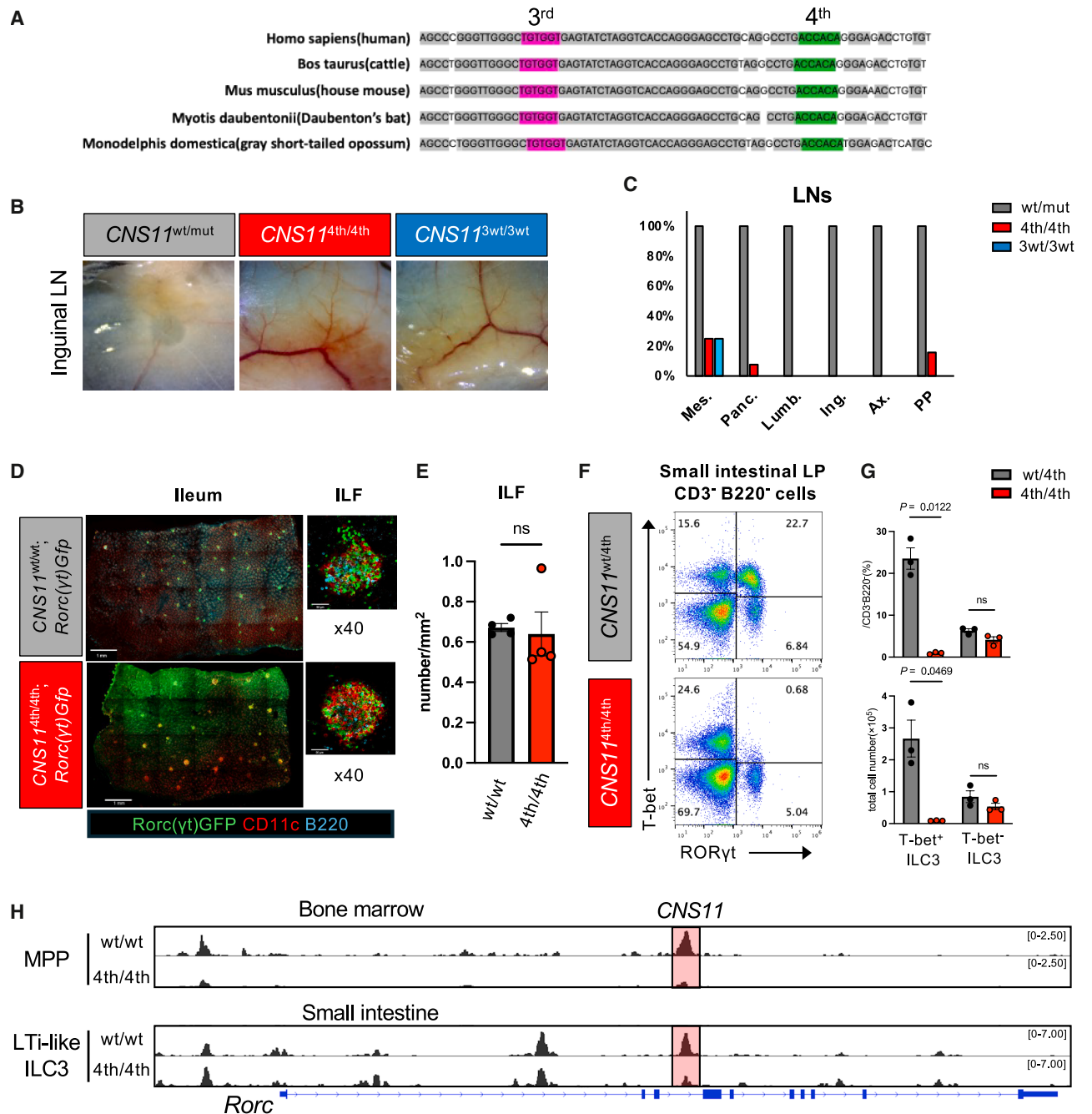


Figure 4. Functional analysis of the 4th Runx-binding sequence within *Rorc* *CNS11* region

(A) Multiple sequence alignment of the *Rorc* *CNS11* region in Homo sapiens, Bos taurus (cattle), Mus musculus (house mouse), Myotis daubentonii (Daubenton's bat), and Monodelphis domestica (gray short-tailed opossum). Runx-binding sites are highlighted.

(B) Mesenteric LN from 6-week-old *CNS11*^{4th/4th}, *CNS11*^{3wt/3wt}, and control *CNS11*^{wt/mut} mice.

(C) LN and PP frequencies in *CNS11*^{4th/4th} (*n* = 4), *CNS11*^{3wt/3wt} (*n* = 4), and control *CNS11*^{wt/mut} (*n* = 3) mice. **Abbreviations:** same as Figure 2B.

(D and E) Ileal whole-mount images of 8-week-old *CNS11*^{wt/wt} and *CNS11*^{4th/4th} mice carrying *Rorc*(yt)*Gfp*. Confocal microscopy (D, left: 10× and right: 40×) shows ILFs with *Rorc*(yt)GFP⁺ (green), CD11c⁺ (red), and B220⁺ (blue) cells. Scale bars: 1 mm (10×), 50 μm (40×) (D). Quantification of ILFs defined as aggregates of CD11c⁺B220⁺ cells (*n* = 4 per group) (E).

(F and G) Flow cytometry of small intestinal LP CD3⁺B220⁺ cells from 4-week-old *CNS11*^{4th/4th} and control *CNS11*^{wt/4th} mice (F). Frequencies and numbers of T-bet⁺ ILC3 and T-bet⁺ ILC3 subsets among CD3⁺B220⁺ populations (G) (*n* = 3 per group).

(H) ATAC-seq analysis at the *Rorc* locus in MPPs from BM and c-Kit⁺CD4⁺LTα-EGFP⁺ LTI-like cells from the small intestinal LP of 8-week-old *CNS11*^{4th/4th} and *CNS11*^{wt/wt} mice carrying *LTA*^{EGFP} knockin allele.

Data are shown as mean ± SEM; representative of three (D) or two (H) experiments or pooled from three experiments (G). Two-tailed *t* test (ns, *p* > 0.05).

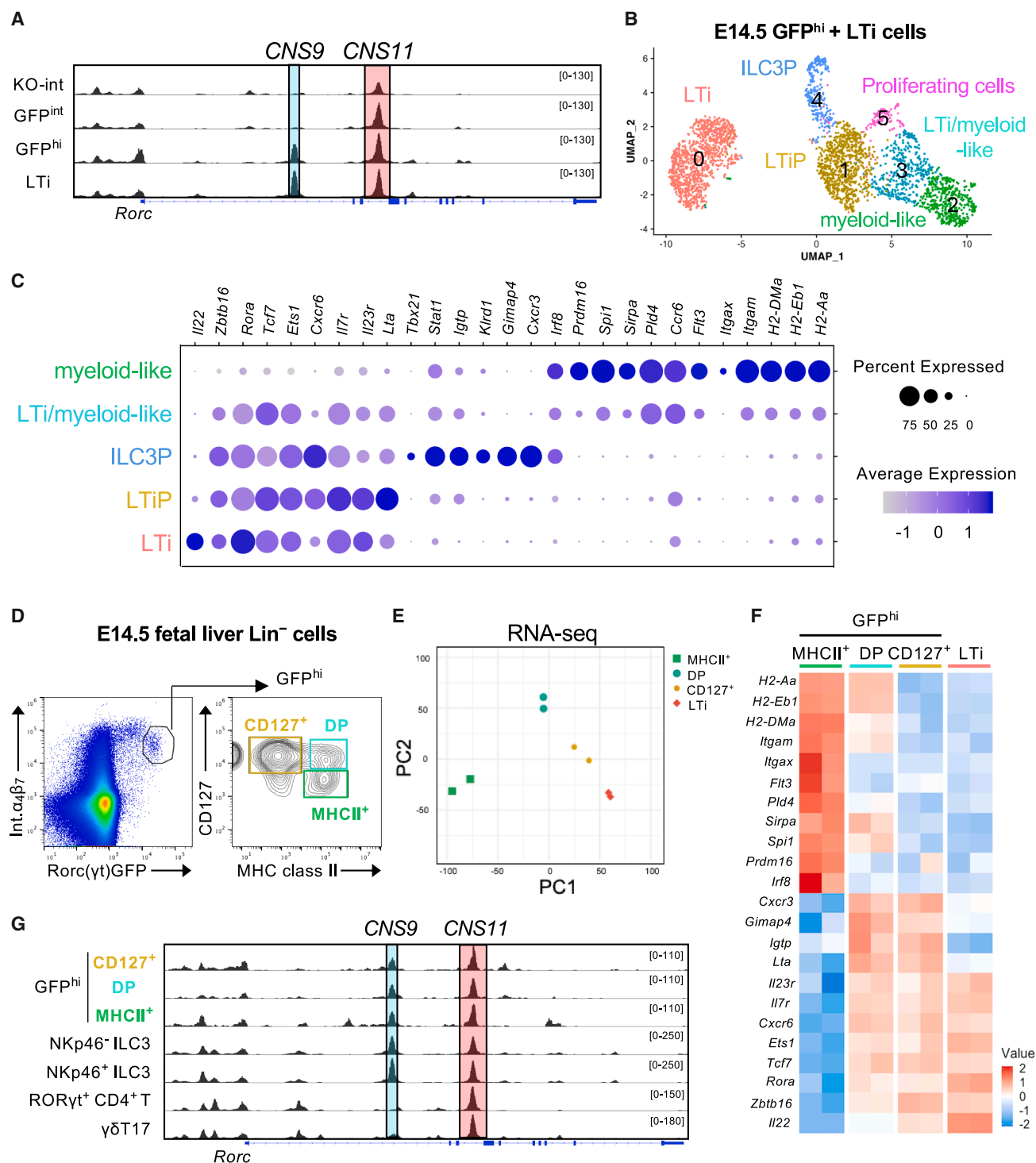


Figure 5. Diversity of RORγt⁺ innate immune cells

(A) ATAC-seq at the *Rorc* locus in fetal LTi and fetal liver (FL) Lin⁻Int.α₄β₇⁺ cells from E14.5 *Rorc*(γt)*Gfp* mice with *Rorc*^{+/-} (GFP^{int}, GFP^{hi}) or *Rorc*^{-/-} (KO-int) backgrounds.

(B and C) Integrated scRNA-seq of E14.5 FL Lin⁻Int.α₄β₇⁺GFP^{hi} cells and fetal LTi cells. Uniform Manifold Approximation and Projection (UMAP) plots: LTi, LTiP, myeloid-like, LTi/myeloid-like, ILC3P, and proliferating cells (B). Dot plot of selected gene expression across five clusters (C).

(D) Flow cytometry of E14.5 FL Lin⁻Int.α₄β₇⁺GFP^{hi} cells.

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findings indicate that the fourth Runx-binding site plays a critical role in establishing *CNS11* chromatin accessibility in precursor cells prior to ROR γ t expression. Furthermore, they suggest that, unlike other ILC3 subsets, LTi-like cells uniquely retain the ability to utilize this residual chromatin accessibility.

Finally, we examined the third Runx-binding site, which is also highly conserved among mammals (Figure 4A). To this end, we generated *Rorc* *CNS11*^{3wt/3wt} mice, in which the first, second, and fourth Runx-binding sites were mutated (Figure S4G). In *Rorc* *CNS11*^{3wt/3wt}; *Rorc*(γ t)*Gfp* mice, LNs and PPs were absent, but ILF formation was partially restored compared with *CNS11*^{mut/mut} mice (Figures 4C, S4H, and S4I). Consistently, the population of T-bet⁺ ILC3s in the ileum was also partially restored (Figures S4J and S4K).

