Lineage-affiliated transcription factors bind the *Gata3 Tce1* enhancer to mediate lineage-specific programs

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The transcription factor GATA3 is essential for the genesis and maturation of the T cell lineage, and GATA3 dysregulation has pathological consequences. Previous studies have shown that GATA3 function in T cell development is regulated by multiple signaling pathways and that the Notch nuclear effector, RBP-J, binds specifically to the *Gata3* promoter. We previously identified a T cell–specific *Gata3* enhancer (*Tce1*) lying 280 kb downstream from the structural gene and demonstrated in transgenic mice that *Tce1* promoted T lymphocyte–specific transcription of reporter genes throughout T cell development; however, it was not clear if *Tce1* is required for *Gata3* transcription in vivo. Here, we determined that the canonical *Gata3* promoter is insufficient for *Gata3* transcriptional activation in T cells in vivo, precluding the possibility that promoter binding by a host of previously implicated transcription factors alone is responsible for *Gata3* expression in T cells. Instead, we demonstrated that multiple lineage-affiliated transcription factors bind to *Tce1* and that this enhancer confers T lymphocyte–specific *Gata3* activation in vivo, as targeted deletion of *Tce1* in a mouse model abrogated critical functions of this T cell–regulatory element. Together, our data show that *Tce1* is both necessary and sufficient for critical aspects of *Gata3* T cell–specific transcriptional activity.

Introduction

The independent lineages of mature hematopoietic cells are initially generated from stem cells that are extrinsically and intrinsically regulated to traverse multiple, distinct developmental stages. A host of tissue- and stage-affiliated transcription factors and signaling pathways plays essential roles in achieving the final differentiated state of each hematopoietic lineage. The appropriate contribution of different factors and signaling pathways to each lineage-specific transcriptional network ultimately determines the developmental fate and activity of each hematopoietic cell type.

Following the circulation of immature hematopoietic cells from the bone marrow to the thymus, early T lineage progenitors (ETPs) are generated and undergo development into double-negative cells (stages DN2 to DN4), in which neither the CD4 nor the CD8 coreceptor is expressed. β-Selection, one of several critical steps during T cell development, occurs at the DN3 stage, and only thymocytes that successfully rearrange the T cell receptor (TCR) β locus (and therefore express a functional pre-Tα/TCRβ complex) are licensed to differentiate further and transition to the DN4 and immature single-positive (SP; $CD4^-CD8^+TCRb^{lo}$) stages. As those immature SP cells become double positive (DP) for the CD4 and CD8 coreceptors, the TCRα locus rearranges. DP cells that express a functional TCRαβ receptor on their cell surface then undergo positive selection and move into the $CD4^+CD8^{lo}$ intermediate stage. $CD4^+CD8^{lo}$ cells are still uncommitted to a specific T cell cytotoxic or helper function, and thus CD4 versus CD8 lineage choice occurs at this stage. Persistent TCR signaling contributes to CD4 lineage fate, and cells differentiate into CD4 SP cells, while cessation of TCR signaling and initiation of IL-7 signaling contribute to CD8 lineage fate. CD4 and CD8 cells then exit the thymus and circulate to peripheral lymphoid organs where they can acquire effector functions as either helper T cells (CD4 lineage) or cytotoxic T cells (CD8 lineage) (reviewed in refs. 1, 2).

Following our original identification of transcription factor GATA3 in chicken, mouse, and human cells (3, 4), we and others showed that it is expressed throughout T cell development, although its level varies significantly between stages, from abundant expression in CD4 cells to quite low expression in CD8 cells (5–11). Numerous studies have demonstrated the crucial importance and essential contributions of GATA3 to different stages of T cell development, in ETP (12), DN1 (13), the DN3-to-DN4 transition (14), CD4 cells (14, 15), and Th2 cells (16, 17). Although GATA3 is dispensable for the initial generation of CD8 cells, it is required for their final maturation, maintenance, and function (18, 19). In addition to the T cell lineage, GATA3 plays important roles in the innate immune system (20–22) and in NK cell development (23, 24). In contrast, B lymphocyte development requires *Gata3* repression (25).

Although its pervasive expression is essential throughout normal T cell development, forced expression or underexpression of GATA3 can trigger pathological consequences (26–30), for example, generating T cell lymphoma in transgenic (Tg) mice (27) or elevated susceptibility to allergic airway inflammation (31, 32). Additionally, GATA3 is aberrantly expressed in Hodgkin's lymphoma (33) and controls cytokine expression, which plays an important role in the pathogenesis of Hodgkin's disease (34). Haploinsufficient *GATA3* mutation in humans leads to HDR syndrome (hypoparathyroidism, sensorineural deafness, and renal disease;

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ref. 35). Collectively, these data highlight the conclusion that normal T cell development requires quantitatively and qualitatively stringent control over GATA3 expression.

We previously reported the identification of a T cell–specific *Gata3* enhancer, which we originally named *TCE-7.1* (referred to hereafter as *Tce1*) (36). *Tce1* is a 7.1-kb segment of the locus located 280 kbp 3′ to the *Gata3* structural gene. We showed that this enhancer induces T lymphocyte–specific transcription of reporter genes throughout T cell development and enhances NK cell expression (36). However, multiple additional mechanistic questions emerged from that study. For example, is *Tce1* necessary for *Gata3* regulation of T cell development in vivo or does it act in a redundant fashion with additional currently unidentified *cis* elements? What is the mechanism of action of *Tce1* (that is, how does *Tce1* differentially regulate the precise differential abundance of GATA3 at multiple T cell developmental stages)? Finally, what transcription factors elicit appropriate *Gata3* transcriptional responses in a stage-specific manner through this enhancer?

Here, we report that homozygous genetic loss of *Tce1* in vivo using CRISPR/Cas9-mediated genome editing impairs at least two critical T cell stages: the initial generation of ETP and the development of CD4 lymphocytes. We also show that *Tce1* contains a critical enhancer sequence defined by a 1.2-kbp core fragment that regulates transcription in developmental stages from ETP through to naive T cells. Previous studies showed that *Gata3* is regulated by transcription factors HEB (37, 38), T cell factor 1 (TCF-1) (39), and RBP-J/CSL (40), all of which bind to the promoter. We show that the binding of those factors to the *Gata3* promoter alone is insufficient to confer T cell–specific activation to *Gata3* but that the same factors also bind avidly within *Tce1*, providing a possible explanation for how *Gata3* regulation mediated through *Tce1* can be responsive to the Wnt (41) and Notch (40) signaling pathways.

Results

Tce1 is necessary for ETP and CD4 T cell development. One of the central questions that emerged from our previous study (36), which showed that *Tce1* was sufficient for reporter gene T cell–specific activation throughout T cell development, was whether *Tce1* is also necessary for *Gata3* activation in vivo. To address this question, we targeted the genomic locus using CRISPR/Cas9 guide RNAs (gRNAs) (42) corresponding to sequences on either side of the 7.1 kbp that define the boundaries of *Tce1* and, at the same time, introduced single-stranded oligonucleotides bearing genomic homology that would additionally incorporate loxP sequences into the two targeted sites (Figure 1A and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI83894DS1). gRNAs were selected after examination of the genome for similar sequences that had the fewest potentially related off-target homologies. In this manner, we anticipated, after injection of the constructs into fertilized murine ova, the generation of germ line mutants in which *Tce1* would be unaltered in function until conditional deletion of the enhancer after Cre recombinase excision. Both gRNA and CRISPR/Cas9 vectors were microinjected into 441 fertilized oocytes of F_2 animals generated by crossing C57BL/6J males already bearing the Mx1-Cre Tg (43) with wild-type SJL females; 77 live pups were screened by PCR for mutations incorporated at the gRNA-targeted sequences.

The results of analysis of these offspring indicated that 3 of the pups displayed evidence for incorporation of both loxP sites (either in *cis* or *trans*), while 3 of the animals had heterozygous deletions and 1 of the animals had homozygous deletions of the entire 7.1-kbp sequence lying between the two gRNA sites (Supplemental Figure 2). Since the two gRNAs targeted for the 5′ and 3′ ends of *Tce1* were injected at the same time, double-strand breaks probably occurred simultaneously at both ends and resulted in deletion of *Tce1* by nonhomologous end joining, as previously reported (44). Sequencing across the two sites in amplified genomic DNA under conditions that would not detect the germ line configuration confirmed deletion of *Tce1* from at least one allele (Supplemental Figure 2). In this study, we focused on the deleted alleles and did not further analyze the potential floxed alleles.

Next, we examined thymocytes, peripheral blood, and spleens for possible alterations in hematopoietic and T cell development and/or *Gata3* mRNA expression that could be detected as a consequence of *Tce1* loss in both homozygous and heterozygous F_3 and subsequent generation mice (Figure 1, B–D, and Supplemental Figure 3), as previously shown (12). As anticipated, homozygous loss of *Tce1* (*Tce1*$^{-/-}$) led to a reduction in the number of ETPs (62% of that of heterozygous controls), and *Gata3* mRNA in the remaining ETPs was only 52% of that of controls (Figure 1B). In contrast, the number of *Tce1*$^{-/-}$ DN2 and DN3 stage cells was unaltered compared with that of controls, although *Gata3* mRNA levels in DN2 thymocytes were reduced (83% of that of heterozygous controls; Figure 1B). This modest reduction in the number of early stage T cells in *Tce1*$^{-/-}$ mice is similar to the phenotype observed in *Gata3* hypomorphic mutants, which express low levels (10%–20% of that of wild-type animals) of a GATA3-EGFP fusion protein in thymocytes of adoptively reconstituted animals (12). In *Tce1*$^{-/-}$ DN4 and DP stages, *Gata3* mRNA was reduced to 55% and 45% of that of controls, respectively, while the total number of cells was normal.

