Runx-dependent and silencer-independent repression of a maturation enhancer in the Cd4 gene

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An intronic silencer, S4, in the Cd4 gene has been shown to be responsible for the helper-lineage-specific expression of CD4; S4 requires Runx complex binding to exert its silencer function against the enhancer-mediated Cd4 activation by modulating the epigenetic state of the Cd4 gene. Here we identify a late-acting maturation enhancer. Bcl11b plays essential roles for activation of both the early-acting proximal enhancer and maturation enhancer of Cd4. Notably, Runx complexes suppress these enhancers by distinct mechanisms. Whereas repression of the proximal enhancer depends on the S4 silencer, the maturation enhancer is repressed by Runx in the absence of S4. Moreover, ThPOK, known to antagonize S4-mediated Cd4 repression, assists Runx complexes to restrain maturation enhancer activation. Distinct modes of S4 silencer action upon distinct enhancers thus unravel a pathway that restricts CD4 expression to helper-lineage cells by silencer-independent and Runx-dependent repression of maturation enhancer activity in cytotoxic-lineage cells.
CD4 and CD8 glycoproteins function as a co-receptor that assists T-cell antigen receptor (TCR) to recognize antigenic peptide presented by major histocompatibility complex (MHC) class II and class I molecules, respectively. In addition, CD4/CD8 molecules serve as useful markers to define thymocyte developmental stages and helper-lineage and cytotoxic-lineage T cells. Signals from pre-TCR complexes in CD4+CD8+ double-negative (DN) thymocyte progenitors induce both CD4 and CD8 expression, resulting in the generation of CD4+CD8+ double-positive (DP) precursor thymocytes. A limited number of DP thymocytes, which have passed a process known as positive selection, differentiate further into mature thymocytes. Post-selection thymocytes expressing MHC-class I (MHC-I) restricted TCRs are specified to differentiate into the cytotoxic-lineage and acquire CD4+CD8+ single-positive (SP) phenotype by terminating CD4 expression, whereas MHC-class II (MHC-II)-mediated TCR engagement generates CD4+CD8− SP thymocytes committed to the helper-lineage by inhibiting CD8 expression.

Such stage-specific and lineage-specific expression of CD4/CD8 co-receptors is regulated at the transcriptional level by a combination of transcriptional silencing and enhancer activity. In this study, we identify the enhancer(s) that regulate CD4 expression. In particular, we show that stage-specific enhancer activity is regulated by a combinatorial action of cis-regulatory elements (cis-REs). For instance, insertion of a 434 bp intronic transcriptional silencer (S4) into a transgene together with a 430 bp proximal enhancer (E4p) and a minimal CD4 promoter (P4) is necessary to recapitulate stage-specific and lineage-specific CD4 expression in reporter transgene expression. CD4 de-repression from CD8+ T cells upon ablation of the S4 sequence also confirmed that E4p is essential to initiate CD4 expression, referred to as a maturation enhancer (E4m), should be present. It has also been shown that stage-specific DNA de-methylation in helper-lineage cells and continuous DNA methylation in cytotoxic-lineage cells are involved in establishment of the heritable active and silent status, and requires E4p and S4 activity, respectively. Thus, CD4 gene regulation has served as an ideal model to study how stage-specific and lineage-specific epigenetic modifications are regulated by cis-regulatory elements. However, the genomic region containing E4m activity remains elusive, as does the mechanism by which E4m activity is regulated.

In this study, we identify the E4m, validated its role in regulating CD4 expression, and isolate Bcl11b as an important activator for E4m. We also show that Runx complexes repress the E4m activity in CD8+ T cells even in the absence of the S4 and find unexpected ThPOK function that prevents premature E4m activation by assisting Runx-mediated E4m repression. Collectively, our results reveal that Runx complexes repress two enhancers, E4p and E4m, in distinct manners, providing a novel insight that revises a silencer-based model for a lineage-specific CD4 expression.