These findings suggest that the third Runx-binding site may contribute to shaping *CNS11* chromatin accessibility, which could partially support LTi-like cell differentiation even in the absence of the other Runx-binding sites.

CNS9 becomes accessible in all innate immune cells in a ROR γ t-dependent manner

We next investigated chromatin regions that gain accessibility during the transition from the GFP^{int} to the GFP^{hi} stage in fetal LTi cell development. ATAC-seq analysis identified a highly conserved intronic region within the *Rorc* locus (Chr3:94,384,238–94,384,700), hereafter referred to as *CNS9*²⁶ (Figure S4A). This region exhibited increased accessibility, coinciding with ROR γ t upregulation during this developmental transition (Figure 5A). *CNS9* remained inaccessible in the ROR γ t-deficient KO-int population, indicating that its accessibility is established in a ROR γ t-dependent manner.

Given the lineage diversity of perinatal ROR γ t⁺ cells—including LTi cells, ILC3s, and ROR γ t⁺ APCs^{11–14}—we hypothesized that the GFP^{hi} population in the fetal liver is heterogeneous, reflecting this diversity. To investigate this, we performed single-cell RNA-seq (scRNA-seq) analysis on E14.5 GFP^{hi} cells from the fetal liver and LTi cells from peripheral tissues. Merged datasets revealed six distinct clusters (Figures 5B, 5C, and S5A). Among these, cluster 1 and cluster 4 were identified as LTi precursors (LTiPs) and ILC3 precursors (ILC3Ps), respectively. Cluster 2 was characterized as ROR γ t⁺ myeloid-like cells based on high expression of antigen presentation genes (e.g., *H2-Aa*, *H2-Eb1*, and *H2-DMa*), DC/myeloid cell-related surface markers (*Itgam* and *Itgax*), and transcription factors (*Spi1* and *Prdm16*¹³). Cluster 3, which had a hybrid character of both cluster 1 and cluster 2, was annotated as LTi/myeloid-like (Figures 5C and S5B).

To validate this heterogeneity, we performed flow cytometry of E14.5 GFP^{hi} cells, identifying three subpopulations based on CD127 and MHC class II (I-A^b) expression (Figure 5D). Bulk RNA-seq analysis of these subpopulations revealed that MHC class II (MHCII)⁺CD127⁺ cells exhibited a distinct gene expression profile, characterized by higher expression of myeloid cell-related genes, such as *Itgam* (Figures 5E and 5F), which corresponds to ROR γ t⁺ myeloid-like cells (cluster 2) in scRNA-seq analysis (Figures 5B and 5C). Given the in-

verse correlation between CD11b and CD127 expression (Figure S5C), CD11b may serve as a useful surface marker for identifying ROR γ t⁺ myeloid-like cells within the GFP^{hi} population in the fetal liver.

ATAC-seq analysis further demonstrated a shared chromatin accessibility pattern at the *Rorc* locus across GFP^{hi} subsets, including ROR γ t⁺ myeloid-like cells and CD127⁺ LTiPs. Accessibility was observed at both *CNS11* and *CNS9* (Figure 5G), suggesting a common *Rorc*(γ t) transcriptional regulatory mechanism in these fetal liver populations.

Because *CNS11*^{mut/mut} mice completely lacked fetal liver ROR γ t⁺ cells, including the CD11b⁺GFP^{hi} population, we reasoned that *CNS11* might also be required for the development of ROR γ t⁺ APC populations. To directly address this, we transferred E14.5 fetal liver cells into irradiated WT mice and subsequently examined the mesenteric lymph nodes (mLNs), where ROR γ t⁺CD11b⁺MHCII⁺ cells—largely composed of ROR γ t⁺ APCs¹³—are enriched. Whereas WT fetal liver cells efficiently reconstituted this population, *CNS11*^{mut/mut} cells failed to do so (Figure S5D). These results indicate that the development of ROR γ t⁺CD11b⁺ MHCII⁺ cells depends on *CNS11*, as does the development of ILC3s and peripheral ROR γ t⁺ CD4⁺ T cells.

Next, to investigate the lineage specificity of *CNS9* accessibility, we performed ATAC-seq analysis on adult small intestinal ILC3—NKp46⁺ ILC3s (T-bet⁺ ILC3s) and NKp46⁺ ILC3s (T-bet⁺ ILC3s)—as well as ROR γ t⁺ T cells. In contrast to *CNS11*, *CNS9* was more accessible in NKp46⁺ ILC3s and NKp46⁺ ILC3s than in ROR γ t⁺CD4⁺ T and γ δ T17 cells (Figure 5G), suggesting that *CNS9* accessibility is specific to LTi, ILC3, and ROR γ t⁺ myeloid-like cells. Thus, *CNS9*-mediated *Rorc*(γ t) transcriptional regulation is a shared feature across all ROR γ t⁺ innate cells beyond the classical ILC3/LTi-lineages.

Rorc CNS9 controls ROR γ t expression in innate lymphoid cells

Within *CNS9*, one RARA/RXRG-binding site and three Runx-binding sites were identified, with the RARA/RXRG-binding site and the second Runx-binding site being conserved between mice and humans (Figures S6A and S6B). Footprinting analysis of fetal LTi cells and adult ILC3s revealed significantly high scores at the RARA/RXRG-binding site and at the last two Runx-binding sites (Figure S6C), suggesting their functional relevance in *Rorc*(γ t) transcriptional regulation.

To elucidate the biological relevance of *Rorc* *CNS9*, we generated three mutant mouse lines: *CNS9* ^{Δ} (Figure S6D), *CNS9*^{*Runx*} (Figure S6E), and *CNS9*^{*RAR*} (Figure S6F). The *CNS9* ^{Δ} allele lacked a 595-bp *CNS9* segment, whereas *CNS9*^{*Runx*} and *CNS9*^{*RAR*} harbor targeted mutations in the second Runx-binding site or the RARA/RXRG-binding site, respectively. In *CNS9* ^{Δ} mice, LN formation remained intact (Figure S6G), indicating that the deleted *CNS9* region is dispensable for LTi cell development and maturation. ILF formation was also preserved, with the normal frequency and number of T-bet⁺ ILC3 in the small

(E and F) RNA-seq analysis of E14.5 FL Lin⁺ Int. α β γ ⁺GFP^{hi} subsets (CD127⁺, DP, and MHCII⁺) and fetal LTi cells ($n = 2$ per group). Principal-component analysis (PCA) (E) and heatmaps showing selected gene expression (F).

(G) ATAC-seq of E14.5 FL subsets, adult ROR γ t⁺ T cells, and ILC3 populations from *Rorc*(γ t)*Gfp* mice. Data are representative of two experiments (A and G) or are pooled from two experiments (E and F).

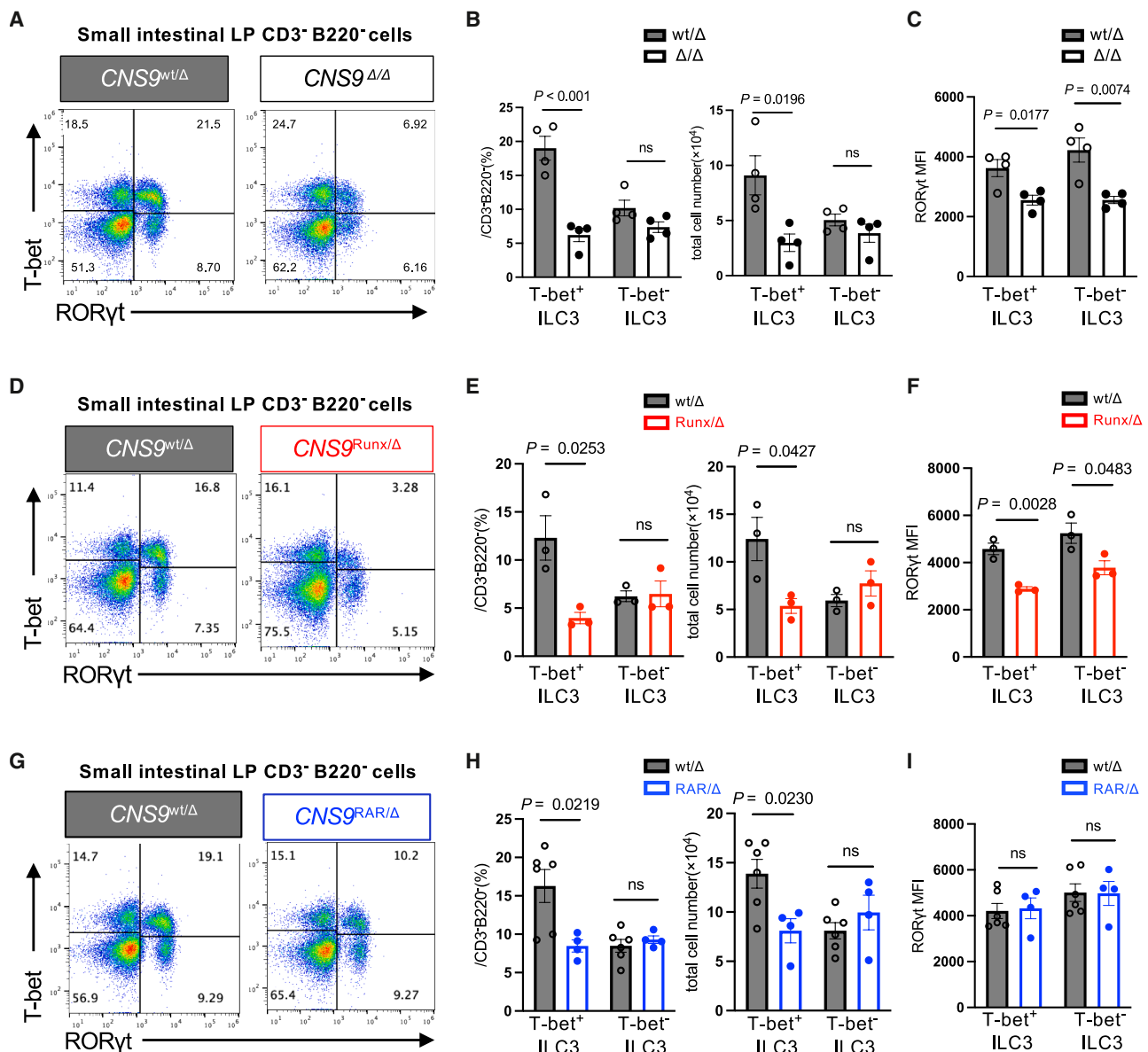


Figure 6. *Rorc* CNS9 controls RORγt expression in innate lymphoid cells

(A–C) Flow cytometry of small intestinal LP CD3⁺ B220⁺ cells from 4-week-old *CNS9*^{Δ/Δ} and control *CNS9*^{wt/Δ} mice (A). Frequencies and numbers of T-bet⁺ ILC3 and T-bet[−] ILC3 among CD3⁺ B220⁺ cells (B). Mean fluorescence intensity (MFI) of intracellular RORγt in T-bet⁺ and T-bet[−] ILC3 subsets (C) (*n* = 4 per group). (D–I) Flow cytometry of small intestinal LP CD3⁺ B220⁺ cells from 4-week-old *CNS9*^{Runx/Δ} and control *CNS9*^{wt/Δ} mice (D–F), *CNS9*^{RAR/Δ}, and control *CNS9*^{wt/Δ} (G–I). Frequencies and numbers of T-bet⁺ and T-bet[−] ILC3 subsets among the CD3⁺ B220⁺ population (E and H). MFI of intracellular RORγt in T-bet⁺ and T-bet[−] ILC3 subsets (*n* = 3 per group) (F and I).