The data shown here demonstrate that T cell development is severely affected in *Tce1*$^{-/-}$ mice. The number of CD4 SP thymocytes was reduced to 30% of that of controls, and *Gata3* mRNA abundance in the surviving CD4 thymocytes was reduced to 42% of that of control values (Figure 1B), while, in contrast, the numbers of CD4 SP thymocytes and mRNA levels of *Gata3* in CD8 T cells were comparable in *Tce1*$^{-/-}$ and heterozygous control mice (Figure 1B). Similarly, the number of CD4 T cells in *Tce1*$^{-/-}$ mice was reduced in the peripheral blood (30% of that of controls, Figure 1C) and spleens (39% of that of controls, Figure 1D) in comparison to those in controls, while the number of peripheral CD8 T cells was unaffected. The CD4 T cell–specific defect observed in *Tce1*$^{-/-}$ mutant mice is consistent with previous studies in which the *Gata3* gene was conditionally ablated by a *Cd4-Cre* Tg (14, 15). The normal level of *Gata3* mRNA found in surviving peripheral CD4 T cells suggests that cells retaining less abundant GATA3 expression cannot survive or that their proliferation is retarded at this stage. To further characterize the peripheral CD4 T cells generated in the absence of *Tce1*, we analyzed expression of CD44 and CD62L, which can distinguish naive T cells from memory T cells (45). While the CD44^{lo}CD62L^{+} naive T cell number was significantly reduced in splenic CD4 T cells isolated from *Tce1*$^{-/-}$ mice when compared with that in heterozygous controls (Supplemental Figure 4), the CD44^{hi}CD62L^{-} memory T cell number was compa-

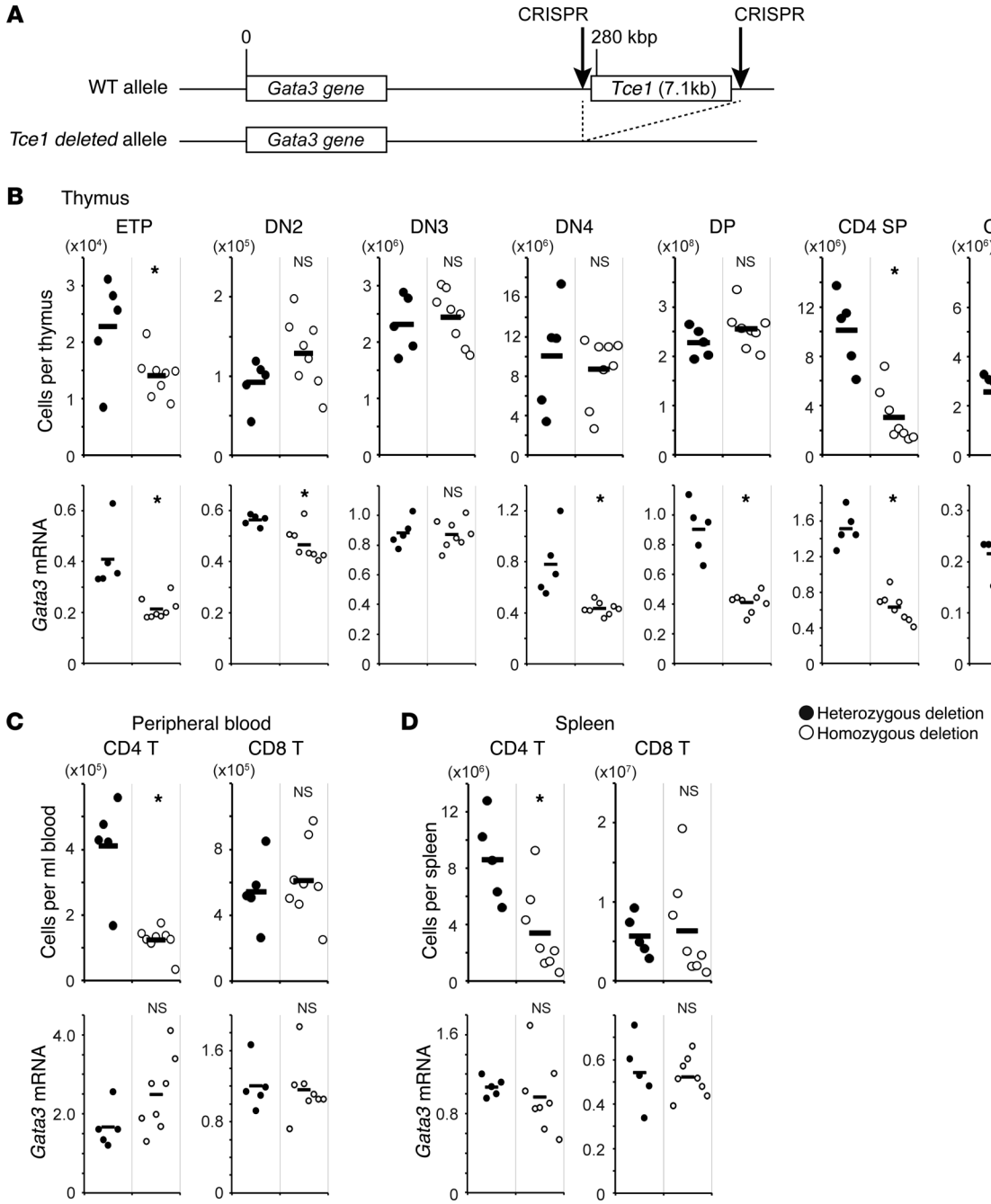

Figure 1. In vivo genome deletion of *Tce1*. (**A**) Two CRISPR/Cas9 plasmids expressing gRNAs that correspond to sequences surrounding the 7.1 kbp that define the boundaries of *Tce1* were coinjected into mouse fertilized oocytes. *Tce1*-deleted mutant allele founder (F_0) mice were intercrossed to obtain homozygous deletion mutant mice. (**B**) Thymocytes, (**C**) peripheral blood cells, and (**D**) splenocytes isolated from F_2 animals at 5 to 6 weeks of age bearing homozygous (white circle) or heterozygous (black circle) deletions of *Tce1* were analyzed for cell surface expression of T cell stage–specific markers. The absolute numbers of $Lin^-cKit^{hi}CD25^-$ (ETP), $Lin^-cKit^{hi}CD25^+$ (DN2), $Lin^-cKit^{lo/-}CD25^+$ (DN3), $Lin^-cKit^{lo/-}CD25^-$ (DN4), $CD4^+CD8^+$ (DP), $CD4^+CD8^-CD3^+TCR\beta^+$ (CD4 SP), and $CD4^-CD8^+CD3^+TCR\beta^+$ (CD8 SP) thymocytes are shown at the top. Each stage of T cells was isolated by flow cytometry and analyzed for the expression of *Gata3* mRNA, as normalized to *Hprt* by qRT-PCR. Each circle represents an individual mouse, and black bars represent the average for each genotype. Data are representative of the summary of 2 independent experiments. $^*P < 0.05$ by Student's *t* test.

rable in the two genotypes. The data suggest that compensatory expansion or accumulation of memory T cells in *Tce1*-deleted mutants must exist. The data clearly demonstrate that CD4 T cell development is severely compromised by deletion of *Tce1* in mice.

Collectively, the data demonstrate that *Tce1* is necessary for *Gata3* gene expression and therefore T cell development in ETP and CD4 stages in the thymus as well as in CD4 T cells in the peripheral blood and spleen. *Tce1* also contributes to *Gata3* expression in DN4 and DP stage cells, even though the number of those cells that accumulate is normal whether *Tce1* is present or absent. We concluded that *Tce1* is a critical regulator of the *Gata3* gene in a subset of thymocytes. Since the phenotypes

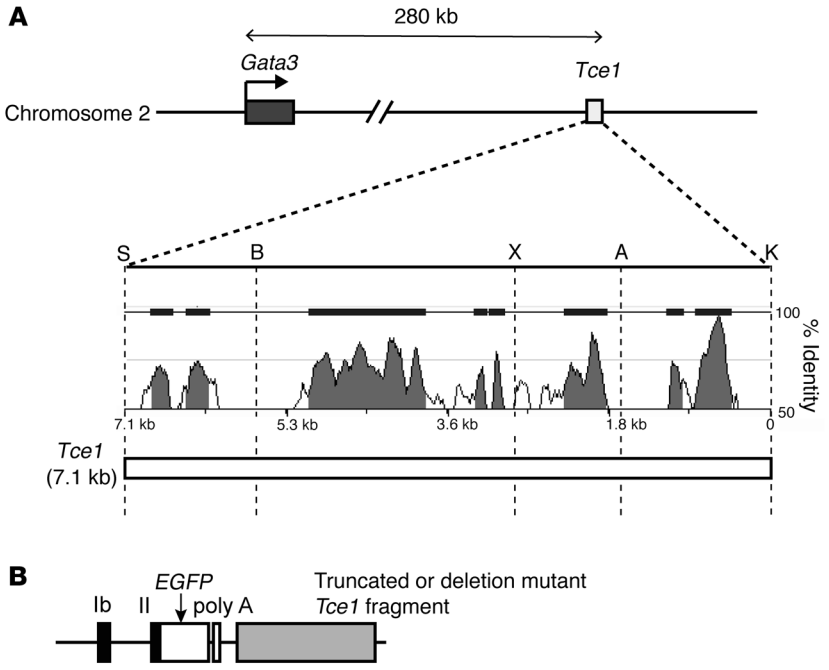

Figure 2. Truncation and deletion mutants of *Tce1*. (**A**) Diagrams of the mouse *Gata3* locus and *Tce1*. Sequence homology between mouse and human *Tce1* sequences is depicted as a Manhattan diagram. Restriction enzyme recognition sites Sal I (S), BstII (B), XhoI (X), ApaLI (A), and KpnI (K) are shown. The genome position of *Tce1* is mm9 chr2, position 9,515,156 to 9,522,270. (**B**) The structure of the EGFP reporter plasmid used in this study is depicted.

observed in *Tce1*$^{-/-}$ mutant mice are milder than those in mice in which the *Gata3* gene itself is ablated and since diminished accumulation of GATA3 transcripts was detected in the affected stages of T cells, a redundant but currently unidentified second enhancer(s) appears to at least partially compensate for the loss of in *Tce1* in vivo (see Discussion).