Characterization of the CD4 maturation enhancer (E4m). In the murine Cd4 locus, the presence of a Cd4 maturation enhancer (E4m) shortly downstream of S4 has been predicted. We found conserved sequences at approximately 1 kb downstream of the S4 region (Supplementary Fig. 2a); moreover, the public ATAC-Seq database showed that this region becomes more accessible specifically in CD4-lineage cells after positive selection, whereas the S4 region becomes more accessible in CD8-lineage cells (Supplementary Fig. 2a). In addition, our chromatin immunoprecipitation (ChIP) combined with sequencing (ChIP-Seq) analysis detected binding of Runx complexes to S4 and its downstream region in total thymocytes. Furthermore, changes of post-translational modifications on K27 of histone 3 for trimethylation (H3K27me3) and for acetylation (H3K27ac), which are known as markers for inactive and active enhancer states, respectively, indicated that H3K27ac at E4p was highest in precursor DP thymocytes, whereas H3K27ac was increased at a putative E4m region in post-selection (CD69+CD24hiTCR8+) thymocytes. In comparison, H3K27me3 modification accumulated at both regions specifically in CD8+ T cells. Together, these features prompted us to test the function of this putative E4m region genetically by deleting the 337 bp core conserved sequences. Accordingly, by using an ES cell line harboring a Cd4Δ/E4m or Cd4Δ/E4p genotype (Supplementary Fig. 2b, c), we established two mouse lines harboring Cd4Δ/E4m and Cd4Δ/E4p alleles, with the latter lacking both E4p and E4m.
Following activation of CD4+CD8− splenic T cells, CD4 expression from the Cd4ΔEth allele decreased to a similar extent as that from the Cd4ΔEth allele (Fig. 2d), in which both S4 and E4m were deleted. However, the percentage of cells retaining CD4 expression was higher in Cd4ΔEth/E4m cells than in Cd4ΔEth/E4m cells, although the percentage of methylated CpG motifs at intronic regions remained higher in these two cell types to a similar extent (Figs 1c and 2c). These results indicated that E4m activity is also necessary to establish a heritable active state at the Cd4 locus in part by activating the DNA de-methylation process together with E4p.

Of note, a percentage of CD4+CD8− cells among the mature thymocyte and lymph node T cell population was decreased in Cd4ΔEth/E4m mice (Fig. 2b, f). To examine whether re-directed differentiation of MHC-II selected cells was involved in the decrease of CD4+ T cells, as was observed in Cd4ΔEth/E4m mice, we analyzed the differentiation of MHC-II selected cells under β2-microglobulin-deficient condition. In Cd4ΔEth/E4m thymocyte population, while CD4−CD8+ cells were undetectable in control β2m−/− : Cd4ΔEth/E4m mice, mature CD4−CD8+ SP thymocytes emerged in B2m−/− : Cd4ΔEth/E4m mice (Fig 3a, b). In the Cd4ΔEth/E4m population, the percentages of CD8
expressing cells were also higher in B2m−/−; Cd4ΔE4m/ΔE4m mice (Fig. 3a). As ThPOK is involved in terminating CD8 expression during the maturation of MHC-II selected cells,

To understand how E4m function is regulated, it is necessary to identify molecules that bind and regulate E4m activity. During analyses of Bcl11b function, we observed that Bcl11b associates with the E4p, S4, and E4m regions in total thymocytes (Fig. 4a). ChIP-qPCR showed that associations of both Runx and Bcl11b with the E4p were dramatically decreased in peripheral T cells (Fig. 4b). Runx binding to S4 was the highest in CD8+ T cells but
was almost lost in CD4+ T cells, with Bcl11b binding to S4 also becoming lower in CD4+ T cells compared to that in other cell subsets (Fig. 4b). Although the association of Runx to E4m was compatible with the three cell subsets, Bcl11b binding to E4m was higher in CD4+ T cells than in CD8+ T cells (Fig. 4b).

Considering such Bcl11b associations with C4 regulatory regions, we examined Bcl11b function in regulating C4 expression by using two Bcl11b mutant models. We recently generated a hypomorphic Bcl11b allele, referred to as Bcl11b<sup>hm</sup>, that produces truncated Bcl11b protein lacking the last zinc-finger domain<sup>23</sup>. In thymi of newborn B<sup>cl11b<sup>hm</sup></sup>-/- mice, which die at two days after birth, CD<sup>4</sup>CD<sup>8</sup> cells were present in the CD24<sup>hi</sup>TCR<sup>hi</sup> population, whereas nearly all CD24<sup>hi</sup>TCR<sup>lo</sup> thymocytes appeared as CD4<sup>+</sup>CD8<sup>+</sup> DP cells in control mice (Fig. 4c, d). In addition, CD4 expression in the TCR<sup>hi</sup>CD8<sup>-</sup> mature thymocyte population became lower, resulting in the generation of the CD4<sup>-</sup>-CD8<sup>-</sup> instead of CD4<sup>+</sup>-CD8<sup>-</sup> subset in Bcl11b<sup>hm</sup>-/- thymi (Fig. 4c, d). We next examined the effect of conditional inactivation of Bcl11b at DP stage by a Cd4-Cre transgene, and observed emergence of the CD4<sup>-</sup>-CD8<sup>-</sup> subset in the CD24<sup>lo</sup>TCR<sup>hi</sup> mature thymocyte population of Bcl11b<sup>fl/fl</sup>; Cd4-Cre<sup>-/-</sup> mice (Fig. 4e). In order to further address whether Bcl11b regulates E4m activity, we then combined E4p-deficiency with Bcl11b inactivation. Under an E4p-deficient background, the CD4 expression level in the mature CD8<sup>-</sup> negative thymocyte population severely decreased upon loss of Bcl11b (Fig. 4e, f). In contrast, CD4 expression level was partially restored after egress from the thymus, with CD4<sup>+</sup> T cells emerging in the peripheral lymphoid tissues of CD4<sup>e4p/ΔE4p</sup>; Bcl11b<sup>fl/fl</sup>; Cd4-Cre<sup>-/-</sup> mice (Fig. 4e, f). However, the CD4 expression in CD4<sup>+</sup> T cells that developed in Bcl11b<sup>fl/fl</sup>; Cd4-Cre<sup>-/-</sup> mice were unstable after activation-induced cell divisions (Fig. 4g). Thus, dysfunction of Bcl11b resulted in a delayed CD4 induction at two transitional stages, one for becoming DP thymocytes and the other for becoming mature CD4 SP thymocytes, which require activation of E4p and E4m, respectively.