Data are pooled from ≥3 independent experiments (B, C, E, F, H, and I); mean ± SEM. Two-tailed *t* test (ns, *p* > 0.05).

intestine (Figures 6A, 6B, and S6G). In contrast, T-bet⁺ ILC3s were significantly reduced (Figures 6A and 6B), accompanied by decreased RORγt expression across all ILC3 subsets (Figure 6C).

In *CNS9*^{Runx/Δ} and *CNS9*^{RAR/Δ} mice, both the frequency and number of the T-bet⁺ ILC3 population were reduced (Figures 6D, 6E, 6G, and 6H). However, while RORγt expression was diminished in *CNS9*^{Runx/Δ} mice (Figure 6F), it remained unchanged in *CNS9*^{RAR/Δ} mice (Figure 6I), indicating that the Runx-binding site plays a more significant role in

regulating RORγt levels. Further analysis of intestinal immunity revealed that IL-22 production in ILC3s—a key factor in maintaining gut epithelial integrity—was unaffected in *CNS9*^{Δ/Δ} mice (Figures S6H and S6I). Consistent with this, dextran sulfate sodium (DSS)-induced colitis severity did not significantly differ between *CNS9*^{Δ/Δ} mice and controls (Figure S6J).

Collectively, these findings demonstrate that *CNS9* acts as a CRE element under the control of Runx and RARA/RXRγ in ILC3s, fine-tuning RORγt expression and T-bet⁺ ILC3 frequencies.

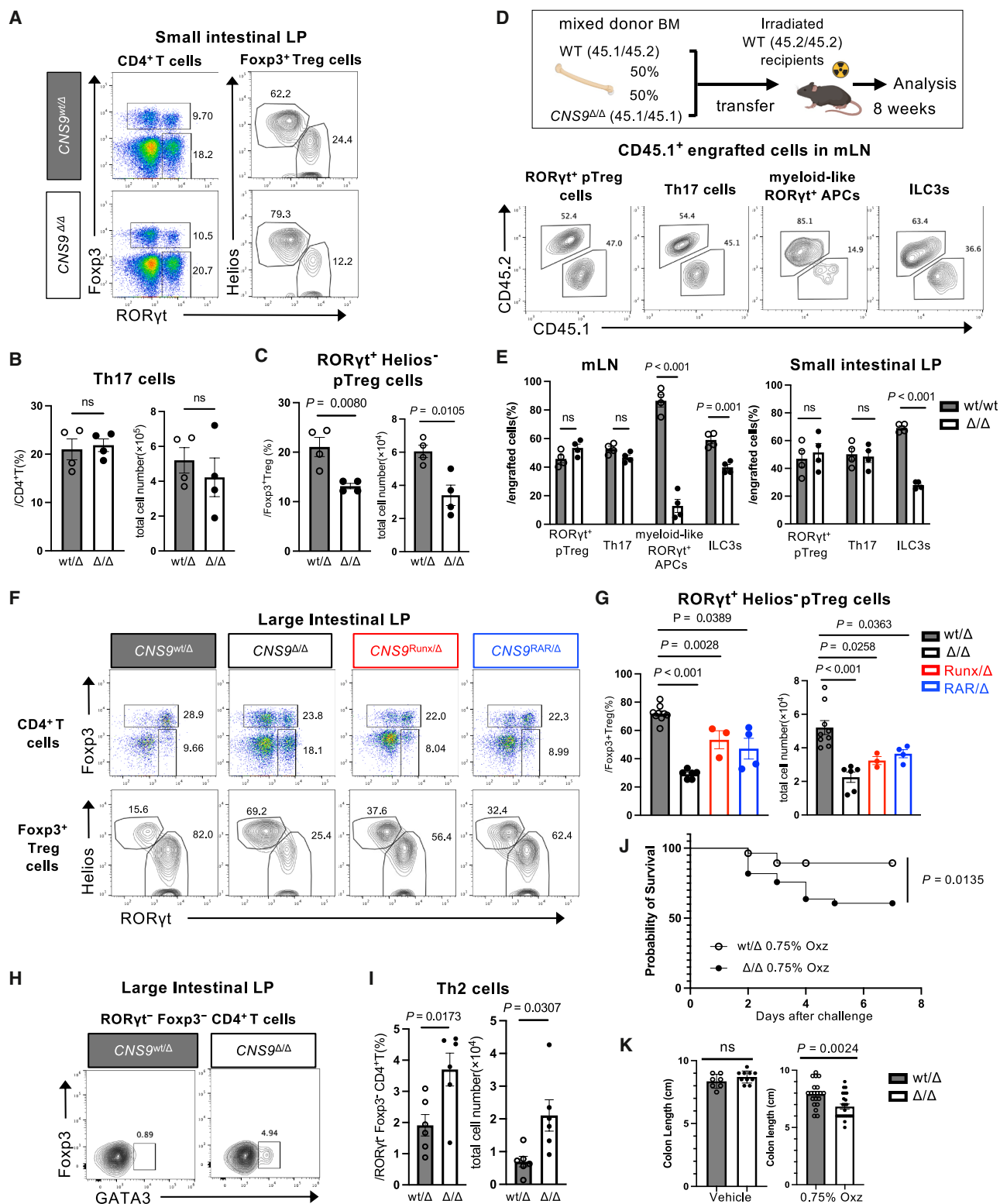


Figure 7. *Rorc* *CNS9* is essential for pTreg cell induction by RORγt⁺ APCs

(A–C) Flow cytometry of small intestinal LP CD4⁺ T cells from 8-week-old *CNS9*^{Δ/Δ} and control *CNS9*^{wt/Δ} mice (A). Frequencies and numbers of RORγt⁺Foxp3⁺ Th17 cells among CD4⁺ T cells (*n* = 4 per group) (B) and RORγt⁺Helios⁻Foxp3⁺ pTreg cells among Foxp3⁺CD4⁺ T cells (*n* = 4 per group) (C). (D and E) Mixed BM transfer experiment. Flow cytometry of RORγt⁺ pTreg cells, Th17 cells, RORγt⁺CD11b⁺MHCII⁺ (myeloid-like RORγt⁺ APCs) and ILC3s in mLN from competitive BM chimeric mice generated by transferring a 1:1 mixture of WT (CD45.1/45.2) and *CNS9*^{Δ/Δ} (CD45.1/45.1) BM cells into WT (CD45.2/CD45.2)

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CNS9 is essential for the induction of ROR γ t⁺ pTreg cells mediated by ROR γ t⁺ APCs

In mice, Th17 cells emerge postnatally in the intestine, depending on colonizing microbes and ROR γ t.^{9,65} Both ROR γ t⁺CD4⁺ T cells and γ δ T17 cells from the small intestinal lamina propria of 8-week-old mice exhibited chromatin inaccessibility at the *CNS9* region (Figure 5G). The frequencies and numbers of both small intestinal Th17 cells (Figures 7A and 7B) and γ δ T17 cells were comparable between *CNS9* Δ/Δ and control mice, although ROR γ t expression in γ δ T17 cells was significantly lower in *CNS9* Δ/Δ mice (Figures S7A–S7C). Although ROR γ t expression levels in ROR γ t⁺Helios[−] pTreg cells—normally induced by commensal bacteria¹⁶—were unaffected, their frequencies and cell numbers were significantly reduced (Figures 7A, 7C, and S7C). These findings raise the possibility that *CNS9* indirectly contributes to the induction of ROR γ t⁺Helios[−] pTreg cells, consistent with its limited accessibility in ROR γ t⁺CD4⁺ T cells.

ROR γ t⁺Helios[−] pTreg cells constitute a major component of colonic Treg cells and regulate both type 2 and 3 immune responses.¹⁶ Microbiota-specific colonic pTreg cells can be directly induced by ROR γ t⁺ APCs.^{13,14,66} In the fetal liver, ROR γ t⁺ myeloid-like cells expressing *Itgam* (encoding CD11b) exhibited an open chromatin state at *CNS9* (Figures 5G and S5C). Based on these observations, we hypothesized that *CNS9* plays a critical role in the development of ROR γ t⁺ APCs.

To test this hypothesis, we examined ROR γ t and CD11b expression in the B220[−]MHCII⁺ population in mLNs of 20- to 25-week-old *CNS9* Δ/Δ mice. Compared with controls, the frequencies, cell numbers, and ROR γ t expression levels of CD11b⁺MHCII⁺ cells were significantly reduced in *CNS9* Δ/Δ mice (Figures S7D–S7F). Furthermore, similar reductions were also observed in *CNS9*^{Runx1 Δ} and *CNS9*^{RAR Δ} mice, suggesting that Runx and RARA/RXRG binding to *CNS9* facilitates *Rorc*(γ t) transcription, thereby supporting the development of ROR γ t⁺CD11b⁺ MHCII⁺ cells.

To determine whether the reduction of ROR γ t⁺ pTreg cells in the small intestine of *CNS9* Δ/Δ mice is cell-intrinsic or secondary to the loss of ROR γ t⁺ APCs, we performed competitive bone marrow (BM) transfer experiments. BM cells were mixed at a 1:1 ratio and transferred into irradiated WT (CD45.2/45.2) recipients (Figure 7D). CD4⁺/CD8⁺ SP and DP thymocytes, as well as splenic CD4⁺/CD8⁺ T cells and B cells, were reconstituted equally from both donor origins (Figure S7G). Similarly, ROR γ t⁺ pTreg cells and Th17 cells were reconstituted at comparable levels regardless of donor origin in mLN and small intestine (Figures 7D and 7E). In contrast, similar to ILC3s, *CNS9* Δ/Δ BM-derived ROR γ t⁺CD11b⁺MHCII⁺ cells in mLN were less competitive than their WT counterparts. Taken together, these results suggest that the reduction of ROR γ t⁺ pTreg cells

observed in *CNS9* Δ/Δ mice is not due to a cell-intrinsic defect but rather represents a secondary effect resulting from the reduced number of ROR γ t⁺ APCs.

We next examined the impact of *CNS9* on colonic ROR γ t⁺ Treg cells. Both the frequency and the numbers of ROR γ t⁺Helios[−] pTreg cells among total Foxp3⁺ Treg cells were significantly reduced in *CNS9* mutant strains (Figures 7F and 7G). These findings indicate that *CNS9* is crucial for the differentiation of ROR γ t⁺ APCs, via Runx and RARA/RXRG-binding sites, and is indispensable for the subsequent induction of ROR γ t⁺ pTreg cells.

The absence of ROR γ t⁺ pTreg cells enhances type 2 immunity and markedly increases susceptibility to 4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone)-induced colitis,¹⁶ a type 2 immunity-mediated model of ulcerative colitis.^{67,68} Consistent with these previous findings, we observed an accumulation of Th2 cells in the colon of *CNS9* Δ/Δ mice (Figures 7H and 7I). In line with this observation, *CNS9* Δ/Δ mice exhibited a significantly exacerbated phenotype in the oxazolone-induced colitis model (Figures 7J and 7K), further highlighting the role of *CNS9* in restraining excessive type 2 immune response in the gut.

Finally, we investigated the potential link between *CNS9* and human immune disorders. A marker SNP, rs11204896, identified in a genome-wide association study (GWAS) analysis of allergic diseases,⁶⁹ is located approximately 4 kb away within the same intron as *CNS9* (Figure S7H). There is no recombination hotspot between *CNS9* and rs11204896, suggesting that these loci may form a haplotype block. This raises the possibility that *CNS9* is a strong candidate for the causative locus underlying allergic disease susceptibility.