Dissection of the molecular architecture that confers Tce1 activity during thymocyte development. Given that homozygous deletion of *Tce1* from the genome resulted in compromised T cell development (Figure 1) and that *Tce1* is capable of driving transcription of a reporter gene at all T cell developmental stages (36), one can imagine several possibilities for how *Tce1* might function. For example, *Tce1* might contain only a single *cis* element that is capable of regulating transcription at all T cell developmental stages in response to modulated signals received from signal-activated transcription factors, as they respond to intracellular or extracellular differentiation signals. Alternatively, the 7.1-kbp *Tce1* might harbor multiple *cis* elements, each of which additively or synergistically contributes to distinct stages of T cell development.

In order to experimentally distinguish among these possibilities, we prepared multiple fluorescent reporter constructs, each directed by the *Gata3 1b* promoter (46) and bearing various *Tce1* fragments (Figure 2B, shown in detail in Figure 3A, Figure 4A, Figure 5A, Figure 6A, and Figure 7A). Tg animals whose transcription was directed by an individual fragment of *Tce1* (e.g., the 1.2-kb fragment shown in Figure 4A) are designated as such (e.g., Tg$^{1.2}$), while deletion of each part of *Tce1* from the whole is designated by a preceding Δ (e.g., Tg$^{\Delta 1.2}$, Figure 5A). The details for plasmid constructions are described in the Supplemental Methods.

We first bisected *Tce1* into two 2.9-kb and 4.2-kb fragments, respectively (Figure 3A). Both fragments contained multiple conserved noncoding sequences (CNSs; Figure 2A) as well as dozens of putative binding sites for T cell–affiliated transcription factors, so an a priori evaluation of their transcriptional potential in T cells was not possible. Each of the EGFP reporter plasmids was injected into fertilized ova to generate founder (F_0) Tg mice (referred to as Tg$^{2.9}$ and Tg$^{4.2}$ mice, respectively), since we had previously demonstrated that T cell *cis* element(s) could be rapidly and efficiently identified by founder analysis (36). One important fact to keep in mind for this analysis is that the *Tce1*-driven EGFP reporter transgenic mouse line (Tg^{Tce1}) is a stable, established Tg line, while Tg$^{2.9}$ mice and all other Tg mice examined in this study are founders. Therefore, it is extremely difficult to quantitatively compare data among different founder animals or between founders and lines.

When we analyzed reporter gene expression in T cells from peripheral blood, we found that EGFP was expressed in CD4 T cells in the majority of Tg$^{2.9}$ mice but not in Tg$^{4.2}$ mice (Table 1 and Supplemental Figure 5A). Furthermore, and as is also true for Tg^{Tce1} mice, EGFP expression was observed in Tg$^{2.9}$ animals at multiple stages, including ETP and CD4 T cells (Figure 3 and Table 2). In addition to αβTCR$^+$ T cells, γδTCR$^+$ thymocytes expressed EGFP (Supplemental Figure 6 and Supplemental Table 1). In contrast, EGFP expression was never detected in thymocytes from Tg$^{4.2}$ mice (Figure 3 and Table 2), and neither group of founders expressed EGFP in other hematopoietic lineages (Supplemental Table 2). These results show that the 2.9-kb fragment, but not the 4.2-kb fragment, is sufficient to direct transcription of reporter genes throughout thymocyte development.

Since the 2.9-kb *Tce1* fragment harbors two clusters of CNSs (Figure 2A), we wondered whether either could function as an independent T cell–specific enhancer and whether both are required for transcription in T cells. Therefore, we bisected the

Table 1. EGFP expression in the peripheral blood of F_0 Tg mice

Tg	No. CD4$^+$EGFP$^+$ Tg mice/no. Tg mice (PCR$^+$)
Tg$^{2.9}$	9/14
Tg$^{4.2}$	0/6
Tg$^{1.2}$	4/7
Tg$^{1.7}$	0/4
Tg$^{\Delta 1.2}$	0/7
Tg$^{\Delta 1.7}$	10/14
Tg$^{\Delta 1.5}$	20/24
Tg$^{\Delta 2.7}$	4/7

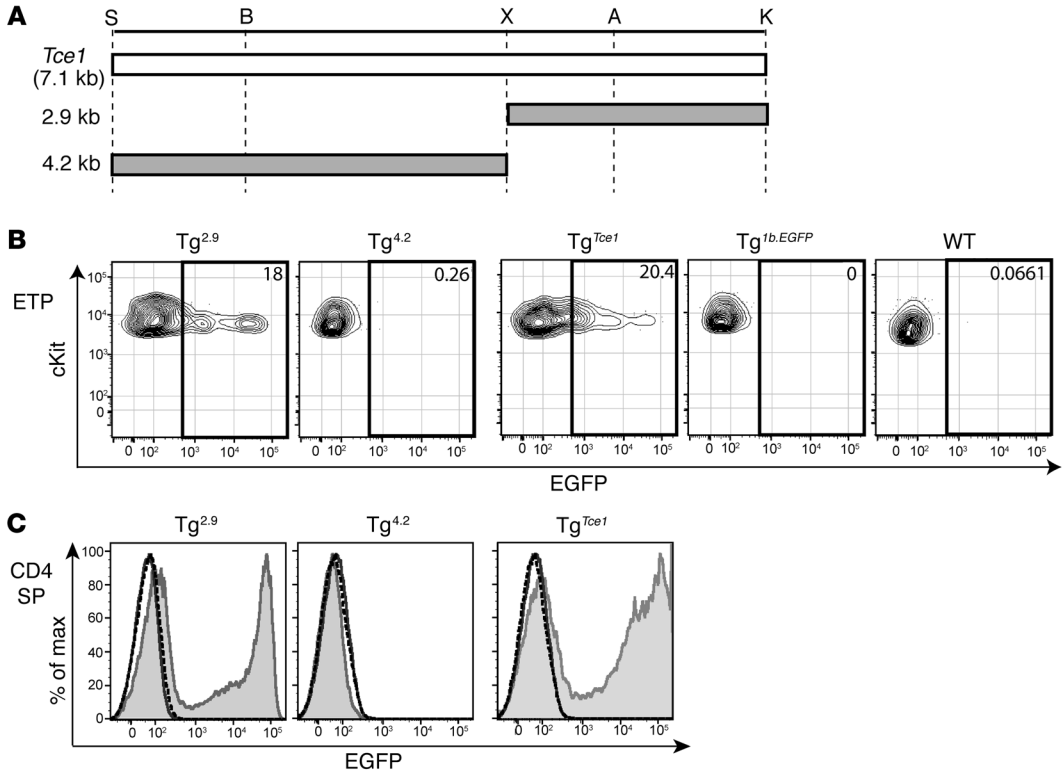

Figure 3. A 2.9-kb fragment of *Tce1* drives thymocyte-specific transcription of reporter genes. (**A**) Illustration of truncated 2.9-kb and 4.2-kb *Tce1* fragments. Restriction enzyme recognition sites are shown. EGFP expression in (**B**) ETP and (**C**) $CD4^+CD8^-TCR\beta^+$ (CD4 SP) cells from $Tg^{2.9}$, $Tg^{4.2}$, Tg^{Tce1}, and $Tg^{1b.EGFP}$ mice and in wild-type thymocytes. Note that $Tg^{2.9}$ and $Tg^{4.2}$ mice are F_0 mice, while Tg^{Tce1} and $Tg^{1b.EGFP}$ mice are from Tg lines established previously. Data are representative of 2 independent experiments; the gray shaded histogram represents expression in $Tg^{2.9}$, $Tg^{4.2}$, or Tg^{Tce1}, respectively; the solid line histogram represents expression in $Tg^{1b.EGFP}$; and the dashed line histogram represents expression in wild-type mice.

2.9-kb fragment into 1.2-kb and 1.7-kb subclones that contained the individual CNSs (Figure 2A and Figure 4A), used these to generate new Tg founders ($Tg^{1.2}$ and $Tg^{1.7}$ mice), and analyzed peripheral blood from those F_0 mice as before. We found that EGFP was expressed in the peripheral blood CD4 T cells in the majority of $Tg^{1.2}$ mice, while EGFP expression was essentially absent in $Tg^{1.7}$ mice (Table 1 and Supplemental Figure 5B). Detailed analyses of their thymocytes revealed that $Tg^{1.2}$ mice again exhibited EGFP expression at multiple stages of thymopoiesis (Figure 4, B and C; Table 2; Supplemental Figure 6B; and Supplemental Table 1), and, as before, EGFP expression was not observed in other kinds of hematopoietic cells (Supplemental Table 2). Taken together, the data show that a 1.2-kb fragment within *Tce1* contains multistage T cell enhancer activity and that this fragment is sufficient to drive the transcription of a reporter gene in all stages of thymocytes.