Characterizing specific activity of E4m. In order to further characterize E4m activity, we addressed how E4m alone behaves
by generating a \(Cd4^{ΔE4p:ΔS4}\) allele, in which only the \(E4m\) among three regulatory regions remains in the \(Cd4\) gene (Fig. 5a). Notably, the percentage of cells expressing \(Cd4\) was higher in CD8-negative cells than in CD8-positive cells in the mature thymocyte population of \(Cd4^{ΔE4p:ΔS4}\) mice (Fig. 5b, c), although it was equally high in both types of cells of \(Cd4^{ΔS4}\) mice. To exclude a possibility that deleted sequences around \(S4\) in the \(Cd4^{ΔE4p:ΔS4}\) allele include a functional element for \(E4m\) activity, we targeted specific mutations onto two Runx-motifs within \(S4\) to abrogate \(S4\) function by a more specific manner\cite{9}, generating a \(Cd4^{ΔE4p:SA4}\) allele (Fig. 5a). In \(Cd4^{ΔE4p:SA4}\) mice, the percentage of cells expressing \(Cd4\) remained higher in CD8\textsuperscript{+} cells compared to CD8\textsuperscript{+} T cells (Fig. 5b, c). Such CD8\textsuperscript{+} cells were almost undetected in B2m\textsuperscript{−/−}: \(Cd4^{ΔE4p:SA4}\) mice (Supplementary Fig. 4), indicating that the majority of CD8\textsuperscript{+} cells in \(Cd4^{ΔE4p:SA4}\) mice consisted MHC-I restricted cells. Given that CD4 expression from the \(Cd4^{ΔE4p:ΔS4}\) allele is likely to depend on \(E4m\), these observations indicate that the \(E4m\) activity is more predominant in the helper-lineage cells than in cytotoxic-lineage cells.

**Regulation of \(E4m\) activity by Runx complexes.** To examine how such helper-lineage dominant activity of \(E4m\) is regulated, we analyzed the function of transcription factors that are known to be involved in lineage-specific expression of the \(Cd4\) gene. Two Runx proteins, Runx1 and Runx3, are essential to control lineage specific \(Cd4\) expression through activating the \(S4\) silencer\cite{9}. The penta-peptide sequences, VWRPY, at the C-terminal end of Runx complexes is the essential and are essential for Runx-mediated \(Cd4\) repression\cite{10,26}. Accordingly, mice lacking the VWRPY motif exhibited full \(Cd4\) de-repression in CD8\textsuperscript{+} T cells, phenocopying the \(Cd4^{ΔS4}\) mice (Figs 5b and 6a). Given a loss of \(S4\) activity by Runx1/Runx3\textsuperscript{ΔV}\textsuperscript{ΔV} mutation, we assumed that CD4 expression in Runx1/Runx3\textsuperscript{ΔV}\textsuperscript{ΔV}: \(Cd4^{ΔE4p:ΔE4p}\) mice was similar to that in \(Cd4^{ΔE4p:ΔS4}\) mice. However, the percentage of CD4-expressing cells was comparable between CD8\textsuperscript{+} and CD8\textsuperscript{−} cells in Runx1/Runx3\textsuperscript{ΔV}\textsuperscript{ΔV}: \(Cd4^{ΔE4p:ΔE4p}\) mice (Fig. 6a). Thus, a discrepancy existed between the effects from loss of \(cis\)-acting \(S4\) and loss of \(trans\)-acting Runx proteins on the \(Cd4^{ΔE4p}\) allele, suggesting a possible involvement of \(S4\)-independent mechanisms through which Runx proteins suppress \(E4m\) activity. We then generated Runx1/Runx3\textsuperscript{ΔV}\textsuperscript{ΔV}: \(Cd4^{ΔE4p:SA4}\) mice and observed an increase of the CD4 expressing population in CD8\textsuperscript{+} T cells (Fig. 6a, b). Our ChIP-qPCR analyses showed that Runx