Together, these findings demonstrate that *CNS9* is essential for the differentiation of ROR γ t⁺ APCs and the induction of ROR γ t⁺ pTreg cells, ultimately restraining excessive immune responses.

DISCUSSION

The Runx/CBF β complex regulates multiple stages of ILC3 development, from HSCs to mature subsets.^{31,38} To define its stage-specific roles, we disrupted Runx-binding motifs with developmental accessibility. This uncovered an early Runx/CBF β -dependent enhancer-priming event at *CNS11* in HSCs and MPPs. Reduced chromatin accessibility in CBF β -deficient precursors and in *CNS11*-mutant precursors with loss of Runx binding provides direct evidence that Runx/CBF β establishes *Rorc* competence prior to ROR γ t induction. *CNS11* is also primed in several mature ILCs and naive CD4⁺ T cells,⁷⁰ but not in B cells or macrophages, suggesting selective licensing of lymphoid subsets for future *Rorc* activation.^{57,58}

recipients (D). Frequencies of WT and *CNS9* Δ/Δ BM-derived cells within engrafted (CD45.1⁺) populations of indicated immune cells in mLN and small intestine ($n = 4$ per group) (E).

(F and G) Flow cytometry of large intestinal LP CD4⁺ T cells from 20- to 25-week-old *CNS9* Δ/Δ , *CNS9*^{Runx Δ} , *CNS9*^{RAR Δ} , and control *CNS9*^{wt Δ} mice (F). Frequencies and numbers of ROR γ t⁺Helios[−] Foxp3⁺ pTreg cells among Foxp3⁺CD4⁺ T cells (control: $n = 9$, *CNS9* Δ/Δ : $n = 6$, *CNS9*^{Runx Δ} : $n = 3$, *CNS9*^{RAR Δ} : $n = 4$) (G). (H and I) Flow cytometry of large intestinal LP ROR γ t⁺ Foxp3[−]CD4⁺ T cells from 8- to 20-week-old *CNS9* Δ/Δ and control *CNS9*^{wt Δ} mice (H). Frequencies and numbers of GATA3⁺ Th2 cells among ROR γ t⁺ Foxp3[−]CD4⁺ T cells ($n = 6$ per group) (I).

(J and K) 0.75% oxazolone (Ox) challenge in 25-week-old *CNS9* Δ/Δ and control *CNS9*^{wt Δ} mice. Kaplan-Meier curve (control: $n = 28$, *CNS9* Δ/Δ : $n = 33$) (J). Colon length 4 days post-challenge in vehicle (control: $n = 7$, *CNS9* Δ/Δ : $n = 10$) and Oxz groups (control: $n = 20$, *CNS9* Δ/Δ : $n = 23$) (K).

Data are pooled from ≥ 3 independent experiments (B, C, E, G, I, and K); mean $\pm$ SEM. Two-tailed t test (ns, $p > 0.05$) or log-rank test (J).

CNS11 function is evolutionarily conserved. In *CNS11*^{4th/4th} mice, disruption of a highly conserved 6-bp Runx-binding site in *CNS11* leads to a near-complete loss of SLOs, except ILFs, implying that this motif enabled the emergence of placental mammal-specific LT_i cells.⁷¹ LT_i-like cells remain intact in *CNS11*^{4th/4th} mice, indicating lineage divergence from fetal LT_i cells and suggesting that LT_i-like cells may represent a more ancestral lymphoid inducer population, consistent with reports of primitive ILCs and GALT in bony fish.^{72,73} *CNS11* also differentially regulates ILC3 subsets. T-bet⁺ ILC3s are lost in *CNS11*^{4th/4th} mice, whereas LT_i-like cells persist. Given that T-bet suppresses RORα,⁵⁷ we propose that T-bet⁺ ILC3s are less able to utilize the limited accessibility of *CNS11*, whereas T-bet[−] LT_i-like cells can rely on RORα-driven activation.

RORγt⁺ APCs also depend on the *CNS11*-*CNS9* hierarchical cascade. We identified fetal RORγt⁺ myeloid-like cells in the E14.5 fetal liver that share regulatory logic and transcriptional profiles with RORγt⁺ APC subsets, whose heterogeneity and developmental pathway are still under debate.^{11–14,66} These cells are absent in *CNS11*^{mut/mut} mice and fail to generate peripheral RORγt⁺CD11b⁺MHC class II⁺ cells upon transfer, indicating that Runx/CBFβ-mediated *CNS11* priming is required for their lineage specification. After RORγt induction, *CNS9*-dependent reinforcement further fine-tunes *Rorc* expression.

Although Th17-related genes are associated with inflammatory bowel disease (IBD) risk,²⁵ *CNS9* deletion did not worsen colitis, whereas this deletion impaired pTreg cell-mediated regulation of type 2 inflammation. The allergic disease-associated SNPs⁶⁹ and *CNS9* form a haplotype block, highlighting a potential role of *CNS9* dysfunction in human allergic susceptibility.

In summary, we define a hierarchical *cis*-regulatory program in which early Runx/CBFβ-dependent *CNS11* priming enables *Rorc* induction, followed by *CNS9*-mediated reinforcement to control differentiation of all RORγt⁺ innate immune cells. This conserved network supports fetal lymphoid organogenesis, adult type 3 immunity, and suppression of type 2 inflammation.

Limitations of the study

Although our study identifies the Runx/CBFβ complex as a central regulator of *Rorc* transcription in RORγt⁺ innate immune cells, the potential involvement of additional transcriptional cofactors remains unresolved. Assessing such cofactors across developmental stages will require assays capable of accurately measuring transcription factor binding in rare precursor populations. Moreover, although *CNS9* and *CNS11* are conserved between mouse and human, determining whether they exert similar functions in human tissues remains challenging due to ethical and technical constraints.

RESOURCE AVAILABILITY

Lead contact

Further information and request for resources and reagents should be directed to the lead contact, Shinichiro Sawa (sawa.shinichiro.353@m.kyushu-u.ac.jp).

Materials availability

All materials generated in this study are available from the lead contact upon request.

Data and code availability

The sequencing data generated in this study have been deposited in the Gene Expression Omnibus (GEO) under accession numbers GEO: GSE278157 and GSE311595 (ATAC-seq), GSE278161 (scRNA-seq), GSE278516 (Bulk RNA-seq_ChIP), GSE311596 (ChIP-seq), GSE311597 (ChIP-seq), and GSE278518 (Bulk RNA-seq_in vitro) and will be publicly available upon publication.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.F., M.W., S.K., and S.S.; methodology, T.F., M.W., R.K., K.T., S.K., M.S., and S.S.; investigation, T.F., M.W., R.K., K.T., S.K., M.S., and S.S.; formal analysis, T.F., M.W., E.S., S.K., A.H., R.S., M.S., and S.S.; resources, I.T.; data curation, T.F., M.W., E.S., and S.S.; writing – original draft, T.F. and S.S.; writing – review & editing, T.F., M.W., E.S., S.K., Y.O., T.Y., and I.T.; visualization, T.F. and M.W.; supervision, S.S.; funding acquisition, T.F., S.S., A.H., and E.S. Available upon publication.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Biotin Anti-mouse TER-119 (clone TER-119)	BioLegend	Cat# 116204; RRID: AB_313705
Biotin Anti-mouse CD3 ϵ (clone eBio500A2)	Thermo Fisher Scientific	Cat# 50-113-78; RRID: AB_842775PE
Biotin Anti-mouse ST2 (clone DIH9)	BioLegend	Cat# 145308; RRID: AB_2565569
Biotin Anti-mouse NKp46	R&D System	Cat# BAF2225; RRID: AB_416729
Biotin Anti-mouse/human B220 (RA3-6B2)	BioLegend	Cat# 103204; RRID: AB_312989
Biotin Anti-mouse CD8 α (clone 53-6.7)	BioLegend	Cat# 100704; RRID: AB_312743
Biotin Anti-mouse CD11c (clone N418)	BioLegend	Cat# 117304; RRID: AB_313773
Biotin Anti-mouse/human CD11b (clone M1/70)	BioLegend	Cat# 101204; RRID: AB_312787
Biotin Anti-mouse CD4 (clone RM4-5)	BioLegend	Cat# 100508; RRID: AB_312711
Biotin Anti-mouse TCRgamma/delta (clone eBioGL3)	Thermo Fisher Scientific	Cat# 13-5711-82; RRID: AB_466668
Biotin Anti-mouse TCR beta (clone H57-597)	Thermo Fisher Scientific	Cat# 13-5961-85; RRID: AB_466820
Biotin Anti-mouse NK1.1 (clone PK136)	Tonbo Bioscience	Cat# 30-5941; RRID: AB_2621653
Biotin Anti-mouse Ly6G (clone RB6-8C5)	Tonbo Bioscience	Cat# 30-5931; RRID: AB_2621652
FITC Anti-mouse CD3 (clone 17A2)	BioLegend	Cat# 100204; RRID: AB_312661
FITC Anti-mouse CD25 (clone PC61.5)	Tonbo Bioscience	Cat# 35-0251; RRID: AB_2621685
AF488 Anti-mouse/human PLZF (clone Mags.21F7)	Thermo Fisher Scientific	Cat# 53-9320-80; RRID: AB_2574444
PE Anti-mouse CD45.1 (clone A20)	Invitrogen	Cat# 12-0453-83; RRID: AB_465676
PE Anti-I-A/I-E (clone M5/114.15.2)	Thermo Fisher Scientific	Cat# 12-5321-82; RRID: AB_465928
PE Anti-mouse NKp46 (clone 29A1.4)	BioLegend	Cat# 137604; RRID: AB_2235755
PE Anti-mouse CD117 (clone 2B8)	BioLegend	Cat# 105808; RRID: AB_313217
PE Rat IgG1 k Isotype Control (clone RTK2071)	BioLegend	Cat# 400408; RRID: AB_326514
PE Anti-mouse ROR γ t (clone Q31-378)	BD	Cat# 562607; RRID: AB_11153137
PE Anti-IL-22 (clone IH8PWSR)	Thermo Fisher Scientific	Cat# 12-7221-82; RRID: AB_10597428
PE Anti-GATA3(clone L50-823)	BD	Cat# 560074; RRID: AB_1645330
PerCP-Cy5.5 Anti-mouse CD45.2 (clone 104)	BioLegend	Cat# 109828; RRID: AB_893350
PerCP-Cy5.5 Anti-mouse TCR β (clone H57-597)	BioLegend	Cat# 109228; RRID: AB_1575173
PerCP-Cy5.5 Anti-mouse CD8 α (clone 53-6.7)	Tonbo Bioscience	Cat# 65-0081; RRID: AB_2621882
PerCP-eFluor710 Anti-mouse CD135 (clone A2F10)	Thermo Fisher Scientific	Cat# 46-1351-82; RRID: AB_10733393
PE-Cy7 Anti-mouse CD127 (clone A7R34)	BioLegend	Cat# 135014; RRID: AB_1937265
PE/Cy7 Anti-mouse CD11c (clone N418)	BioLegend	Cat# 117318; RRID: AB_493568
PE/Cy7 Anti-mouse/human CD11b (clone M1/70)	BioLegend	Cat#101216; RRID: AB_312799
PE/Cy7 Anti-mouse CD45.2 (clone 104)	BioLegend	Cat#109830; RRID: AB_1186098
PE Anti-mouse/human CCR6 (clone140706)	R&D systems	Cat# FAB590P100; RRID: AB_2244251