We next asked whether this 1.2-kb *Gata3 Tce1* fragment was also necessary for the transcription of a reporter gene in T cells. To answer this question, we prepared another EGFP reporter plasmid in which the 1.2-kb sequence was deleted from full-length *Tce1* (Figure 5A) and generated additional F_0 Tg mice ($Tg^{\Delta 1.2}$ mice). None of the F_0 $Tg^{\Delta 1.2}$ mice expressed EGFP in peripheral CD4 T cells (Table 1 and Supplemental Figure 5C) or in thymocytes (Figure 5, B and C), except possibly at the DN4 stage (Table 2). We also analyzed $Tg^{\Delta 1.7}$, $Tg^{\Delta 1.5}$, and $Tg^{\Delta 2.7}$ mice and found that all of them expressed EGFP in peripheral CD4 T cells (Tables 1 and 2). Therefore, the EGFP expression in those mice confirmed that these sequences were not required for *Tce1* enhancer activity in peripheral CD4 T cells. These data show that the 1.2-kb T cell enhancer fragment within *Tce1* is also necessary for reporter gene transcription in T cells and, taken together with the data shown in Figure 1, that this fragment functions as an enhancer core element in vivo for *Gata3* T cell–specific transcription. The data also show that the extended *Gata3* promoter, either alone or when combined with inactive fragments of *Tce1*, is not sufficient for T cell activation.

Transcription of Gata3 in Th2 cells requires cooperation of multiple elements within Tce1. GATA3 is necessary for the differentiation and function of Th2 cells (5), and we have shown that *Tce1* can

Table 2. EGFP expression at each thymocyte developmental stage in F_0 Tg mice

Tg	No. $EGFP^+$ cells by stage in Tg mice/no. Tg mice (PCR^+)						
	ETP	DN2	DN3	DN4	DP	CD4 SP	CD8 SP
$Tg^{2.9}$	7/8[A]	6/8[A]	8/8[A]	8/8[A]	8/8[A]	8/8[A]	8/8[A]
$Tg^{4.2}$	0/6	0/6	0/6	0/6	0/6	0/6	0/6
$Tg^{1.2}$	4/7	2/7	3/7	4/7	4/7	4/7	4/7
$Tg^{1.7}$	0/4	0/4	0/4	0/4	0/4	0/4	0/4
$Tg^{\Delta 1.2}$	0/7	0/7	0/7	3/7	0/7	0/7	0/7

[A]Eight of nine $Tg^{2.9}$ mice whose EGFP expression in peripheral T cells was positive were analyzed.

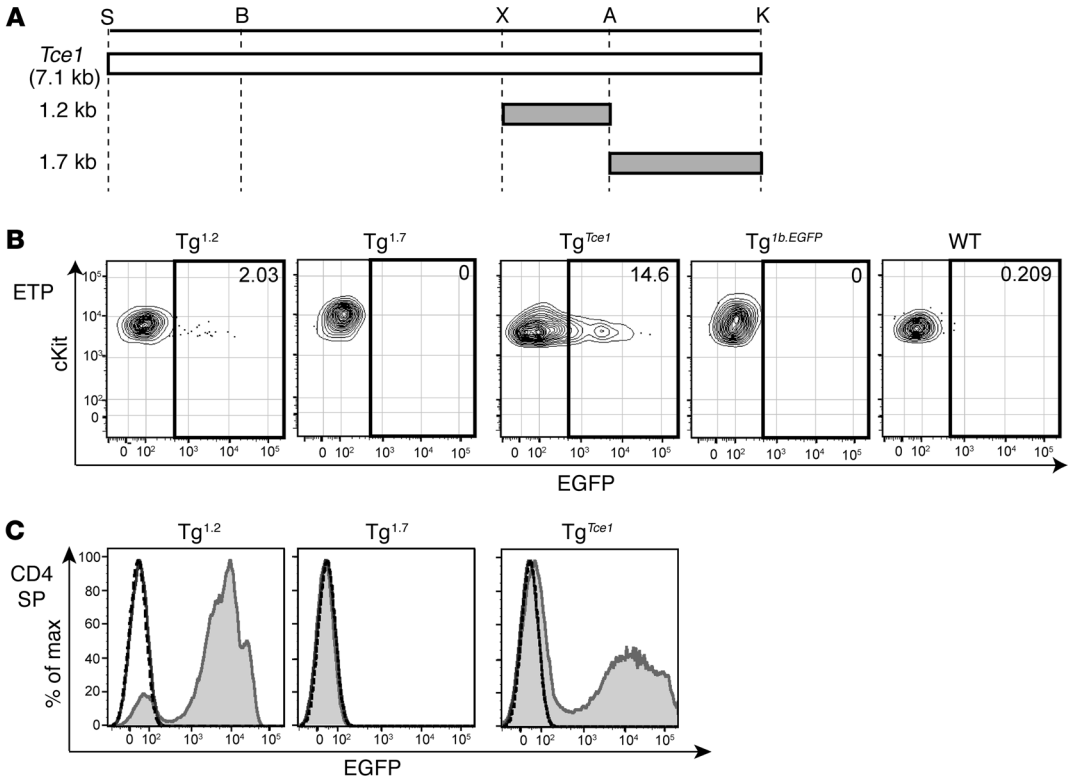

Figure 4. A 1.2-kb fragment of *Tce1* drives thymocyte transcription of reporter genes. (**A**) Illustration of truncated 1.2-kb and 1.7-kb *Tce1* fragments. Restriction enzyme recognition sites are shown. EGFP expression in (**B**) ETP and (**C**) $CD4^+CD8^-TCR\beta^+$ (CD4 SP) cells from $Tg^{1.2}$, $Tg^{1.7}$, Tg^{Tce1}, $Tg^{1b.EGFP}$, and wild-type mice. Note that $Tg^{1.2}$ and $Tg^{1.7}$ mice represent expression in F_0 Tg mice. Data are representative of 2 independent experiments. The gray shaded histogram represents expression of $Tg^{1.2}$, $Tg^{1.7}$, or Tg^{Tce1}, respectively; the solid line histogram indicates the expression of $Tg^{1b.EGFP}$; and the dashed line histogram represents EGFP expression in wild-type thymocytes.

direct the transcription of a reporter gene in peripheral Th2 cells as well as in thymocytes, although significant induction of EGFP at the Th2 stage was not observed, as it is in endogenous GATA3 (36). This observation indicates that *Tce1* activity alone cannot account for all GATA3 expression in Th2 cells and that at least one additional *cis* element may be required to achieve full GATA3 abundance. In order to clarify the mechanism by which *Tce1* contributes to GATA3 expression in Th2 cells, we analyzed EGFP expression of reporter Tg mice bearing *Tce1* fragments and found that naive CD4 cells from both $Tg^{2.9}$ and $Tg^{1.2}$ mice expressed EGFP (Figure 6 and Table 3). We then stimulated naive CD4 T cells under Th2-polarizing conditions in vitro (see Methods) and, surprisingly, discovered that EGFP expression was eliminated in almost all of the $Tg^{2.9}$ and $Tg^{1.2}$ Th2 cells, comparable to reporter expression in $Tg^{4.2}$ and $Tg^{1.7}$ mice (Figure 6 and Table 3). These data show that neither of the initially bisected larger *Tce1* fragments is sufficient for the transcription of the EGFP reporter gene in Th2-polarized cells, while *Tce1* itself does confer such activity. We concluded that multiple individual *cis* elements, located within both the 2.9-kb and 4.2-kb fragments, must collaborate to activate *Gata3* Th2 cell–specific transcription.

Given that the 1.2-kb enhancer core fragment of *Tce1* is necessary for reporter transcription throughout thymocyte development, we asked whether it is also necessary for Th2 transcription. We determined that neither naive CD4 T cells nor Th2-polarized cells that were developed from $Tg^{\Delta 1.2}$ mice exhibited EGFP fluorescence (Figure 7B and Table 3). Thus, the 1.2-kb core enhancer region is necessary, but not sufficient, for reporter gene transcription in Th2 cells.

In order to identify which elements within *Tce1* are involved in the regulation of Th2 transcription, we generated Tg mice bearing internal deletions of *Tce1* (Figure 7A) and analyzed their expression in naive CD4 T cells and in Th2 cells (Figure 7B). Since the data indicate that sequences within the 4.2-kb fragment are involved in Th2 transcription and this fragment contains several CNSs (Figure 2A), we examined reporter gene expression after individually deleting either the 2.7-kb ($Tg^{\Delta 2.7}$) or the 1.5-kb ($Tg^{\Delta 1.5}$) segments of *Tce1*. As shown in Figure 7B and Table 3, naive CD4 T cells from $Tg^{\Delta 2.7}$ and $Tg^{\Delta 1.5}$ mice both expressed EGFP. After those cells were stimulated to undergo Th2 differentiation, both genotypes retained EGFP expression (Table 3). These results indicate that the 2.7-kb and 1.5-kb segments of *Tce1* are both at least partially redundant for Th2 stimulatory activity and that the 2.7-kb fragment may contribute more robustly than the 1.5-kb fragment to Th2 stimulatory activity in collaboration with the 1.2-kb *Tce1* enhancer core fragment.