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**Fig. 4 Requirement for Bcl11b in activating \(Cd4\) at two stages.** a ChIP-Seq tracks at the \(Cd4\) gene showing binding of Bcl11b in total thymocytes. b ChIP-qPCR analyses for Runx (left) and Bcl11b (right) bindings to the \(Cd4\) proximal enhancer (\(E4p\)), \(Cd4\) silencer (\(S4\)), and \(Cd4\) maturation enhancer (\(E4m\)) in DP thymocytes, peripheral CD4\textsuperscript{+} and CD8\textsuperscript{−} T cells. c Contour plots showing CD4 and CD8 expression in indicated thymocyte subsets and lymph node (LN) T cells of mice with indicated genotypes. Numbers in quadrants indicate respective cell percentages. Representative results of at least three independent analyses. d Summary of percentage of indicated cell types in indicated cell populations of Bcl11b\textsuperscript{+/+} and Bcl11b\textsuperscript{−/−} newborn mice. Means ± SD. ***p < 0.001 (unpaired student t test, two-sided). e Contour plots showing CD4 and CD8 expression in indicated thymocyte subsets and lymph node (LN) T cells of mice with indicated genotypes. Numbers in quadrants indicate respective cell percentages. Representative results of at least three independent analyses. f Statistical summary of percentage of CD4\textsuperscript{+} cells in CD8\textsuperscript{−} mature thymocytes and CD8\textsuperscript{−} lymph node (LN) T cells of \(Cd4^{ΔE4p:ΔE4p}\) mice and \(Cd4^{ΔE4p:ΔE4p}\), Bcl11b\textsuperscript{+/-}, CD4-Cre mice. Means ± SD. ***p < 0.001 (unpaired student t test, two-sided). g Dot plots showing CD4 expression and cell divisions five days after in vitro stimulation of sorted CD4\textsuperscript{+} T cells from mice with indicated genotypes. One representative of three experiments.
association with the E4m was unaffected by eliminating Runx-motifs in S4, whereas Runx binding to S4 was abrogated (Fig. 6c). Together, these observations indicated that Runx proteins are likely to repress E4m activity in a VVWRPY-dependent manner through their S4-independent association with E4m.

Lack of canonical Runx-motifs within E4m suggests that recruitment of Runx complexes to E4m might be mediated via protein-protein interaction. As both Bcl11b and Satb1 are functional activators for the E4m enhancer and interact with Runx proteins23,27, we wished to test whether Runx association with E4m is affected by lack of Bcl11b and Satb1. However, the severe reduction of mature thymocytes in Bcl11bΔ/Δ; Satb1Δ/Δ; Cd4-Cre mice (Supplementary Fig. 5a) made it possible to perform ChIP-qPCR analyses only in DP thymocytes. In this setting, Runx binding to S4, E4p, and E4m was not significantly changed by the loss of Bcl11b and Satb1 proteins (Supplementary Fig. 5b), suggesting the presence of another factor(s) that recruits Runx complexes to E4m.

Repressive regulation of E4m by ThPOK. As ThPOK was shown to be essential for the regulation of lineage-specific CD4 expression by countering against S4-mediated Cd4 repression16,28, we next examined whether ThPOK is involved in the regulation of E4m activity by generating Cd4ΔE4p:S4M/ΔE4p:S4M; Thpokgfp/gfp mice that lack ThPOK expression. Notably, loss of ThPOK resulted in an increase of CD4 expressing cells in mature CD8+ thymocyte and peripheral CD8+ T cell populations (Fig. 7a, b). Increase of the CD4+ subset by lack of ThPOK had already been observed at the CD24hiTCRβhi stage composed of freshly selected thymocytes and in Thpok-gfp- cells that represent MHC-I-signalized ThPOK non-expressing cells. These genetic results suggested that ThPOK represses E4m activity from the early initiation phase. Similar early induction of CD4 in CD24hiTCRβhi thymocytes from the Cd4ΔE4p:S4M allele was also observed by Runx1/Runxa3ΔA1V mutation (Fig. 7c), suggesting that the mechanism by which Runx represses E4m activity became non-functional through the lack of ThPOK expression. Our ChIP-qPCR using peripheral CD4+ T cells showed that ThPOK associated with E4m in the absence of the S4 (Supplementary Fig. 5c), and Runx association with E4m in pre-selection DP thymocytes was not affected by loss of ThPOK (Fig. 7d). Interestingly, level of H3K27ac at E4m region tended to be higher in CD8+ mature thymocytes in the absence of ThPOK, although it was lower than that in control CD4 SP thymocytes (Supplementary Fig. 5d). These observations unraveled an unexpected ThPOK function that assists Runx-mediated repression of E4m activity after recruitment of Runx to the E4m enhancer.