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Pacific Blue Anti-mouse/human Helios (clone 22F6)	BioLegend	Cat# 137220; RRID: AB_10690535
BV421 Anti-mouse Integrin $\alpha_4\beta_7$ (clone DATK32)	BD	Cat# 747758; RRID: AB_2872226
BV421 Anti-mouse CD117 (clone 2B8)	BD	Cat# 562609; RRID: AB_11154585
BV421 Anti-mouse ROR γ t (clone Q31-378)	BD	Cat# 562894; RRID: AB_2687545
VF450 Anti-mouse KLRG1 (clone 2F1)	Tonbo Bioscience	Cat# 75-5893; RRID: AB_2621966
VF450 Anti-mouse CD11c (clone N418)	Tonbo Bioscience	Cat# 75-0114; RRID: AB_2621937
BV570 Anti-mouse CD4 (clone RM4-5)	BioLegend	Cat# 100542; RRID: AB_2563051
BV711 Anti-mouse I-A/I-E (clone M5/114.15.2)	BioLegend	Cat# 107643; RRID: AB_2565976
BV711 Anti-mouse CD3 ϵ (clone 145-2C11)	BioLegend	Cat# 100349; RRID: AB_2565841
BV711 Anti-mouse CD45.1 (clone A20)	BioLegend	Cat# 110739; RRID: AB_2562605
BV786 Anti-mouse CD19 (clone 1D3)	BD	Cat# 563333; RRID: AB_2738141
APC Anti-mouse CD3 (clone 17A2)	BioLegend	Cat#100236; RRID: AB_2561456
APC Anti-mouse NK1.1(clone PK136)	BioLegend	Cat#108710; RRID: AB_313397
APC Anti-mouse IL-17A (clone TC11-18H10.1)	BioLegend	Cat# 506916; RRID: AB_536018
APC Anti-mouse T-bet (clone 4B10)	BioLegend	Cat# 644814; RRID: AB_10901173
APC Anti-mouse/human CD11b (clone M1/70)	BioLegend	Cat# 101212; RRID: AB_312795
APC Anti-Foxp3 (clone FJK-16s)	Thermo Fisher Scientific	Cat# 17-5773-82; RRID: AB_469457
APC Rat IgG1 k Isotype Control (clone eBRG1)	Thermo Fisher Scientific	Cat# 17-4301-81; RRID: AB_470177
APC Anti-mouse Ly-6A/E (clone D7)	Sony Biotechnology	Cat# 1140560; RRID: AB_1727552
AF647 Anti-TCF7 (clone C63D9)	Cell Signaling Technology	Cat# 6709; RRID: AB_2797631
AF700 Anti-mouse I-A/I-E (clone M5/114.15.2)	BioLegend	Cat# 107622; RRID: AB_493727
Normal Rabbit IgG	Cell Signaling	Cat# 2729; RRID: AB_1031062
Purified Rat IgG2a, k Isotype Ctrl Antibody	BioLegend	Cat# 400501; RRID: AB_326523
ROR alpha antibody	GeneTex	Cat# GTX79267; RRID: AB_11168482
ROR gamma(t) antibody	Invitrogen	Cat# 14-6988-82; RRID: AB_1834475
Pan-Cb β antibody	Tenno et al. ³⁶	N/A
Purified anti-mouse B220 (RA3-6B2)	BioLegend	Cat# 103202; RRID: AB_312986
Purified anti-mouse CD11c (N418)	BioLegend	Cat# 117302; RRID: AB_313770
Purified Chicken anti-GFP	Invitrogen	Car# A10262; RRID: AB_2534023
Alexa 647 Donkey anti-Rat	Invitrogen	Cat# A78947; RRID: AB_2910635
PE Goat anti-Armenian hamster	Invitrogen	Cat# 12-4112-83; RRID: AB_465915
Alexa 488 Goat anti-chicken	Invitrogen	Cat# A11039; RRID: AB_2534096

Chemicals, peptides, and recombinant proteins

RPMI 1640 with L-Gln	Nacalai Tesque	Cat#:0264-56
DMEM (1.0g/L Glucose) with L-Gln and Sodium Pyruvate	Nacalai Tesque	Cat#: 08456-36
DMEM-high glucose	Sigma-Aldrich	Cat#: D5796
MEM Non-Essential Amino Acids Solution (100X)	Thermo Fisher Scientific	Cat#:11140-050
Sodium Pyruvate (100 mM)	Thermo Fisher Scientific	Cat#:11360-070
L-Glutamine	Sigma-Aldrich	Cat#: G7513
EmbryoMax® 2-Mercaptoethanol (100X)	Sigma-Aldrich	Cat#: ES-007-E

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
EmbryoMax® 1M HEPES Buffer Solution, Liquid, 1M	Sigma-Aldrich	Cat#: TMS-003-C
Penicillin-streptomycin	Thermo Fisher Scientific	Cat#:15140122
0.5mol/l-EDTA Solution (pH 8.0)	Nacalai Tesque	Cat# 06894-14; CAS: 6381-92-6
cOmplete protease inhibitor cocktail tablet	Roche	Cat#: 04693116001
Opti-MEM™Reduced Serum Medium, powder	Thermo Fisher Scientific	Cat#: 22600050
Collagenase D	SIGMA	Cat#11088882001
DNaseI	SIGMA	Cat#25D-10G; CAS# 9003-98-9
Percoll™ system	Cyvita	17089109
Fetal bovine serum (FBS)	Cyvita	SH30910.03
Alt-R® Recombinant Cas9 V3	iDT	Cat# 1081060
Alt-R® CRISPR-Cas9 tracrRNA	iDT	Cat# 1072534
DAPI	DOJINDO	Cat# D523
Fixable Viability Dye eFluor 780	Thermo Fisher Scientific	Cat# 65-0865-14
Fixable Viability Dye eFluor 506	Thermo Fisher Scientific	Cat# 65-0866-14
BV605 Streptavidin	BioLegend	Cat# 405229
BV650 Streptavidin	BioLegend	Cat# 405232
Fluoromount-G solution.	SouthernBiotech	Cat# 0100-35
G-mount	GenoStaff	Cat# GM-01
Recombinant Mouse IL-23	R&D System	Cat# 1887-ML-010/CF
Recombinant Mouse IL-15	Pepro Tech	Cat# 210-15-10
Recombinant Mouse IL-2	R&D System	Cat# 402-ML-020/CF
Recombinant Mouse IL-7	R&D System	Cat# 407-ML-005/CF
Recombinant Mouse IL-7	Pepro Tech	Cat# 217-17-10UG
Recombinant Mouse SCF	Pepro Tech	Cat# 250-03-10UG
Brefeldin A Solution	Thermo Fisher Scientific	Cat# 00-4506-51
4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (Oxazolone)	SIGMA	Cat# 862207-1G-A; CAS:15646-46-5
Dextran Sulfate Sodium (DSS) salt molecular weight, 36.000–50.000	MP Biomedicals	Cat# MFCD00081551; CAS:9011-18-1
Isoflurane	RIKEN	IsofluraneRIKEN; CAS: 26675-46-7
All-trans retinoic acid	Sigma-Aldrich	Cat# R2625; CAS: 302-79-4
Formaldehyde Solution	Nacalai Tesque	Cat# 16223-55; CAS: 50-00-0
Acetate	SIGMA	Cat# 179124; CAS: 67-64-1
Corn oil	SIGMA	Cat# 47112; CAS: 47112
Critical commercial assays		
Foxp3/Transcriptional Factor Staining Buffer Set	Thermo Fisher Scientific	Cat#00-5523-00
ATAC-Seq Kit	Active Motif	Cat#53150
Chromatin Immunoprecipitation(ChIP) Assay Kit	Sigma-Aldrich	Cat#17-295
NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs)	NEW ENGLAND Biolabs	Cat#E6440
FuGENE® 6 Transfection Reagent	Promega	Cat# E2691
Dual-Glo® Luciferase Assay System	Promega	Cat# E2920
SMART-Seq® HT PLUS Kit	Takara Bio	Cat#R400749
SMART-Seq v4 Ultra Low Input RNA Kit	Takara Bio	Cat#634889
AMPure XP beads	Beckman Coulter	Cat#A63880

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chromium Next GEM single cell 3' library & Gel beads kit v3.1	10x Genomics	Cat#1000128
Chromium Next GEM single cell G chip kit	10x Genomics	Cat#1000127
Chromium i7 multiplex kit	10x Genomics	Cat#120262
High Sensitivity DNA kit	Agilent	Cat#5067-4626
Qubit 1X dsDNA HS Assay Kit	Thermo Fisher Scientific	Cat#Q33230

Deposited data

ATAC-seq	Kearly et al. ⁵⁹ ; Weizman et al. ⁵⁴ ; Ito et al. ⁵⁵ ; Gao et al. ⁵⁶ ; and Habash et al. ⁶⁰	GSM8430804; GSM2785745; GSE270373; GSM7036250; GSM8493112
ChIP-seq	Liu et al. ⁶² and Wang et al. ⁶⁴	GSM7656352 and GSM4979627
CUT&Tag	Itokawa et al. ⁶¹	GSM5723333
CUT&RUN	Mahajan et al. ⁶³	GSM4551384
ATAC-seq for fetal liver cells, ILC3s	This study	GSE278157
Single-cell RNA-seq	This study	GSE278161
Bulk RNA-seq_ChIP	This study	GSE278516
Bulk RNA-seq_vitro	This study	GSE278518
ChIP-seq (CBF β) for E12.5 fetal liver cells	This study	GSE311597
ChIP-seq (CBF β) for MNK3 cells	This study	GSE311596
ATAC-seq for Vav1Cre Cbfb ^{flox/flox}	This study	GSE311595
ATAC-seq for CNS11 mutants	This study	GSE311595

Experimental models: Cell lines

Tst-4-DLL1 stroma cell	Watanabe et al. ⁷⁴	N/A
MNK-3 Mouse Innate Lymphoid Cell Line	Sigma-Aldrich	SCC628
HEK293T cells	Abcam	ab255449

Experimental models: Organisms/strains

Mouse: C57BL/6J	The Jackson Laboratory Japan	Strain#000664
Mouse: C57BL/6J SJL- <i>Ptprca</i> ^a <i>Pepc</i> ^b /BoyJ	The Jackson Laboratory	Strain#002014
Mouse: BDF1 (C57BL/6N x DBA/2)	Japan SLC	N/A
Mouse: <i>Runx3</i> ^{tdTomato}	Kojo et al. ³²	N/A
Mouse: <i>Cbfb</i> ^{flox}	Naoue et al. ⁵²	N/A
Mouse: <i>Il7r(Cd127)</i> ^{Cre}	Schlenner et al. ⁵¹	N/A
Mouse: <i>Rorc</i> (γ t) <i>Gfp</i>	Lochner et al. ¹⁵	N/A
Mouse: <i>Vav1</i> ^{Cre}	Ogilvy et al. ⁷⁵	Jackson Laboratory Strain#000664
Mouse: <i>Rag1</i> ^{-/-}	Mombaerts et al. ⁷⁶	Jackson Laboratory Strain#002216
Mouse: <i>Rorc</i> ^{-/-}	This study	N/A
Mouse: <i>Rorc</i> CNS9 ^A	This study	N/A
Mouse: <i>Rorc</i> CNS9 ^{R_{unx}}	This study	N/A
Mouse: <i>Rorc</i> CNS9 ^{R_{AR}}	This study	N/A
Mouse: <i>Rorc</i> CNS11 ^{mut}	This study	N/A
Mouse: <i>Rorc</i> CNS11 ^{4th}	This study	N/A
Mouse: <i>Rorc</i> CNS11 ^{3wt}	This study	N/A
Mouse: <i>Lta</i> ^{GFP}	This study	N/A