We also asked whether the 1.7-kb fragment of *Tce1* contributes to transcription in Th2 cells by generating additional deletion mutants ($Tg^{\Delta 1.7}$). Naive CD4 T cells from $Tg^{\Delta 1.7}$ mice expressed EGFP (Figure 7B and Table 3), as expected. Th2-polarized stim-

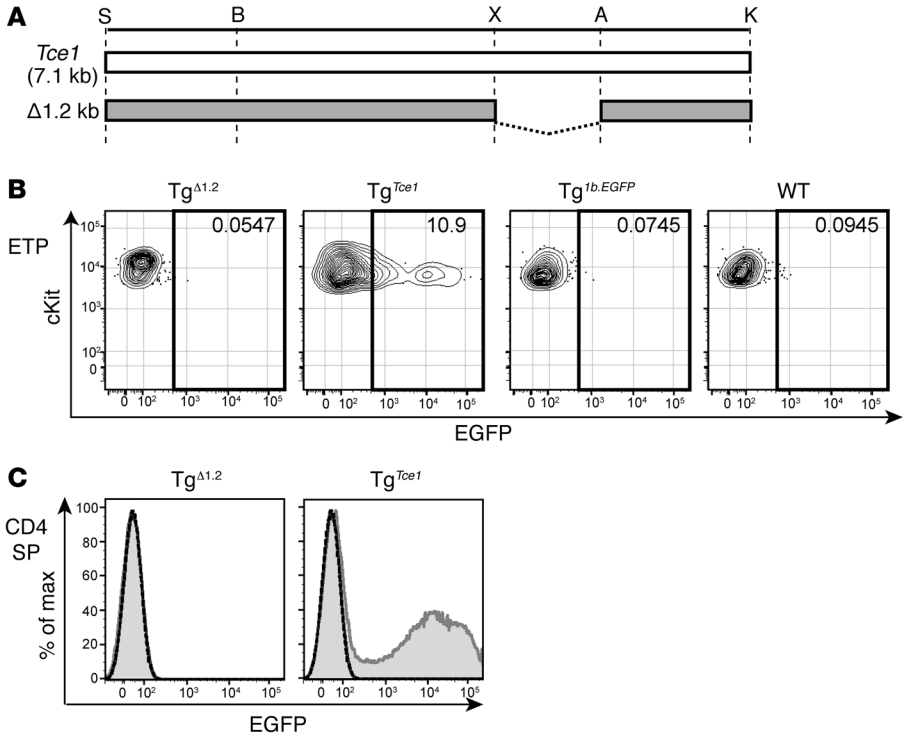

Figure 5. The *Tce1* 1.2-kb fragment is necessary for thymocyte reporter gene transcription. (**A**) Illustration of the 1.2-kb deletion mutant *Tce1* fragment. Restriction enzyme recognition sites are shown. EGFP expression in (**B**) ETP and (**C**) $CD4^+CD8^-TCR\beta^+$ (CD4 SP) cells from $Tg^{\Delta1.2}$, Tg^{Tce1}, $Tg^{1b.EGFP}$, and wild-type mice. Note that $Tg^{\Delta1.2}$ mice represent expression in F_0 mice, while Tg^{Tce1} and $Tg^{1b.EGFP}$ mice are from previously described Tg lines. Data are representative of 2 independent experiments. The gray shaded histogram represents expression of $Tg^{\Delta1.2}$ or Tg^{Tce1}, respectively; the solid line histogram indicates expression of $Tg^{1b.EGFP}$; and the dashed line histogram represents the expression in wild-type mice.

ulated T cells also expressed EGFP, although the number of mice that were $EGFP^+$ at the Th2 stage was reduced (Table 3). These results suggest that the 1.7-kb region may also modestly contribute to Th2-specific transcription.

We concluded that both the 1.2-kb *Tce1* enhancer core and either the 2.7-kb or the 1.5-kb fragments within the adjacent 4.2-kb fragment are required for transcription of reporter genes in Th2 stage cells. These results imply that the transcriptional mechanisms mediated by *Tce1* during T cell development are complex, and at least partially redundant through activities residing wholly within *Tce1*, and, therefore, that the amalgam of *cis* contributions to T cell enhancement of *Gata3* transcription contained within *Tce1* may more closely resemble a locus control region (47) or super-enhancer (48) (i.e., an extended genomic domain harboring clusters of constituent enhancers) rather than a classical monotonic enhancer activity.

TCF-1, HEB, and RBP-J regulate Gata3 transcription by binding within Tce1. Several transcription factors have been proposed as direct or indirect upstream regulators of *Gata3* in T cells. For example, TCF-1, a major regulator of T cell developmental activity, which is required for early stages of thymopoiesis (39), appears to not be required for DN2 to DN4, DP, and SP stages (49) but is reinduced for the acquisition of Th2 fate. E-box transcription factor HEB has similarly been implicated as a critical determinant of T cell developmental decisions (37, 38), and T cell development has been shown to critically require Notch pathway input at multiple stages (40, 50–52). Since *Tce1* contains dozens of putative transcription factor–binding sites, including sites for all 3 of these proposed upstream regulators, we next examined which of these factors might regulate *Gata3* through *Tce1*.

TCF-1 belongs to the TCF/LEF transcription factor family that binds to DNA through an HMG box and is strongly expressed in T cells (53). TCF-1 has been shown to be essential for multiple stages of T cell development and for *Gata3* transcription (39, 49, 54–56). Enforced expression of TCF-1 induces *Gata3* in $Thy1^+CD25^+$ T lineage cells when developed in vitro (39). While researchers have reported that TCF-1 binds to the *Gata3* promoter (39), it is unknown whether TCF-1 binds to *Tce1* to regulate *Gata3* expression in T cells. *Tce1* contains multiple TCF/LEF-binding sites that are conserved between mice and humans (Figure 8A and Supplemental Figure 7), so we investigated TCF-1's occupancy at those sites by ChIP assays. The binding of TCF-1 to *Axin2*, a known target gene of TCF-1 in T cells (39), was used as a positive control (Figure 8B).

We found that TCF-1 bound to site e (Figure 8A and Supplemental Table 3), located within the 1.2-kb enhancer core element, in total thymocytes (Figure 8B). Since more than 80% of thymocytes are DP cells, we also examined whether TCF-1 binds to this enhancer at other T cell developmental stages. As shown in Figure 8B, TCF-1 bound to sequences within fragment e (Figure 8A and Supplemental Table 3) in both DN and CD4 thymocytes. Although TCF-1 occupies the *Gata3* promoter in DN stage cells (39), we previously demonstrated that a YAC, which includes both *Gata3* promoters (*1a* and *1b*) but not *Tce1*, is insufficient to drive *Gata3* transcription in DN cells (36), an observation reconfirmed here (e.g., Figure 3B shows the failure to express EGFP in transgenic mice bearing only the *Gata3* 1b promoter, $Tg^{1b.EGFP}$). Therefore, TCF-1 appears to directly control *Gata3* expression through the *Tce1* enhancer.

During late T cell development, TCF-1 functions to promote specification to the CD4 lineage by regulating Th-POK in DP cells, while TCF-1 and Runx3 collectively repress *Cd4* in cells that are committed to the CD8 lineage (49, 57). Because the absence of TCF-1 during DP through $CD4^+CD8^{lo}$ intermediate cells does not affect the expression level of *Gata3* (49), TCF-1 may not be

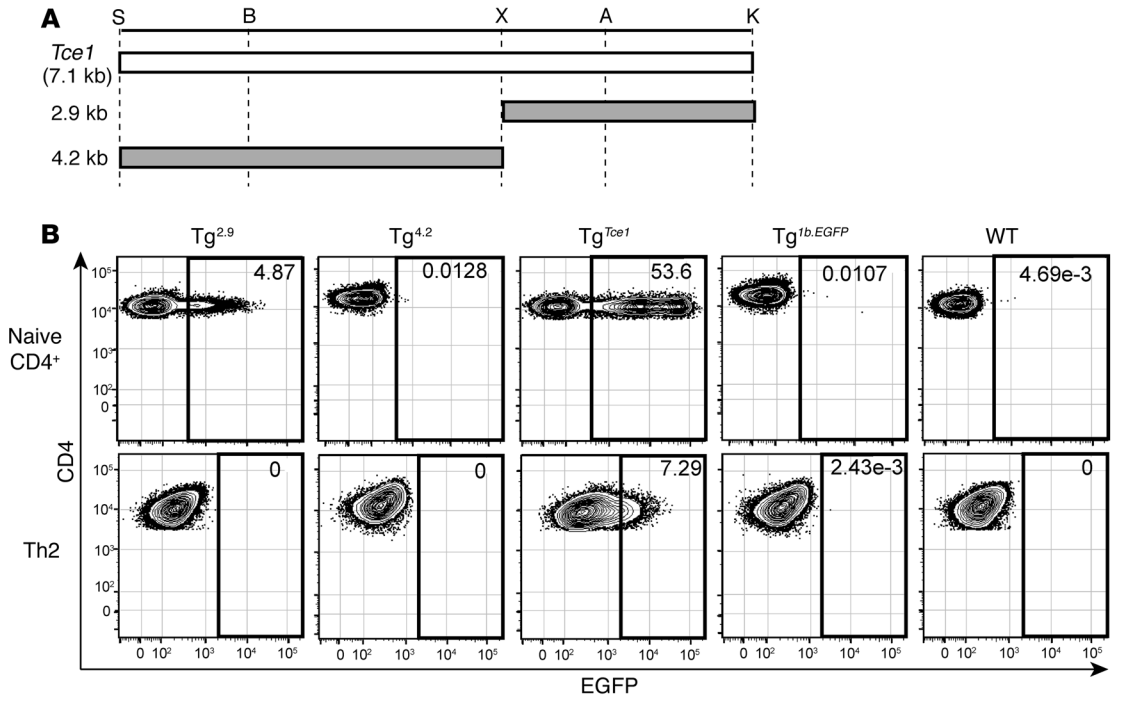

Figure 6. No fragment of *Tce1* is alone sufficient for Th2-specific transcription. (**A**) Illustration of truncated 2.9-kb and 4.2-kb *Tce1* fragments. Restriction enzyme recognition sites are shown. (**B**) EGFP expression in naive CD4$^+$ T cells and Th2 cells that were generated under Th2-polarizing conditions using in vitro T cell differentiation. Note that $Tg^{2.9}$ and $Tg^{4.2}$ mice are F_0 Tg mice. Data are representative of 2 independent experiments.

a principal component in the induction of *Gata3* at those stages. Instead, TCF-1 may function through *Tce1* to maintain the expression of *Gata3* after the CD4/CD8 lineage determination decision, perhaps by differentially increased binding to sequences within fragment f (Figure 8A and Supplemental Table 3) to reinforce CD4 commitment (Figure 8B). The binding of TCF-1 to fragments e and f (Figure 8A and Supplemental Table 3) is consistent with previously published ChIP-seq data from total thymocytes (GSE46662 and Supplemental Figure 7) (58).