Discussion

By generating several mutant Cd4 alleles, our study provides novel insights into how two enhancers, E4p and E4m, regulate Cd4 expression (Supplementary Fig. 6). E4p and E4m cooperate to establish a stably heritable active state in the helper-lineage T cells by activating the DNA de-methylation process. It is unclear whether such property is endowed to specific enhancers or is a general feature of enhancers. We showed that a synthetic Eth enhancer derived from the Thpok gene could compensate the E4p activity that induces DNA de-methylation. This suggests that specific sequences within the E4p are unlikely to be absolutely essential to control DNA de-methylation at the Cd4 locus. Despite their differences in sequences, it is possible that Eth and E4p poise similar epigenetic codes that can be recognized by common machinery that induces DNA de-methylation. Recent work reported accumulation of 5hmC, which is generated by TET family proteins through DNA de-methylation process29, near the Thpok proximal enhancer core region in the CD4 SP thymocytes30, suggesting that TET proteins are involved in DNA de-methylation for activation of the Thpok gene. Thus, once distinct protein complexes bound on a distinct enhancer marks the target locus with such epigenetic codes, a sequential reaction that induces DNA de-methylation might be carried out. Alternatively, E4p/E4m enhancers and Eth were bound by same nuclear protein(s) that recruit machinery to induce DNA de-methylation. Bcl11b and Satb1 may represent candidates for such shared factors, as

Fig. 5 Helper-lineage dominant activity of E4m. a Schematic structures of Cd4, Cd4ΔS4, Cd4ΔE4p:S4M, and Cd4ΔE4p:S4M alleles are shown as in Fig. 2a. Mutations at two Runx sites within S4 are marked as X in the Cd4ΔE4p:S4M allele. b Dot plots showing CD4 and CD8 expression in indicated thymocyte subsets of mice with indicated genotypes. Numbers in quadrants indicate respective cell percentages. Right histograms showing CD4 expression in CD8+ thymocytes from the Cd4ΔE4p:S4M allele. c Summary of percentage of CD4+ cells in CD8− and CD8+ mature (CD24loTCRβhi) thymocyte populations of Cd4Δ4+/− (Wt), Cd4ΔS4Δ5+/− (ΔS4), Cd4ΔE4p:S4M/ΔE4p:S4M (ΔE4p:S4M), and Cd4ΔE4p:S4M/ΔE4p:S4Δ (ΔE4p:S4Δ) mice. Means ± SD. **p < 0.01, ***p < 0.001 (unpaired student t test, two-sided)
both proteins are involved in the activation of E4m in the Cd4 gene and the PE enhancer in the Thpok gene. This also suggests that activation of the Thpok gene might involve DNA demethylation. Release of Thpok silencing from Cd8+ T cells by an inhibitor for maintenance methyltransferase and the low Thpok expression level effected by a lack of Tet2/3 proteins support the involvement of DNA de-methylation in Thpok activation.

In B2m−/−: Cd4E4m/E4m mice, a re-directed CD4−CD8+ cell subset is generated in the thymus in part due to impaired ThPOK induction; however, these CD4−CD8+ thymocytes failed to expand sufficiently to generate a visible population in the periphery. As these cells are MHC-II restricted, the lack of TCR signaling by loss of Cd4 expression could impair their survival capacity. However, previous reports have shown that mice expressing a low level of ThPOK retain re-directed CD4−CD8+ MHC-II restricted cells in the periphery. It is possible that aberrant CD4 expression kinetics unique to Cd4E4m/E4m mice may generate CD4−CD8+ MHC-II restricted cells that acquire IL-7-dependent survival capacity similar to normal MHC-I restricted CD8 SP thymocytes with a low level of IL-7Ra expression. An imbalance between retaining an IL-7-responsive property and inefficient receptor expression might thus inhibit the expansion of CD4−CD8+ MHC-II restricted cells in Cd4E4m/E4m mice.

Our genetic results clearly showed that Runx proteins are necessary for S4-independent repression of E4m activity in CD8+ T cells. Previous research showed that mutant Runt protein, a counterpart of Runx protein in Drosophila, lacking DNA binding activity by point mutations in the Runt-domain, retains the activity to repress the segment polarity gene engrailed (en), indicating that recognition of the Runx-motif is not absolutely necessary for Runx-mediated repression. Once Runx proteins are included into nuclear protein complexes on E4m, TLE/Groucho co-repressor family proteins are likely to be recruited and suppress E4m activity in a CD8-lineage-predominant manner. In contrast, although Runx complexes also associate with the E4p enhancer even in the absence of S4, inhibition of E4p activity requires S4 sequences. Thus, the modes of Runx action to repress the two enhancers in the Cd4 locus are different, although the VWRPY motif in Runx proteins are shared by two modes. It is possible that protein complexes formed on the S4 region are required specifically to repress transcriptional activators on the E4p enhancer. Alternatively, based on the different position of the two enhancers with respect to S4, topological regulation such as prevention of chromatin looping between E4p and the P4 promoter may require S4-dependent regulation.