Oligonucleotides

crRNA-1 for <i>Rorc</i> ^{-/-} ; 5'-ACUUCUCAUGACUGAGAACU-3'	This study (iDT)	N/A
crRNA-2 for <i>Rorc</i> ^{-/-} ; 5'-AGUCAUGAGAAGUAAGUGAA-3'	This study (iDT)	N/A
crRNA for <i>Lta</i> ^{EGFP} ; 5'-CUUUAGAAUCUACAGUGCAA-3'	This study (iDT)	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
crRNA-1 for <i>Rorc</i> <i>CNS11</i> ^{mut} , 5'-AGAAGGTGGGGGAUCGAGUG-3'	This study (iDT)	N/A
crRNA-2 for <i>Rorc</i> <i>CNS11</i> ^{mut} , 5'-ACACAGGUUCCUGUGGUC-3'	This study (iDT)	N/A
crRNA for <i>Rorc</i> <i>CNS11</i> ^{4th} , 5'-ACACAGGUUCCUGUGGUC-3'	This study (iDT)	N/A
crRNA for <i>Rorc</i> <i>CNS11</i> ^{3wt} , 5'-UACUCCAACACGCCCAACCC-3'	This study (iDT)	N/A
crRNA-1 for <i>Rorc</i> <i>CNS9</i> ^Δ , 5'- CCAUGCCAAUCCAAAGCAG-3'	This study (iDT)	N/A
crRNA-2 for <i>Rorc</i> <i>CNS9</i> ^Δ , 5'-UUCACCGUCAUUUGCUGCCA-3'	This study (iDT)	N/A
crRNA for <i>Rorc</i> <i>CNS9</i> ^{Runx} , 5'-GGAAGAGGUAGGGAGGCCUG-3'	This study (iDT)	N/A
crRNA for <i>Rorc</i> <i>CNS9</i> ^{RAR} , 5'-ACCAGCAAUGGGUCAGGUAU-3'	This study (iDT)	N/A
ssODN for <i>Rorc</i> ^{-/-}	This study (Eurofingenomics)	N/A
ssODN for <i>Rorc</i> <i>CNS9</i> ^Δ	This study (Eurofingenomics)	N/A
ssODN for <i>Rorc</i> <i>CNS9</i> ^{Runx}	This study (Eurofingenomics)	N/A
ssODN for <i>Rorc</i> <i>CNS9</i> ^{RAR}	This study (Eurofingenomics)	N/A
ssODN for <i>Rorc</i> <i>CNS11</i> ^{4th}	This study (Eurofingenomics)	N/A
ssODN for <i>Rorc</i> <i>CNS11</i> ^{3wt}	This study (Eurofingenomics)	N/A
<i>CNS11</i> -F for ChIP-qPCR	5'-AGCGAAAGGAAACATGCAG -3'	N/A
<i>CNS11</i> -R for ChIP-qPCR	5'-AAGAGAAGGGCTGCGAACAC -3'	N/A
Negative control region-F for ChIP-qPCR	5'-CCCAGGCAAACCTCAAAGTAC -3'	N/A
Negative control region-R for ChIP-qPCR	5'-TGAGTCCCTTACGCCTCTTG -3'	N/A
Recombinant DNA/Plasmid		
Donor plasmid for <i>Rorc</i> <i>CNS11</i> ^{mut} in pEX-A2J2 vector	This study (Eurofingenomics)	N/A
Donor plasmid for <i>Lta</i> ^{GFP} in pcDNA3.1(-) vector <i>pLta/p2a-Egfp-SV40polyA</i>	This study (Eurofingenomics)	N/A
MSCV-tTA-IRES-GFP vector	Addgene	#18783
pEX-A2J2 vector	Eurofingenomics	N/A
Plasmid containing <i>Rora</i> cDNA in pcDNA3.1(+) vector	This study	N/A
Plasmid containing <i>Rorc</i> (<i>γt</i>) cDNA in pcDNA3.1(+) vector	This study	N/A
Plasmid containing <i>Runx1</i> cDNA in pcDNA3.1(+) vector	This study	N/A
Plasmid containing <i>Runx3</i> cDNA in pcDNA3.1(+) vector	This study	N/A
Plasmid containing wild-type <i>Rorc</i> <i>CNS11</i> sequence (Chr3:94387969–94388515) in pGL4.27 vector	This study	N/A
Plasmid containing <i>Rorc</i> <i>CNS11</i> ^{mut} sequence in pGL4.27 vector	This study	N/A
pGL4.27 vector	Promega	Cat# E845A; Accession# DQ904458.1
pcDNA3.1(-) vector	Thermo Fisher Scientific	V79520
pcDNA3.1(+) vector	Thermo Fisher Scientific	V79020
Software and algorithms		
Zen Black edition	Zeiss	https://www.zeiss.co.jp/microscopy/products/microscope-software/zen.html

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
GraphPad Prism 9	GraphPad	https://www.graphpad.com
FlowJo software	BD	https://www.flowjo.com
Seurat 4.0	Hao et al. ⁷⁷	https://satijalab.org/seurat/
R 4.2.1	N/A	https://www.r-project.org/

EXPERIMENTAL MODEL AND STUDY PARTICIPANTS DETAILS

Mice

Runx3^{tdTomato} mice,³² *Cbfb*^{fllox} mice,⁵² *Il7r(Cd127)*^{Cre} mice,⁵¹ *Vav1*^{Cre} mice,⁷⁵ *Rag1*^{-/-} mice,⁷⁶ and *Rorc(γt)Gfp* mice¹⁵ have been described. B6.SJL-*Ptprca*^a*Pepcb*^b/BoyJ been described. B6.SJL- participants details]rl Inderom the Jackson Laboratory. C57BL6/J mice were obtained from CLEA Japan. *Rorc*^{-/-} mice, *LTA*^{EGFP} mice, *Rorc* *CNS9*^Δ mice, *Rorc* *CNS9*^{R^{unx}} mice, *Rorc* *CNS9*^{RAR} mice, *Rorc* *CNS11*^{mut} mice, *Rorc* *CNS11*^{4th} mice and *Rorc* *CNS11*^{3wt} mice were generated by CRISPR/Cas9-mediated genome editing as described in method details. All mice were bred and maintained under specific pathogen-free conditions. All animal experiments were approved by the Presidents of NCGM and Kyushu University following consideration by the Institutional Animal Care and Use Committee of NCGM (approval ID: no.2024-A025) and Kyushu University (approval ID: A23-440, A23-442, A23-427, A25-215, A25-399, and A25-400), respectively, and were carried out in accordance with institutional procedures, national guidelines, and the relevant national laws on the protection of animals. All experiments used age- and sex-matched controls of both sexes. Mice ages ranged from E12.5 to 20 weeks.

METHOD DETAILS

DSS-induced colitis

Dextran Sulfate Sodium (DSS) salt (molecular weight, 36,000–50,000; MP Biomedicals) was dissolved in the drinking water at a concentration of 3% (wt/vol). Male mice at 3 weeks of age were exposed to DSS for 5 days, followed by a recovery period of 6 days with normal drinking water. The disease progression was monitored by measurement of the body weight.

4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (Oxazolone)-Induced Colitis

Sensitization Solution: Oxazolone (SIGMA #862207-1G-A) was dissolved in a sensitization buffer (acetate and corn oil, both from SIGMA, in a 4:1 v/v ratio) to prepare a 3% (wt/vol) oxazolone sensitization solution. The solution was mixed thoroughly by gentle vortexing. Challenging Solution: Oxazolone was dissolved in 50% ethanol to prepare a 0.75% (wt/vol) oxazolone challenging solution, which was mixed thoroughly by gentle vortexing. Sensitization: On day 0, a 2 cm × 2 cm area on the mouse's back was shaved using an electronic clipper. Then, 150 μl of the sensitization solution was applied to the shaved skin. Challenge: On day 6, sensitized animals were anesthetized using an isoflurane inhalation anesthetic system (RIKEN, Muromachi). A 3.5F catheter was inserted into the murine colon (approximately 3–4 cm proximal to the anus), and 100 μl of the challenging solution was slowly administered over 10–30 seconds. Disease Monitoring and Analysis: Disease progression was monitored by measuring body weight. For analysis, mice were sacrificed on day 10 (four days after the challenge).

Bone marrow and fetal liver cell transplantation

For bone marrow (BM) transplantation, BM cells were harvested from the tibias of 5–6-week-old CD45.1⁺/CD45.1⁺ *Rorc* *CNS9*^{Δ/Δ} and CD45.1⁺/CD45.2⁺ WT mice. *Rorc* *CNS9*^{Δ/Δ} and WT BM cells were mixed at a 1:1 ratio. For host preparation, sex-matched 6-week-old C57BL/6J mice (CD45.2⁺/CD45.2⁺) were lethally irradiated with 9.5 Gy using a Gammacell40@ Exactor (Best Theratronics). One day after irradiation, host mice were anesthetized with Isoflurane and received an intravenous injection of 5 × 10⁶ BM cells in 100 μL saline via the orbital vein. Two months after BM transplantation, mice were sacrificed for analysis. For fetal liver transplantation, fetal liver cells were harvested from E14.5 CD45.1⁺/CD45.1⁺ *Rorc* *CNS11*^{mut/mut} and CD45.1⁺/CD45.1⁺ WT mice. For host preparation, 6-week-old male C57BL/6J mice (CD45.2⁺/CD45.2⁺) were lethally irradiated with 9.5 Gy. One day after irradiation, host mice were anesthetized with Isoflurane and received an intravenous injection of 5 × 10⁵ fetal liver cells in 100 μL saline via the orbital vein. Two weeks after fetal liver transplantation, mice were sacrificed for analysis.

Adoptive CD4⁺ T cell transfer

For adoptive CD4⁺ T cell transfer, CCR6⁺CD4⁺ T cells were FACS-sorted from 3-week-old *Rorc* *CNS11*^{mut/mut} and WT mice. A portion of sorted cells was subjected to intracellular staining for RORγt and Foxp3 to confirm the absence of contaminating RORγt⁺Foxp3⁺ CD4⁺ T cells. Subsequently, *Rag1*^{-/-} host mice were anesthetized with Isoflurane and received an intravenous injection of 1 × 10⁵ CCR6⁺CD4⁺ T cells suspended in 100 μL saline via the orbital vein. Two weeks after transfer, recipient mice were sacrificed for analysis.

Generation of knock-in mice by CRISPR/Cas9-mediated genome editing

Construction of knock-in donor plasmids

For *Rorc* *CNS11*^{mut/mut} mice, the donor plasmid sequence was cloned into pEX-A2J2 vector (Eurofingenomics, Tokyo, Japan). The 558bp 5'-arm (from 583 upstream of the 1st Runx-binding site in *Rorc* *CNS11*) and 522bp 3'-arm (from the start codon to 580 bp downstream of the 4th Runx-binding site in *Rorc* *CNS11*) were cloned into the vector to complete knock-in targeting plasmids. For *Lta*^{EGFP} mice, the donor plasmids *pLta/p2a-Egfp-SV40polyA* were constructed to include the P2A, EGFP, and SV40 polyA sequences. The *Egfp-SV40polyA* sequence was cloned from the MSCV-tTA-IRES-GFP vector (Addgene plasmid #18783) into the pCDNA3.1(-) vector (Thermo Fisher Scientific) via PCR. To complete the knock-in targeting plasmids, 5'-arm spanning 1.0 kb just before the stop codon and a 3'-arm spanning from the stop codon to 1.0 kb downstream of exon 4 were cloned into *pLta/p2a-Egfp-SV40polyA* donor plasmid.