Transcription factor HEB (59) is an E-box protein belonging to the bHLH family. HEB, like TCF-1, has been implicated in the regulation of various stages of T cell development (38, 60–62). Since the absence of HEB results in changes in *Gata3* levels during T cell development (37, 38) and *Tce1* contains several E-boxes (putative HEB-binding sites), we asked whether HEB binds to any of those sites. ChIP assays conducted using an anti-HEB serum confirmed HEB binding to the *Tcrb* locus, a known HEB target gene (Figure 8C and ref. 63). We determined that HEB also binds to region e within the 1.2-kb enhancer core element in DN, DP, and CD4 stage cells (Figure 8C). *Gata3* expression increases in HEB-deficient DN3 cells generated from fetal liver hematopoietic progenitors grown on OP9-DL1 feeder layers, and a model proposing how HEB negatively regulates *Gata3* has been advanced (37). In contrast, the induction of *Gata3* during the DP-to-SP transition is perturbed in the absence of HEB and E2A (38). While HEB forms a homodimer or heterodimer with other E-box proteins (such as E2A) to transactivate target genes (59), it can also heterodimerize with inhibitor of DNA binding (Id) proteins, which prevent E-box factors from binding to DNA (64, 65). Since Id3 is expressed in thymocytes and plays an important regulatory role there (66), it seems possible that HEB-E2A as well as HEB-Id3 heterodimers could directly contribute to precision tuning of *Gata3* expression through *Tce1*.

Notch is a transmembrane receptor, and Notch signaling is essential, not only for T cell development, but also for cell fate decisions and cellular proliferation in numerous other tissues (52, 67, 68). After Notch ligand binds to Notch receptor, the cleaved receptor (the intracellular domain of Notch [ICN]) translocates to the nucleus to form a ternary complex with mastermind and transcription factor RBP-J, converting it from a transcriptional repressor to an activator (69, 70). During early T cell development and Th2 differentiation, Notch signaling induces *Gata3* expression

Table 3. EGFP expression in naive CD4$^+$ T cells and Th2 cells of F_0 Tg mice

Tg	No. EGFP$^+$ Tg mice/no. Tg mice (PCR$^+$)	
	Naive CD4$^+$	Th2
$Tg^{2.9}$	8/8[A]	1/8[A]
$Tg^{4.2}$	0/6	0/6
$Tg^{1.2}$	4/7	1/7
$Tg^{1.7}$	0/4	0/4
$Tg^{\Delta 1.2}$	0/7	0/7
$Tg^{\Delta 2.7}$	5/7	3/7
$Tg^{\Delta 1.5}$	9/9[B]	9/9[B]
$Tg^{\Delta 1.7}$	9/9[C]	6/9[C]

[A]Eight of nine $Tg^{2.9}$ mice, [B]nine of twenty $Tg^{\Delta 1.5}$ mice, and [C]nine of ten $Tg^{\Delta 1.7}$ mice whose EGFP expression in peripheral T cells was positive were analyzed.

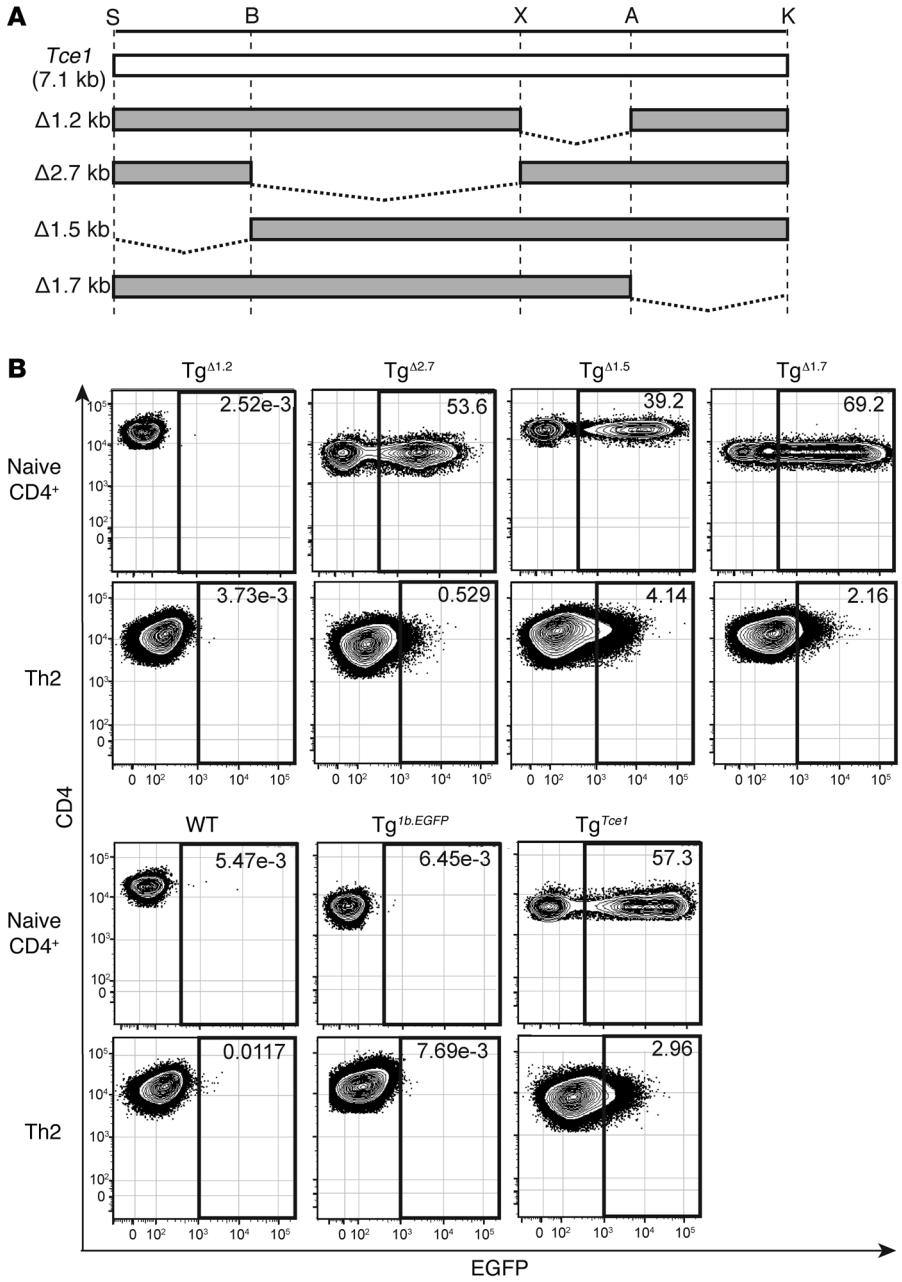

Figure 7. The 1.2-kb *Tce1* fragment is necessary for Th2 reporter gene transcription. (**A**) Illustration of 1.2-kb, 2.7-kb, 1.5-kb, and 1.7-kb deletion mutant *Tce1* fragments. Restriction enzyme recognition sites are shown. (**B**) EGFP expression in naive CD4+ T cells and Th2 cells from F_0 $Tg^{\Delta1.2}$, $Tg^{\Delta2.7}$, $Tg^{\Delta1.5}$, and $Tg^{\Delta1.7}$ mice as well as $Tg^{1b.EGFP}$, Tg^{Tce1}, and wild-type mice. Data are representative of 2 independent experiments.

(40, 50, 71, 72). Although ICN and CSL/RBP-J have been shown to bind to the *Gata3 1a* promoter in Th2 cells (40, 50), that binding is clearly insufficient for T cell–specific *Gata3* expression (36), and it is unknown whether the same factors bind to *Tce1*. We therefore examined the association of CSL/RBP-J with predicted binding sites within *Tce1*. CSL/RBP-J binding to the *Cd25* locus, a robust direct Notch target gene (51), was used as a positive control with an anti–RBP-J antibody in ChIP assays (Figure 8D). We found that CSL/RBP-J bound to sequences within fragments d, e, and g (Figure 8A and Supplemental Table 3) of *Tce1* in DN cells but not in DP cells (Figure 8D). This result is consistent with the observations that Notch signaling is required for early T cell development and for induction of *Gata3* in ETP (67, 68, 73) but the absence of Notch signaling does not perturb late T cell development (68). Thus, these results suggest that Notch signaling regulates *Gata3* expression through *Tce1* during early T cell development but perhaps not during later stages.

Taken together, these data show that at least 3 critical T cell–affiliated transcription factors, TCF-1, HEB, and CSL/RBP-J, occupy binding sites within *Tce1* at different developmental stages. Since all 3 have been shown to vitally affect T cell development, it seems likely that these factors and their associated signaling pathways directly modulate *Gata3* expression through their binding to multiple consensus sites within *Tce1*.