An S4-independent regulation of lineage specificity of E4m activity challenges the previous silencer-based model that has been proposed to explain how lineage-specific Cd4 expression is
ThPOK is necessary for Runx-mediated repression of E4m activity. a Flow cytometry analyses for CD4, CD8 and Thpok-gfp expression during T cell development in Cd4ΔE4p:S4M/ΔE4p:S4M mice in the presence or absence of ThPOK. Numbers in quadrants, indicated gate, and the region in histograms indicate respective cell percentages. b Summary of percentage of CD4+ cells in CD8+ mature thymocytes and CD8+ splenic T cells of Cd4ΔE4p:S4M/ΔE4p:S4M, Thpok+/+/gfp and Cd4ΔE4p:S4M/ΔE4p:S4M, Thpok-gfp/gfp mice. Means ± SD. *** p < 0.001 (unpaired student t test, two-sided). c Histogram showing CD4 expression in freshly selected (CD24hiTCRβhi) thymocytes of Cd4ΔE4p:S4M/ΔE4p:S4M, Thpok+/+/gfp and Cd4ΔE4p:S4M/ΔE4p:S4M, Thpok-gfp/gfp mice. One representative of two independent analyses. d Graph showing summary of three independent ChIP-qPCR experiments for Runx bindings to E4m in pre-selection DP thymocytes from Cd4ΔE4p:S4M/ΔE4p:S4M, Thpok+/+/gfp and Cd4ΔE4p:S4M/ΔE4p:S4M, Thpok-gfp/gfp mice. Means ± SD

Methods

Generation of mutant Cd4 alleles. In order to generate the target vector for the Cd4ΔE4th allele, we first generated an Eth DNA fragment by overlap PCR. In the Eth
sequences, core enhancer sequences from the thymic enhancer (TE) and the proximal enhancer (PE) from the Thpok locus were conjugated (Supplementary Fig. 1a). In order to make the vectors E4p with Eib, the Eib DNA fragment was ligated into the SaII site of the target vector (a gift from Mark Chong) that was used to generate the E4pΔEi allele. Transfection of target vectors into M1 ES cells and identification of ES clones underwent homologous recombination by PCR were performed. During a process of removal of the neomycin resistant gene (neo+) by transient transfection of a Cre expression vector, pM-Cre, Cre clones harboring C4dΔ1/ΔEi or C4dΔ1/mice were generated from the ES clones harboring the C4dΔ1/mice allele. In order to make the target vector for the C4dΔ1/mice allele in which mouse genomic sequences, core enhancer sequences from corresponding to 124835251-124835615 in NCB137/mmm were deleted (Supplementary Fig. 2b), we first amplified the 12483833-1283251 region by PCR to add BglII site at 5’ end and SpeI site at 3’ end. In order to make a longer 3’ homology region, a DNA fragment prepared from the pCassette vector by SpeI/BglII digestion, and were ligated together with a BglII/BglIII 3’ homology region into the HindIII/KpnI cleaved pUC18 vector. In order to generate ES clones harboring the C4dΔ1/mice and C4dΔ1/mice allele, was targeted by retroviral Cre transduction, followed by PCR that distinguished pattern of recombination between three loxP sites (Supplementary Fig. 2c). ES clones harboring the C4dΔ1/mice or C4dΔ1/mice were similarly generated by transfection of the target vector that remove S4 core region (124835896-124836384 in NCB137/mmm) into C4dΔ1/mice ES clone, respectively. ES clones harboring the C4dΔ1/mice were generated by transfection of the target vector, which was used to target mutation into the Runx sites within the S4, into the C4dΔ1/mice ES clone.

**Mice.** Runx1+/- mice, Runx3+/- mice, Thpok+/- mice and Bcl11b+/- mice have been described. 5’-TCTGGAACTG-3’ and 5’-TCCTCCTCAGACCGCTTTT-3’ were used as the primer set for PCR amplification from FACS-sorted cells was subjected to quantitative ChIP-qPCR, FACS-sorted or MACS (Mitenyi Biotec)-separated 0.5 to 3 x 10^6 cells were used to prepare chromatin DNA. Cells were cross-linked by incubation in a 1% of formaldehyde solution for 10 min with gentle rotation at room temperature. Nuclear were separated and were sonicated using a model XL2000 ultrasonic cell disruptor (MICROSON). Sonicated chromatin was incubated overnight at 4 °C with anti-Histone H3K27ac (clone D5E4, Cell Signaling Technology), anti-Histone H3K27me3 (clone C6M811), 5 ug of anti-Cbf antibody (A300-385A, Bethyl Laboratories) or anti-ThPOK polyclonal antibody (23) that were pre-conjugated with Dynabeads M-280 Sheep anti-Rabbit IgG (Thermo Fisher Scientific). After washing beads, immunoprecipitates were eluted from beads into elution buffer. Eluted immunoprecipitates were incubated at 65 °C overnight for reverse-crosslinking. Input DNA and ChIP DNA were treated with RNaseA (Thermo Fisher Scientific) and Proteinase K (Thermo Fisher Scientific), and then purified by ChiP DNA Clean and ConcentratorTM kit (ZYMOM RESEARCH). Quantitative PCR was performed using the QuantStudio 3 Real-Time PCR system (Applied Biosystems) with SYBR Green detection system. Primers sequences for quantitative PCR are listed in the Supplementary Table 1.