Microinjection

For *Rorc* *CNS11*^{mut} and *Lta*^{EGFP} mice, donor plasmids were isolated with NucleoBond Xtra Midi Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and purified by standard phenol/chloroform extraction respectively. The DNA solution was diluted by de-ionized distilled water. crRNA for target sequence and tracrRNA were synthesized by IDT (Iowa, USA), and recombinant Cas9 protein was purchased from IDT. The gRNA (50ng/μL; a complex of crRNA and tracrRNA), Cas9 protein (100 ng/μl) and donor plasmids (4 ng/μl) water were co-injected into male pronuclei of fertilized oocytes created by *in vitro* fertilization with eggs of BDF1 (C57BL/6N x DBA/2) females (Japan SLC, Hamamatsu, Japan) and sperms of C57BL/6Ncr (Japan SLC). After the injected oocytes were cultured overnight *in vitro*, two-cell embryos were transferred into the oviduct of pseudopregnant ICR females (CLEA Japan, Tokyo, Japan).

Electroporation

For *Rorc*^{-/-}, *Rorc* *CNS9*^Δ, *Rorc* *CNS9*^{R_{unx}}, *Rorc* *CNS9*^{R_{AR}}, *Rorc* *CNS11*^{4th} and *Rorc* *CNS11*^{3wt} mice, crRNAs for target sequence and tracrRNA were synthesized by IDT. Recombinant Cas9 protein was purchased from IDT. 120bp Single stranded-donor oligo DNA (ssODN) which contains 50-60bp 5' and 3' arms were synthesized by Eurofingenomics (Tokyo, Japan). For *Rorc*^{-/-} mice, *Rorc* *CNS9*^Δ, *Rorc* *CNS9*^{R_{unx}}, *Rorc* *CNS9*^{R_{AR}} and *Rorc* *CNS11*^{4th} mice, the mixture of crRNA (0.61 pmol/μl), tracrRNA (0.61 pmol/μl) and Cas9 protein (30 ng/μl) in OPTI-MEM (Invitrogen) were electroporated into male pronuclei of fertilized oocytes created by *in vitro* fertilization with eggs of wild type BDF1 (C57BL/6N x DBA/2) females (Japan SLC, Hamamatsu, Japan) and sperms of wild type C57BL/6Ncr (Japan SLC). For *Rorc* *CNS11*^{3wt} mice, fertilized *CNS11*^{mut/wt} oocytes were created by *in vitro* fertilization with eggs of wild type BDF1 females and sperms of *Rorc* *CNS11*^{mut/mut} mice. After the electroporation, oocytes were cultured overnight *in vitro*, two-cell embryos were transferred into the oviduct of pseudopregnant ICR females (CLEA Japan, Tokyo, Japan).

Isolation of cells from tissue

Fetal liver and fetal thymus were separated under a stereo microscope, and single-cell suspension was generated by robustly pipetting with a 1000-μl pipette. The cell suspension was then filtered through a 100 μm strainer and resuspended in FACS buffer (2% FBS/0.05% NaN₃/PBS). Fetal body was obtained by removal of head, liver and mediastinal organ including thymus under a microscope and was then finely dissected. The specimens were incubated in DMEM (Nacalai Tesque) containing 1mg/mL Collagenase D (SIGMA) and 0.33 mg/mL DNase I (SIGMA) at 37°C for 15 min. Dissociated cells were collected at 15-min intervals, twice in total and strained with a 100 μm nylon mesh and were subjected to density gradient centrifugation using 40%/80% Percoll™ system (Cytiva 17089109). The cells at intermediated layer were collected by centrifuge and resuspended in FACS buffer. The collected adult intestines were cut into 1 cm pieces and washed with PBS. After opening the intestines along the longitudinal axis, they were incubated in 30mM EDTA-containing PBS at room temperature for 15 minutes and were immediately washed with EDTA-free PBS to remove epithelium. The tissues were then digested by incubating at 37°C in DMEM (Nacalai Tesque) containing 1 mg/mL Collagenase D (SIGMA) and 0.33 mg/mL DNase I (SIGMA) for 30 min. Dissociated cells were collected every 30 min and strained with a 100μm strainer, and then lymphocytes were further enriched on a 40%/80% Percoll gradient. Mesenteric LNs were isolated under a microscope and were then finely dissected. The specimens were incubated in DMEM (Nacalai Tesque) containing 1 mg/mL Collagenase D (SIGMA) and 0.33 mg/mL DNase I (SIGMA) at 37°C for 15 min. Dissociated cells were collected at 15-min intervals, twice in total and strained with a 100 μm nylon mesh. Bone marrow was isolated from the femur and tibia (four bones in total), crushed using the syringe plunger flange, filtered through a 100 μm cell strainer, and resuspended in FACS buffer.

Flow cytometry and cell sorting

For primary staining against mouse cells, antibodies were used at concentration of 1:200. For secondary staining, fluorescence-conjugated streptavidin was used at concentration of 1:250. Dead cells were removed by staining with Fixable Viability Dye eFluor™ 780 or Fixable Viability Dye eFluor™ 506 at concentration of 1:1000 (Thermo Fisher). For intranuclear staining, cells were fixed with Foxp3/Transcriptional Factor Staining Buffer Set (Thermo Fisher) according to the manufacturer's instruction. In short, cells were fixed for 15 min at room temperature and intranuclear staining were performed for 30 min at 4°C. (Foxp3 and Helios were stained overnight). For the analysis of IL-22 producing cells, single-cell suspensions obtained from small intestine lamina propria were stimulated in DMEM (Nacalai Tesque) containing IL-23 (40ng/ml, R&D systems) in the presence of Golgi-stop with Brefeldin A Solution (Thermo Fisher) for 3h at 37°C. After washing the cells, cells were surface labeled with the antibodies listed, followed by cell fixation using the same method as for intranuclear staining. Intracellular staining was performed at 4°C overnight. Flow cytometric analysis and cell sorting were performed with MA900 (Sony Imaging Products & Solutions) respectively. Data were analyzed using FlowJo software (BD). The gating strategy for HSCs, MPPs, aLPs~ILCPs, ILC2s, and LTi-like cells from *CNS11*^{mut/mut} and *CNS11*^{4th/4th} mice

subjected to ATAC-seq is as follows: The lineage markers for the following fetal liver cells were defined using MA900 (Sony Imaging Products &1, TCR β , CR $\gamma\delta$, B220, ST2, CD4. Fetal liver HSC/MPP: Lin⁻ Sca-1^{high} c-Kit^{high}, Fetal liver aLP~ILCP: Lin⁻CD127⁺Int. $\alpha_4\beta_7$ ⁺Flt3⁻ excluding Sca-1^{high}c-Kit^{high} double positive cells. The lineage markers for the following bone marrow cells were defined using Ter119, B220, GR-1, TCR β , TCR $\gamma\delta$, CD4, CD8 α , CD3e, NK1.1, CD11b, CD11c. Bone marrow HSC: Lin⁻ Sca-1^{high}c-Kit^{high}Flt3⁻, Bone marrow MPP: Lin⁻ Sca-1^{high}c-Kit^{high}Flt3⁺, Bone marrow aLP~ILCP: Lin⁻CD127⁺Int. $\alpha_4\beta_7$ ⁺Flt3⁻CD25⁻ excluding Sca-1^{high}c-Kit^{high} double positive cells, small intestine ILC2: CD3e⁻B220⁻CD11b⁻CD11c⁻MHC classII⁻NK1.1⁻KLRG1⁺, small intestine LT α -like cells: CD3e⁻B220⁻TCR β ⁻LT α -EGFP⁺c-Kit⁺CD4⁺.

***In vitro* cell culture of FL precursor**

Stock of Tst-4-DLL1 stroma cells⁷⁴ were a gift from Hiroshi Kawamoto (University of Kyoto, Kyoto, Japan). All experiments were performed in RPMI 1640 (Nacalai Tesque) containing 10%FBS (Cytiva), 1% penicillin-streptomycin (Gibco, Thermo Fisher), 1% MEM Non-Essential Amino Acids Solution (Gibco, Thermo Fisher) and 1mM sodium pyruvate (Gibco, Thermo Fisher) and were maintained in 37°C incubator with 5% CO₂. The stromal cells were plated at 30,000 cells per well in a 24-well plate the day before and incubated overnight. Before adding the sorted lymphocytes, the supernatant was replaced with fresh medium containing IL-7 (20ng/ml, Pepro Tech) and stem cell factor (20ng/ml, Pepro Tech). Fetal liver cells were lineage-depleted with CD3e, B220, Ter119, ST2, and NKp46 antibodies and then sorted. 1000 cells from each precursor population were plated and allowed to grow for 10-12 days with the supernatant being changed every other day. CD45.2⁺ cells were considered for analysis of hematopoietic progeny.

***In vitro* culture of MNK3 cells**

For the MNK-3 cell line culture, the culture protocol was according to a previous report.⁴⁵ In short, the MNK-3 cells were cultured in DMEM (Sigma-Aldrich) with 10% fetal bovine serum, 2 mM L-Glutamine (Sigma-Aldrich), 1 mM sodium pyruvate (Gibco, Thermo Fisher), 55 μ M 2-mercaptoethanol (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), and 100 μ g/ml penicillin and streptomycin. To maintain the property of MNK-3 cells, 10 ng/ml IL-7 (R&D System), 10 ng/ml IL-2 (R&D System), 10 ng/ml IL-15 (Pepro Tech), and 10 ng/ml retinoic acid (Sigma-Aldrich) were added to the culture.

Luciferase reporter assay

The *CNS11* region (Chr3:94387969–94388515) was inserted into the KpnI/NheI site of pGL4.27. To mutate the Runx binding motifs, the sequences TGGTGT (Chr3:94388011–94388016), ACCACA (Chr3:94388068–94388073), TGGTGT (Chr3:94388314–94388319), and ACCACA (Chr3:94388354–94388359) were modified to GAATTC, CAACAC, GTGTTG, and GAATTC, respectively. HEK293T cells were transfected with FuGENE® 6 Transfection Reagent (Promega) according to the manufacturer's protocol. Briefly, 1×10^5 HEK293T cells were transfected with 0.1 μ g of the pGL4.27 vector, 0.01 μ g of Renilla as an internal control, and 0.01–0.1 μ g of either the empty pcDNA3.1(+) vector or pcDNA3.1(+) containing each transcription factor. After incubation at 37°C with 5% CO₂ for 60 hours, cells were harvested, and luciferase activity was measured using the Dual-Glo® Luciferase Assay System (Promega) according to the manufacturer's instructions with an EnSpire plate reader.

ChIP assay

Formaldehyde (37%) was added directly to the culture medium at a final concentration of 1%, and 1×10^7 MNK3 cells were incubated for 10 min at 37 °C. Chromatin immunoprecipitation (ChIP) was performed using a ChIP Assay Kit (Millipore) according to the manufacturer's instructions. Antibodies against ROR α (5 μ g), ROR γ t (5 μ g), or control IgG were used. Immunoprecipitated DNA was analyzed by qPCR to assess binding of the indicated antibodies to CNS11 and a negative control region. medium.

Histological analysis

1cm of terminal ileums were isolated and washed with EDTA-free PBS. After opening the ileums along the longitudinal axis, they were incubated in 30mM EDTA-containing PBS at room temperature for 15 minutes and were immediately washed with EDTA-free PBS to remove epithelium. Tissues were submerged in 4% paraformaldehyde for 30 minutes at 4°C then rinsed and kept in PBS for one day. For whole mount immunohistochemistry, tissues were immersed in PBS-TS (0.1% TritonX-100/1% FBS in PBS) then blocked in 10% FBS/PBS-TS for overnight at 4°C. Tissues were subsequently incubated in PBS-TS containing primary antibody (1:100) at 4°C overnight. The sections were further rinsed with PBS-TS and incubated with secondary antibody diluted with PBS-TS (1:250) for 1h at room temperature. Followed by PBS-TS rinse, the tissues were nuclear stained with DAPI (Dojindo Molecular Technologies, Inc) for 5min at room temperature and further mounted on slides with Fluoromount-G solution (SouthernBiotech). Fluorescence images of 1024x1024 pixel resolution were obtained with LSM780 confocal microscope (Carl Zeiss) and processed with Zen Black software (Carl Zeiss) to yield maximum intensity projection.