Discussion

Here, we report that a complex enhancer, encoded by the distant *Tce1* activator of transcription factor *Gata3*, is necessary for T cell development and is critical for the generation of ETP and for CD4 development, as demonstrated by CRISPR/Cas-mediated genome editing. This analysis also illuminated the mechanism of action of *Tce1* during T cell development and identified several transcription factors that are responsible for engaging this enhancer activity in T cells.

Many studies have shown that GATA3 is required at multiple stages for normal T cell development (1, 2). Its abundance varies significantly between stages and is tightly controlled (5–11, 74). Although several transcription factors have been proposed as upstream regulators of *Gata3* by demonstration that they bind near the *Gata3 1a* and/or *1b* promoters (39, 40, 50, 75, 76), a functional requirement for any of those binding sites has not been confirmed by in vivo mutagenesis. Furthermore, the data shown here and previously clearly demonstrate that the promoters alone are insufficient to activate *Gata3* expression in T cells (36), while *Tce1* mediates this activity.

Tce1 contains a 1.2-kb enhancer core element, which is sufficient to drive transcription of a reporter gene from the ETP stage through to naive T cells. While this core element is also required for Th2 transcription, it is insufficient alone for directing *Gata3* transcription in those cells. Additional studies demonstrated that

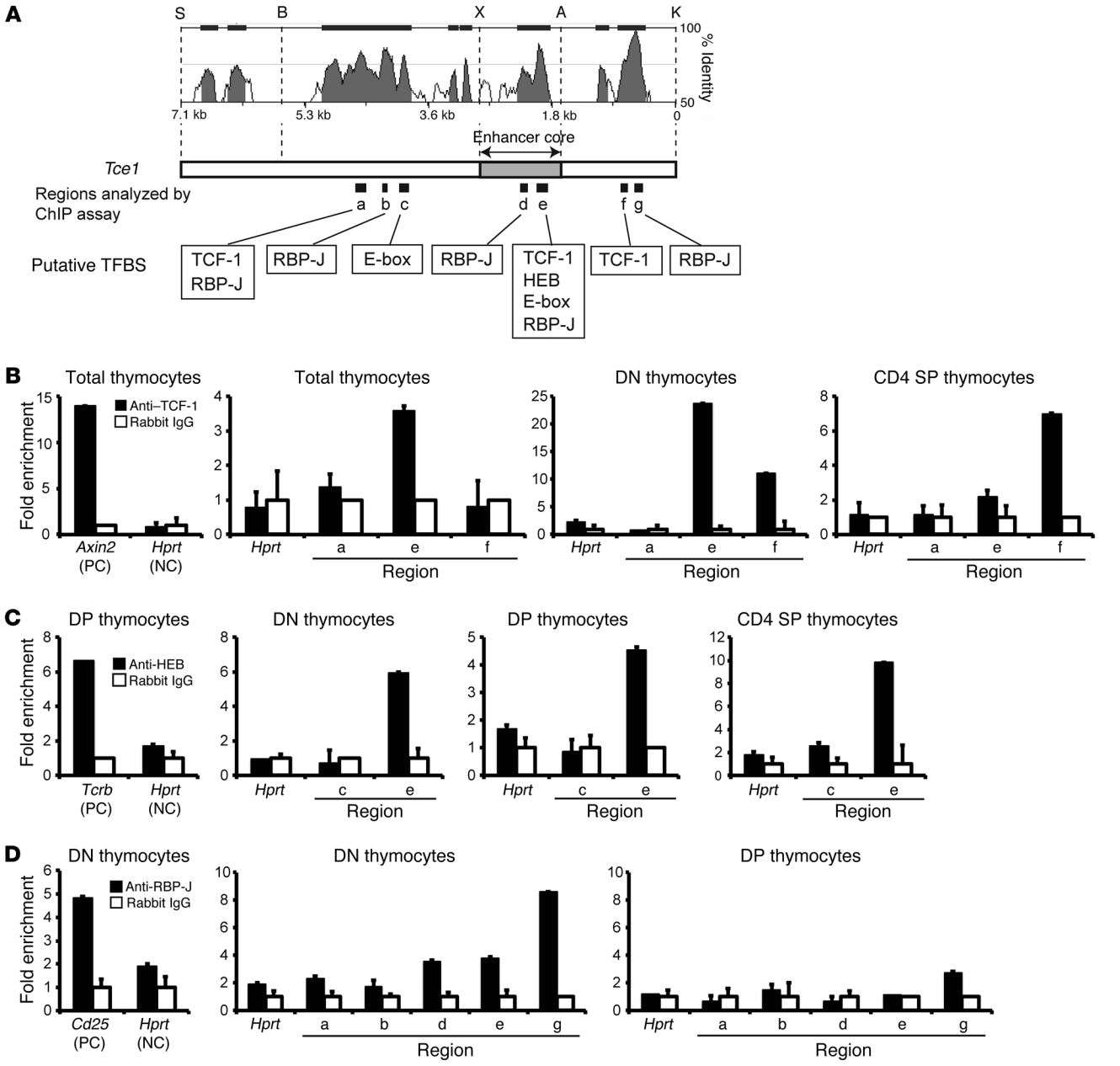

Figure 8. Transcription factors TCF-1, HEB, and RBP-J bind to multiple sites within *Tce1*. (**A**) The segments of highest homology between corresponding human and mouse DNA sequences within *Tce1* were examined in ChIP assays, and putative transcription factor–binding to these sites were examined. Restriction enzyme sites are shown. The results of ChIP assays comparing immunoprecipitation with either control IgG or (**B**) anti–TCF-1–, (**C**) anti-HEB–, or (**D**) anti–RPB-J–specific antibodies. The graph on the left of each row is the ChIP result from a published positive control locus (PC) and negative control locus (NC) for each antibody (39, 51, 63). The type of cells used for each assay is shown at the top of each graph. The location of each cluster of binding sites that was analyzed by ChIP assay is shown in **A**. The error bars represent the mean ± SD from triplicate qPCR samples. Data are representative of 2 independent experiments.

adjoining 2.7-kb or 1.5-kb fragments within *Tce1* must collaborate with the core 1.2-kb fragment to confer Th2-specific enhancement.

During T cell development, multiple distinct combinations of transcription factors are required to regulate specific target genes and guide progenitor cells toward a T lineage fate. Transcription factors TCF-1, HEB, and RBP-J are each known to be important for early stages of T cell development (reviewed in ref. 77), and the data herein indicate that each of these regulatory proteins binds to one or more sites within *Tce1*, each in a developmental stage-specific manner. The demonstration that in vivo deletion of *Tce1* results in reduced ETP numbers implies that those factors are involved in *Gata3* regulation through this enhancer. Notch signaling has been proposed to activate *Gata3* in ETP, although the *cis* element that is responsible for this activation by Notch has not been previously identified. These data indicate that Notch signaling can activate *Gata3* through *Tce1* in ETP stage cells.

HEB and TCF-1 are important for CD4 lineage differentiation (38, 49). E proteins HEB and E2A function as gatekeepers to

maintain cells at the DP stage until a functional αβTCR is generated (62). E proteins are also required for CD4 lineage choice and for preventing MHC class II–restricted thymocytes from entering the CD8 lineage. During this transition from the DP stage to the SP stage, the absence of E-box proteins or E-box inhibitors Id2 and Id3 results in upregulated or downregulated *Gata3* expression, respectively (38). The finding that genetic ablation of *Tce1*, which harbors in vivo binding sites for HEB, impaired CD4 T cell development suggests that HEB, possibly acting as an HEB-E2A heterodimeric activator, positively regulates *Gata3* expression through *Tce1* during the DP-to-SP transition.

In the absence of TCF-1 and LEF-1, CD4 development is impaired and MHC class II–restricted thymocytes are redirected to the CD8 lineage, although their absence does not significantly change the levels of *Gata3* in DP and $CD4^+CD8^{lo}$ cells (49). TCF-1 and LEF-1 may contribute to the regulation of *Gata3* through *Tce1* in order to maintain abundant *Gata3* expression at the CD4 stage. Alternatively, once bound to *Tce1*, TCF-1 and LEF-1 may play an architectural role to facilitate the recruitment of other factors, since TCF-1 and LEF-1 contain an HMG domain that has been shown to bend DNA (78).

Interestingly, the finding that phenotypes in *Tce1* knockout mice were relatively more subtle than in hematopoietic lineage–specific *Gata3* conditional knockout mice (12) implies that there may be a partially redundant enhancer activity somewhere within the extended *Gata3* locus (and lying outside of the 1.5 Mb already surveyed; ref. 36). Although *Tce1* is sufficient for transcription of a reporter gene throughout T cell development, we previously concluded that additional *cis* elements might be required for some stages (for example, for transition from the ETP stage to the DN3 stage or to promote Th2 differentiation) because of differences in the expression pattern of reporter genes and endogenous *Gata3* mRNA (36). The result of *Tce1* deletion by CRISPR/Cas-mediated genome editing in this study supports the redundant *Gata3* T cell enhancer hypothesis.