In vitro T-cell culture. Sorted CD4+ T cells were labeled with CFSE (ThermoFisher Scientific) and were cultured in custom ordered Dulbecco's Modified Eagle Medium (D-MEM, KOHJIN BIO) supplemented with 10% heat inactivated FBS (HyClone). 2.0 x 10^6 cells were stimulated in 96-well flat-bottomed plate, which was pre-coated with 2 μg/ml anti-CD3ε antibody (553068, BD Biosciences) and 2 μg/ml soluble anti-CD28 antibody (553295, BD Biosciences), for two days, and were maintained in D-MEM supplemented with 10 mg/ml recombinant mouse IL-2 (402-ML, R&D system) for 2 to 4 days.

**Data availability.** ChiP-seq data that support the findings of this study have been deposited with accession GSE90949 and GSE90794, respectively. All other relevant data are available from the authors.

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**References**

1. Zamoyta, R. CD4 and CD8: modulators of T-cell receptor recognition of antigens and of immune responses? Curr. Opin. Immunol. 10, 82–87 (1998).
2. Ellmeier, W., Sawada, S. & Littman, D. R. The regulation of CD4 and CD8 coreceptor gene expression during T cell development. Annu. Rev. Immunol. 17, 523–554 (1999).
3. Hoggquist, K. A. & Jameson, S. C. The self-obscision of T cells: how TCR signaling thresholds affect fate ‘decisions’ and effector function. Nat. Immunol. 15, 815–823 (2014).
4. Sawada, S., Scarborough, J. D., Killeen, N. & Littman, D. R. A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. Cell 77, 917–929 (1994).
5. Sui, G., Wurster, A. L., Duncan, D. D., Soliman, T. M. & Hedrick, S. M. A transcriptional silencer controls the developmental expression of the CD4 gene. EMBO J. 13, 3570–3579 (1994).
6. Zou, Y. R. et al. Epigenetic silencing of CD4 in T cells committed to the cytotoxic lineage. Nat. Genet. 29, 332–336 (2001).
7. Leung, R. K. et al. Deletion of the CD4 silencer element supports a stochastic mechanism of thymocyte lineage commitment. Nat. Immunol. 2, 1167–1173 (2001).
8. Taniguchi, I., Ellmeier, W. & Littman, D. R. The CD4/CD8 lineage choice: new insights into epigenetic regulation during T cell development. Adv. Immunol. 83, 55–89 (2004).
9. Taniguchi, I. et al. Differential requirements for Runx proteins in CD4 expression and epigenetic silencing during T lymphocyte development. Cell 111, 621–633 (2002).
10. Woolf, E. et al. Runx3 and Runx1 are required for CD8 T cell development during thymopoiesis. Proc. Natl Acad. Sci. USA 100, 7731–7736 (2003).
11. Chong, M. M. et al. Epigenetic propagation of CD4 expression is established by CD4 proximal enhancer in helper T cells. Genev Dev. 24, 659–669 (2010).
12. Sellars, M. et al. Regulation of DNA methylation dictates CD4 expression during the development of helper and cytotoxic T cell lineages. Nat. Immunol. 16, 746–754 (2015).
13. He, X. et al. CD4-CD8 lineage commitment is regulated by a silencer element at the ThPOK transcription-factor locus. Immunity 28, 346–358 (2008).
14. Muroi, S., Tanaka, H., Miyamoto, C. & Taniguchi, I. Cutting edge: fine-tuning of Thpok gene activation by an enhancer in close proximity to its own silencer. J. Immunol. 190, 1397–1401 (2013).
15. Tsagaratou, A. et al. TET proteins regulate the lineage specification and TCR-mediated expansion of iNKT cells. Nat. Immunol. 18, 45–53 (2017).
16. Muroi, S. et al. Cascading suppression of transcriptional silencers by ThPOK seals helper T cell fate. Nat. Immunol. 9, 1113–1121 (2008).
17. Henson, D. M., Chou, C., Sakurai, N. & Egawa, T. A silencer-proximal intronic region is required for sustained CD4 expression in postselection thymocytes. J. Immunol. 192, 4620–4627 (2014).
18. Shalyueva, D., Stampfel, G. & Stark, A. Transcriptional enhancers: from properties to genome-wide predictions. Nat. Rev. Genet. 15, 272–286 (2014).