Bulk RNA-seq assay

Bulk RNA-seq was performed on 50-200 sorted cells using the SMART-Seq HT PLUS Kit (Takara Bio). Cells were directly sorted into the lysis buffer and the extracted RNA was then reverse transcribed into cDNA using oligo (dT) priming and SMART technology, ensuring full-length cDNA synthesis. The cDNA was amplified through PCR and subsequently converted into Illumina-compatible sequencing libraries using the kit's integrated library preparation components. Library quality and concentration were assessed

using Bioanalyzer High Sensitivity DNA kit (Agilent) and Qubit dsDNA HS Assay Kit (ThermoFisher), respectively. The libraries were pooled and sequenced with Novaseq6000 (Illumina) or DNBSEQ-G400 (MGI) sequencers.

Single-cell RNA-seq

Droplet based scRNA-seq was performed using the Chromium single cell gene expression platform (10x Genomics). Single cell-derived gene expression library was generated using Chromium Next GEM Single Cell 3' Reagent kits v3.1 following the manufacturer's protocol with some modifications. Briefly, 3,000–10,000 cells were sorted using PBS as sheath fluid, directly into the reagent mix for generating gel beads-in-emulsion (GEM). GEM reverse transcription, cDNA amplification, library construction was performed according to the manufacturer's guide. Library quality and concentration was assessed using Bioanalyzer High Sensitivity DNA kit (Agilent) and Qubit dsDNA HS Assay Kit (Thermo Fisher), respectively. The libraries were pooled and sequenced with Novaseq6000 (Illumina) or DNBSEQ-G400 (MGI) sequencers. E14.5 LT α and fetal liver GFP^{hi} cells were isolated from six fetuses to generate libraries "RORgt_LT α " and "RORgt_high", respectively, which have been deposited in Gene Expression Omnibus (GEO) under the dataset accession GSE278161.

ATAC-seq assay

150–10000 cells were used for ATAC-seq following the manufacturer's instructions (Active Motif). Cells were pelleted and washed with 100 μ L of ice-cold PBS. They were then lysed with 100 μ L of ATAC Lysis Buffer and were spun down at 1000g for 10–15 min at 4°C. The nuclei were incubated in 50 μ L of Tagmentation Master Mix at 37°C for 30 min in a thermomixer set at 800 RPM. Accessible DNA region in nuclei was probed with Tn5 Transposase that inserts sequencing adapters into open regions. The tagmented DNA was amplified by i7 and i5 indexed primers and purified by SPRI beads. Library quality and concentration were assessed using Bioanalyzer High Sensitivity DNA kit (Agilent) and Qubit dsDNA HS Assay Kit (ThermoFisher), respectively. The libraries were pooled and sequenced with HiSeq2500 (Illumina) sequencer.

ChIP-seq assay

5 x 10⁶ E12.5 Lin[−] fetal liver cells from WT and *CNS11*^{mut/mut} were used for ChIP-seq analysis with anti-CBF β antibody established previously^{34,38} with some modifications. After 10 min of fixation in 1% formaldehyde PBS at room temperature, the reaction was stopped by the addition of 1.5M glycine solution (final concentration of 0.15 M), and the cells were lysed in lysis buffer1 (50 mM HPES-KOH pH7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, and 0.25% Triton X-100) supplemented with cComplete protease inhibitor cocktail tablet (Roche). The nuclei were pelleted and then washed with lysis buffer 2 (10mM Tris-HCl (pH 8.0), 200mM NaCl, 1mM EDTA, 0.5mM EGTA). Washed nuclei were resuspended in lysis buffer 3 (10mM Tris-HCl (pH 8.0), 100mM NaCl, 1mM EDTA, 0.5mM EGTA, 0.1% sodium deoxycholate, 0.5% N-lauroylsarcosine sodium salt) and sonicated using the MODEL Q55 Sonicator® (QSONICA). The fragmented chromatin was immunoprecipitated with anti-CBF β antibody. After reverse crosslinking and purification steps, the precipitated DNA was re-sonicated with the Covaris M220 to produce 200bp DNA fragments. Sequence libraries were constructed with NEBNext Ultra II DNA Library Prep Kit and NEBNext Multiplex Oligos for Illumina (New England Biolabs). The libraries were pooled and sequenced with NextSeq2000 (Illumina) sequencer.

Chromatin integration labeling (ChIL)-seq assay

A total of 5,000 MNK3 cells per well were cultured in 96-well ibidi plates. ChIL-seq was performed as previously described (Harada et al.⁴⁴). Library synthesis and amplification were conducted using a SMART-Seq v4 Ultra Low Input RNA Kit (TaKaRa) with minor modifications. All reagents were prepared separately, and reactions were performed according to the manufacturer's protocol using ChIL Read2 primers together with Ad1 and Ad2 primers. Amplified DNA was purified with AMPure XP beads (Beckman Coulter). The quality of the resulting cDNA was evaluated using a TapeStation 4200 system (Agilent Technologies) and quantitative PCR (qPCR). Sequencing was performed on an Illumina NovaSeq 6000.

Bulk RNA-seq data processing and analysis

Raw RNA-seq reads were trimmed and filtered for quality control using fastp. The clean reads were mapped to the GRCm39 reference genome using HISAT2 and then read counts for each sample were obtained using featureCounts. For heatmaps and Principal Component Analysis (PCA), normalized RNA-seq z-score transformed data were plotted using the ggplot2 R package.

Single-cell RNA-seq data processing

The raw sequencing data were demultiplexed and aligned to mouse reference genome mm10, and UMI counting was performed using Cell Ranger v6.0.1 (10x Genomics). Further quality control and secondary analysis was conducted using the Seurat R Package v4.0.⁷⁷ To exclude low-quality cells, barcodes with < 500 gene count (nFeature_RNA) or > 5% proportion of mitochondrial genes (percent.mt) were filtered. The data were normalized and scaled using standard functions in Seurat. Two thousand variable genes were selected for downstream analysis using *FindVariableFeatures* function of Seurat package. Data scaling was performed using the ScaleData function. Principal component analysis (PCA) was performed using the scaled data of variable genes. The top 50 PCA components were used for clustering in this study unless otherwise specified. Graph-based clustering of the PCA reduced data was performed by computing a shared nearest neighbor with the *FindNeighbors* function followed by clustering with the Louvain Method using the *FindClusters* function. The resolution for Louvain clustering, based on a shared nearest neighbor graph, was determined for

each analysis between 0.2–0.4. Dimension reduction plots were created by Uniform Manifold Approximation and Projection (UMAP). Differentially expressed genes for each cluster and cell cycle estimation for each barcode were determined by the *FindAllMarkers* and *CellCycleScoring* functions, respectively. After a sensible clustering was obtained, each cluster was briefly annotated by the marker gene expression and quality parameters (nFeature_RNA, nCount_RNA, percent.mt, and/or cell cycle). Then, one cluster with low nFeature_RNA and mixed marker gene expression was considered as a low-quality cluster and filtered. The filtered data were processed again through the same analytical pipeline.

Merged analysis of E14.5 LTⁱ and GFP^{hi} single-cell datasets

A dataset of the E14.5 LTⁱ cells (RORgt_LTⁱ) and a dataset of the E14.5 fetal liver GFP^{hi} cells (RORgt_high) were merged by the *merge* function of the Seurat package. The merged data was processed as described above. Clusters with low nFeature_RNA and mixed marker gene expression were removed from the subsequent analysis as low-quality clusters. The clusters which contain a considerable number of cells were defined as “LTⁱ+GFP^{hi}” and isolated for downstream analyses. The processing of this LTⁱ+GFP^{hi} resulted in identification of six clusters.

ATAC-seq data processing and analysis

Raw paired-end ATAC-seq reads were trimmed and filtered for quality control using Cutadapt with the adapter sequence “CTGTCTCTTATACATCT”. The trimmed reads were then aligned to the mouse genome (mm10 or CNS11^{mut}-modified mm10) using Bowtie2 v2.2.5 with the parameter -X 2000. Non-uniquely mapping reads and reads that aligned to the mitochondrial genome were filtered out using Samtools. Only concordantly mapped reads were used for further analysis. Normalized coverage tracks (bigWig) were generated with deepTools bamCoverage using CPM normalization. Peaks were called using Genrich with the following parameters: -j (ATAC-seq mode), -q 0.01 (FDR threshold), -y (exclude mitochondrial reads), and -e chrM (exclude mitochondrial chromosome). For footprinting analysis, TOBIAS was used. First, the ATACCorrect step was performed. Footprint scores were calculated using TOBIAS FootprintScores. Binding positions of individual transcription factors and differential binding between two conditions were carried out using TOBIAS BINDetect with JASPAR CORE Position Frequency Matrix (PFM) motifs. Principal Component Analysis (PCA) was performed using R and ggplot2 to visualize the data distribution. For ATAC seq analysis of liver ILC1, lung ILC2, macrophages, splenic naïve CD4⁺ T cells, and splenic B cells, we examined the deposited datasets GSM2785745,⁵⁴ GSE270373,⁵⁵ GSM8493112,⁶⁰ GSM7036250,⁵⁶ and GSM8430804,⁵⁹ respectively, available on the Gene Expression Omnibus (GEO).

ChIP-seq data processing and analysis

Raw paired-end ChIP-seq reads were trimmed for adapters and filtered for quality using Cutadapt. Trimmed reads were aligned to the mouse genome (mm10 or CNS11^{mut}-modified mm10) with Bowtie2 (v2.2.5). Non-uniquely mapping reads and reads that aligned to the mitochondrial genome were filtered out using Samtools. Normalized coverage tracks (bigWig) were generated with deepTools bamCoverage using CPM normalization.

ChIL-seq data processing and analysis

Raw single-end ChIL-seq reads were trimmed for adapters and filtered for quality using Cutadapt. Trimmed reads were aligned to the mouse genome (mm10) with Bowtie2 (v2.2.5). Non-uniquely mapping reads and reads that aligned to the mitochondrial genome were filtered out using Samtools. Normalized coverage tracks (bigWig) were generated using deepTools bamCoverage with CPM normalization. Reads were extended to 75 bp (–extendReads 75) prior to coverage calculation.

Publicly available ChIP-seq, CUT&Tag and CUT&RUN data analysis

For the ChIP-seq analysis of naïve CD4⁺ T cells (lymph nodes) and bone marrow-derived macrophages, the CUT&Tag analysis of bone marrow-derived HSCs, and the CUT&RUN analysis of splenic B cells, we reanalyzed the publicly available datasets GSM7656352,⁶² GSM4979627,⁶⁴ GSM5723333,⁶¹ and GSM4551384⁶³ from the Gene Expression Omnibus (GEO).

Visualization of genomic loci

UCSC Genome Browser (<https://genome.ucsc.edu/>)⁷⁸ was used for the visualization of genomic loci. The corresponding human locus was obtained by the convert function of the browser. The recombination rate track for the average of paternal and maternal is calculated in deCODE.⁷⁹

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism v9.5 software. All error ranges represent standard error of the mean, unless otherwise stated. For two-group comparison, unpaired two-tailed t-test was used. Probabilities of the null hypothesis $p < 0.05$ were judged to be statistically significant.