Enhancer redundancy has been well documented elsewhere. Some such elements exhibit no significant phenotypic effects after loss of the enhancer (e.g., refs. 79, 80). Other studies conclude that enhancers can specify both redundant and essential roles (e.g., ref. 81), as appears to be the case with *Tce1*, whose loss confers clear deficits in T cell homeostasis, while not completely abrogating T cell function. One mechanism that has been proposed for enhancer redundancy is the presence of "shadow enhancers" (refs. 82, 83, and reviewed in ref. 84). According to this hypothesis, two or more enhancers for any given gene may direct similar expression patterns, and those enhancers may appear to be redundant under normal laboratory conditions. However, under stressful conditions (e.g., elevated temperature, food scarcity), environmental influences leading to disrupted function of a single enhancer may perturb normal gene expression. Thus, those shadow enhancers are thought to provide robustness to environmental or genetic perturbation and stress, and the thymus is known to be particularly sensitive to such stresses (85). Therefore, reliable, robust transcription of *Gata3* during the ETP and CD4 stages of T cell development may require both *Tce1* and a putative shadow enhancer in order to generate appropriate thymic cellular output under conditions that are not usually encountered in the laboratory.

Alternatively, the interaction among multiple *cis* elements, each containing overlapping but slightly different activity, may be required to produce authentic patterns of gene expression, as previously reported (e.g., refs. 86, 87). For example, *Hoxd* genes are important for developing hand and foot digits, and *cis* elements for regulation of the *Hoxd* genes are distributed over an 800-kb "gene desert." Each element appears to associate with the genes as well as other *cis*-regulatory elements and function to drive *Hoxd* gene transcription either quantitatively or qualitatively. Serial deletion of the *Hoxd cis* elements reveals incomplete redundancy within the cluster (87). The gene desert surrounding the *Gata3* locus contains multiple *cis* elements, not just *Tce1*, as well as multiple enhancers that regulate the gene in numerous other tissues (refs. 46, 88–91 and our unpublished observations). Intriguingly, Pazin and colleagues demonstrated that a region 736 kb 3′ to the *Gata3* structural gene possesses enhancer-like activity in a T cell–derived cell line using an episomal vector (92). Furthermore, recent genome-wide association studies have revealed that multiple, distinct disease-associated SNPs are located either within or near the *GATA3* structural gene and are associated with leukemia (93–95), Hodgkin's lymphoma (96), rhinitis (97), and asthma (98); furthermore, another asthma-associated SNP is located in a gene desert lying 1 Mb 3′ to *GATA3* (99). The underlying hypothesis in all of these studies is that those SNPs may reside in yet-to-be-defined *cis*-regulatory elements and may affect *GATA3* expression in those T cell–related and/or aberrant *GATA3* expression-related diseases. Therefore, *Tce1* and other currently undefined *cis* elements may contain partially overlapping activity and interact with one another to coordinately regulate *Gata3* transcription during T cell development.

Tce1, which consists of the enhancer core and accessory *cis* elements, is necessary for ETP and CD4 T cell development and *Gata3* expression. We showed that TCF-1, HEB, and RBP-J (as well as other factors that were not examined in this study) occupy this enhancer and regulate *Gata3* during T cell development. Understanding *Tce1* function provides an essential pathway to elucidating the direct temporal regulation of *Gata3*, a critical, central regulatory protein that is required for T cell development. The present study should help to better define and further integrate transcriptional networks that regulate *Gata3* during T cell development. Further studies addressing *Gata3* regulation and function would provide important advances in our understanding of T cell development and should lead to clarification of the mechanisms underlying the molecular basis for the multiple diseases of hematopoietic origin in which GATA3 is implicated, such as leukemia and asthma.

Methods

Mice. The locus surrounding *Tce1* was edited using CRISPR/Cas9, as described previously (42). gRNA sequences corresponding to sequences surrounding the 7.1 kbp that define the boundaries of *Tce1* (Supplemental Figure 1A) were cloned into the BbsI site of the bicistronic expression vector px330 (Addgene; the gift of Feng Zhang, Broad Institute of MIT and Harvard, Cambridge, Massachusetts, USA; ref. 100), which expresses Cas9 and single gRNA. The two CRISPR/Cas9-gRNA plasmids were coinjected with single-stranded oligos containing loxP sequences, an EcoRI site, and 60-bp homology arms (Supplemental Figure 1B) into the pronuclei of fertilized eggs. The injected zygotes

were transferred into the oviduct of pseudopregnant ICR females at 1.0 to 1.5 dpc. Mice were genotyped by PCR using primers shown in Supplemental Figure 1A. The deletion of *Tce1* was confirmed by DNA sequencing of the PCR product (Supplemental Figure 2).

Tg^{Tce1} and $Tg^{Ib.EGFP}$ mice were established as previously described (36), and Tg lines were used in this study. Founder Tg mice containing various truncated *Tce1* fragments were generated using standard techniques in the University of Michigan Transgenic Animal Model Core. Plasmid DNAs were microinjected into (C57BL/6J × SJL) F_2 fertilized eggs. Those founder Tg mice were used for analysis instead of establishing Tg lines.

Plasmid construction. Details are provided in the Supplemental Methods.

Flow cytometry. To analyze expression of the EGFP reporter gene in the various Tg founder mice, single-cell suspensions of thymocytes, bone marrow cells, splenocytes, lymph node cells, or peripheral blood were incubated with Fc Block (BD Bioscience). Before incubation, splenocytes and peripheral blood were hemolyzed with ammonium chloride. Cells were stained with a variety of antibodies as previously described (36), washed, and analyzed on a FACSCanto II, Fortessa, or FACSAria III (BD Biosciences). Dead cells were excluded by DAPI or propidium iodide. Immature T cells were analyzed as previously described (12). The following antibodies were purchased from BioLegend, eBioscience, and/or BD: B220 (RA3-6B2; BioLegend and eBioscience), CD3 (17A2; BioLegend and eBioscience), CD8a (53-6.7; BioLegend and eBioscience), CD11b (M1/70; BioLegend and eBioscience), CD11c, (N418; BioLegend and eBioscience), CD19 (1D3; BioLegend and eBioscience), CD25 (PC61.5; BioLegend and eBioscience), cKit (2B8; BioLegend and eBioscience), Gr1 (RB6-8C5; BioLegend and eBioscience), NK1.1 (PK136; eBioscience), TCRβ (H57-597; BioLegend and eBioscience), TCRγ/δ (GL3; BioLegend, eBioscience, and BD), TER119 (TER-119; BioLegend and eBioscience), and Thy1.2 (53-2.1; BioLegend).

For every experiment in which EGFP expression was analyzed, a LinearFlow Green Flow Cytometry Intensity Calibration Kit (Molecular Probes) was used. These calibration beads were excited by 488-nm laser, and fluorescence measurements were performed in the same manner as those for EGFP in order to confirm that the intensity of EGFP PMT voltage was at comparable levels in all experiments. Acquired data were analyzed using FlowJo software (Tree Star Inc.).

To sort each stage of thymocytes for ChIP assay, cells were incubated with Fc Block and stained with PE-Cy7-anti-CD4 (clone RM4-5; BioLegend), APC-anti-CD8a (clone 53-6.7; BioLegend), and PerCP-Cy5.5-anti-TCRβ (clone H57-597; BioLegend). DAPI was used as a dead cell marker. DN ($CD4^-CD8^-$) cells, DP ($CD4^+CD8^+$) cells, and CD4 SP ($CD4^+CD8^-TCR\beta^+$) cells were sorted in a FACSAria III (BD Bioscience).

Quantitative RT-PCR. Quantitative RT-PCR (qRT-PCR) was performed as described previously (74).

In vitro CD4 T cell differentiation. $CD4^+CD25^-$ lymph node cells were purified using the Dynal Mouse CD4-Negative Isolation Kit (Invitrogen) in combination with affinity-purified anti-mouse CD25 antibody (PC61.5; eBioscience or BioLegend) and cultured as previously described (36). Briefly, isolated CD4 cells were stimulated with plate-bound anti-CD3ε antibody (10 μg/ml; 145-2C11; BD Biosciences) and anti-CD28 antibody (10 g/ml; 37.51; BD Biosciences). To induce Th2-polarizing conditions, 10 μg/ml anti–IFN-γ antibody (XMG1.2; BD Biosciences), 10 μg/ml anti–IL-12 antibody (C17.8; BD Biosciences), 10 ng/ml recombinant human IL-2 (200-02, PeproTech), and 10 ng/ml recombinant mouse IL-4 (554432, BD Pharmingen) were added. On day 4 of culture, stimulation was stopped and the cells were diluted. On day 6, cells were restimulated with plate-bound anti-CD3ε and anti-CD28 antibodies (3 μg/ml each) for 6 hours. Cells were harvested, and we analyzed EGFP expression by flow cytometry as previously described (36). A small fraction of harvested cells was used for RNA extraction to confirm in vitro T cell differentiation by measuring the expression of cytokine mRNAs (*Il4* and *Ifng*) and endogenous *Gata3* mRNA by qRT-PCR (data not shown).

ChIP. Details are provided in the Supplemental Methods.

Prediction of putative transcription factor–binding sites. A search for putative transcription factor–binding sites in *Tce1* was performed using rVISTA 2.0 (http://rvista.dcode.org) (101) or rVISTA (http://genome.lbl.gov/vista/rvista/submit.shtml) (102).

Statistics. Data were analyzed by 2-tailed Student's *t* test. A *P* value of less than 0.05 was considered significant.

Study approval. All animal experiments were approved by and conducted in accordance with the guidelines of the Committee on the Use and Care of Animals of the University of Michigan or the Animal Experiment Committee of Tsukuba University.

Author contributions

SO devised and executed experiments and wrote the paper. SM and HO performed and interpreted experiments. HO and ST devised and interpreted experiments. MH executed experiments. CJK performed experiments and wrote the paper. TH devised and performed experiments and wrote the paper. JDE designed experimental strategy, interpreted experiments, and wrote the paper.

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