19. Rui, J., Liu, H., Zhu, X., Cui, Y. & Liu, X. Epigenetic silencing of CD8 genes by ThPOK-mediated deacetylation during CD4 T cell differentiation. J. Immunol. 189, 1380–1390 (2012).
20. Wang, L. et al. The zinc finger transcription factor Zhub7b represses CD8-lineage gene expression in peripheral CD4^+ T cells. Immunity 29, 876–887 (2008).
21. Park, J. H. et al. Signaling by intrathymic cytokines, not T cell antigen receptors, specifies CD8 lineage choice and promotes the differentiation of cytotoxic-lineage T cells. Nat. Immunol. 11, 257–264 (2010).
22. Tani-ichi, S. et al. Interleukin-7 receptor controls development and maturation of late stages of thymocyte subpopulations. Proc. Natl Acad. Sci. USA 110, 612–617 (2013).
23. Kojo, S. et al. Priming of lineage-specifying genes by Bcl11b is required for lineage choice in post-selection thymocytes. Nat. Commun. 8, 702 (2017).
24. Levanon, D. et al. Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/Groucho corepressors. Proc. Natl Acad. Sci. USA 95, 11590–11595 (1998).
25. Aronson, B. D., Fisher, A. L., Blechman, K., Caudy, M. & Gergen, J. P. Groucho-dependent and -independent repression activities of Runx domain proteins. Mol. Cell Biol. 17, 5581–5587 (1997).
26. Seo, W. et al. Roles of WVRPY motif-mediated repression of Runx proteins during T-cell development. Immunol. Cell Biol. 90, 827–830 (2012).
27. Kakugawa, K. et al. Essential roles of SATB1 in specifying T lymphocyte subsets. Cell Rep. 19, 1176–1188 (2017).
28. Wildt, K. F. et al. The transcription factor zhub7b promotes CD4 expression by antagonizing runx-mediated activation of the CD4 silencer. J. Immunol. 179, 4405–4414 (2007).
29. Pastor, W. A., Aravind, L. & Rao, A. TETonic shift: biological roles of TET proteins in DNA demethylation and transcription. Nat. Rev. Mol. Cell Biol. 14, 341–356 (2013).
30. Tsagaratou, A. et al. Dissecting the dynamic changes of 5-hydroxymethylcytosine in T-cell development and differentiation. Proc. Natl Acad. Sci. USA 111, E3306–E3315 (2014).
31. Tanaka, H. et al. Epigenetic Thpok silencing limits the time window to choose CD4(+) helper-lineage fate in the thymus. EMBOJ 32, 1183–1194 (2013).
32. Polic, B., Kunkel, D., Scheffold, A. & Rajewsky, K. How alpha beta T cells deal with induced TCR alpha ablation. Proc. Natl Acad. Sci. USA 98, 8744–8749 (2001).
33. Egawa, T. & Littman, D. R. ThPOK acts late in specification of the helper T cell lineage and suppresses Runx-mediated commitment to the cytotoxic T cell lineage. Nat. Immunol. 9, 1131–1139 (2008).
34. Vander Zwan, C. J., Wheeler, J. C., Li, L. H., Tracey, W. D. & Gergen, J. P. A DNA-binding-independent pathway of repression by the Drosophila Runt protein. Blood. Cells Mol. Dis. 30, 207–222 (2003).
35. Zullo, J. M. et al. DNA sequence-dependent compartmentalization and silencing of chromatin at the nuclear lamina. Cell 149, 1474–1487 (2012).
36. Taniuchi, I., Sunshine, M. J., Festenstein, R. & Littman, D. R. Evidence for distinct CD4 silencer functions at different stages of thymocyte differentiation. Mol. Cell 10, 1083–1096 (2002).
37. Nishimura, M. et al. WVRPY motif-dependent and -independent roles of AML1/Runx1 transcription factor in murine hematopoietic development. Blood 103, 562–570 (2004).
38. Yarmus, M. et al. Groucho/transducin-like Enhancer-of-split (TLE)-dependent and -independent transcriptional regulation by Runx3. Proc. Natl Acad. Sci. USA 103, 7384–7389 (2006).
39. Naoe, Y. et al. Repression of interleukin-4 in T helper type 1 cells by Runx3/Chb beta binding to the I4 silencer. J. Exp. Med. 204, 1749–1755 (2007).

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S.K., N.Y. and M.T. performed phenotypic analyses and ChIP. S.M. generated mouse strains. I.T. designed experiments and wrote the manuscript.

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