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

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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-TCα/TCRβ complex) are licensed to differentiate further and transition to the DN4 and immature single-positive (SP; CD4–CD8+TCRβlo) 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 TCRβ receptor on their cell surface then undergo positive selection and move into the CD4+CD8+ intermediate stage. CD4+CD8lo 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). Hypoinsufficient GATA3 mutation in humans leads to HDR syndrome (hypoparathyroidism, sensorineural deafness, and renal disease;
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 naïve 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 F1 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 F1 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 naïve T cells from memory T cells (45). While the CD44+/CD62L− naïve 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+CD62L− memory T cell number was compa-
ETP and CD4 stages in the thymus as well as in CD4 T cells in the peripheral blood and spleen.

_Tc e 1_ also contributes to _Gata3_ expression in DN4 and DP stage cells, even though the number of those cells that accumulate is normal whether _Tc e 1_ is present or absent. We concluded that _Tc e 1_ is a critical regulator of the _Gata3_ gene in a subset of thymocytes. Since the phenotypes

rable in the two genotypes. The data suggest that compensatory expansion or accumulation of memory T cells in _Tc e 1_-deleted mutants must exist. The data clearly demonstrate that CD4 T cell development is severely compromised by deletion of _Tc e 1_ in mice.

Collectively, the data demonstrate that _Tc e 1_ 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. _Tc e 1_ also contributes to _Gata3_ expression in DN4 and DP stage cells, even though the number of those cells that accumulate is normal whether _Tc e 1_ is present or absent. We concluded that _Tc e 1_ is a critical regulator of the _Gata3_ gene in a subset of thymocytes. Since the phenotypes

**Figure 1. In vivo genome deletion of _Tc e 1_.** (A) Two CRISPR/Cas9 plasmids expressing gRNAs that correspond to sequences surrounding the 71 kbp that define the boundaries of _Tc e 1_ were coinjected into mouse fertilized oocytes. _Tc e 1_-deleted mutant allele founder (F0) mice were intercrossed to obtain homozygous deletion mutant mice. (B) Thymocytes, (C) peripheral blood cells, and (D) splenocytes isolated from F2 animals at 5 to 6 weeks of age bearing homozygous (white circle) or heterozygous (black circle) deletions of _Tc e 1_ 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^-^CD3^-^TCRβ^-^ (CD4 SP), and CD4^-^CD8^-^CD3^-^TCRβ^-^ (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.
observed in Tce1Δ 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 contribute 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., Tg1.2−), while deletion of each part of Tce1 from the whole is designated by a preceding Δ (e.g., TgΔ1.2, 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 (F0) Tg mice (referred to as Tg2.9 and Tg4.2 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 (TgTce1) is a stable, established Tg line, while Tg2.9 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 Tg2.9 mice but not in Tg4.2 mice (Table 1 and Supplemental Figure 5A). Furthermore, and as is also true for TgTce1 mice, EGFP expression was observed in Tg2.9 mice at multiple stages, including ETP and CD4 T cells (Figure 3 and Table 2). In addition to αβ TCR+ T cells, γδ TCR+ thymocytes expressed EGFP (Supplemental Figure 6 and Supplemental Table 1). In contrast, EGFP expression was never detected in thymocytes from Tg4.2 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 F0 Tg mice

<table>
<thead>
<tr>
<th>Tg</th>
<th>No. CD4+EGFP+ Tg mice/no. Tg mice (PCR)</th>
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<tbody>
<tr>
<td>Tg1.2</td>
<td>1/2</td>
</tr>
<tr>
<td>Tg1.7</td>
<td>0/6</td>
</tr>
<tr>
<td>Tg1.5</td>
<td>4/7</td>
</tr>
<tr>
<td>Tg1.7</td>
<td>0/4</td>
</tr>
<tr>
<td>Tg1.12</td>
<td>0/7</td>
</tr>
<tr>
<td>Tg1.17</td>
<td>10/14</td>
</tr>
<tr>
<td>Tg1.15</td>
<td>20/24</td>
</tr>
<tr>
<td>Tg1.2</td>
<td>4/7</td>
</tr>
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</table>
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 T cell enhancer activity in peripheral CD4 T cells. These data show that the 1.2-kb T cell enhancer fragment within \textit{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 \textit{Gata3} T cell–specific transcription. The data also show that the extended \textit{Gata3} promoter, either alone or when combined with inactive fragments of \textit{Tce1}, is not sufficient for T cell activation. Transcription of \textit{Gata3} in Th2 cells requires cooperation of multiple elements within \textit{Tce1}. \textit{GATA3} is necessary for the differentiation and function of Th2 cells (5), and we have shown that \textit{Tce1} can

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\textsuperscript{1.2} and Tg\textsuperscript{1.7} mice), and analyzed peripheral blood from those F\textsubscript{0} mice as before. We found that EGFP was expressed in the peripheral blood CD4 T cells in the majority of Tg\textsuperscript{1.2} mice, while EGFP expression was essentially absent in Tg\textsuperscript{1.7} mice (Table 1 and Supplemental Figure 5B). Detailed analyses of their thymocytes revealed that Tg\textsuperscript{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 \textit{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.

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\textsuperscript{1.2} and Tg\textsuperscript{1.7} mice), and analyzed peripheral blood from those F\textsubscript{0} mice as before. We found that EGFP was expressed in the peripheral blood CD4 T cells in the majority of Tg\textsuperscript{1.2} mice, while EGFP expression was essentially absent in Tg\textsuperscript{1.7} mice (Table 1 and Supplemental Figure 5B). Detailed analyses of their thymocytes revealed that Tg\textsuperscript{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 \textit{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 \textit{Gata3} \textit{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 \textit{Tce1} (Figure 5A) and generated additional F\textsubscript{0} Tg \textit{ΔTg\textsuperscript{1.2}} mice. None of the F\textsubscript{0} Tg\textsuperscript{ΔTg\textsuperscript{1.2}} mice 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 \textit{Tce1} enhancer activity in peripheral CD4 T cells. These data show that the 1.2-kb T cell enhancer fragment within \textit{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 \textit{Gata3} T cell–specific transcription. The data also show that the extended \textit{Gata3} promoter, either alone or when combined with inactive fragments of \textit{Tce1}, is not sufficient for T cell activation.

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

Table 2. EGFP expression at each thymocyte developmental stage in F\textsubscript{0} Tg mice

<table>
<thead>
<tr>
<th>Tg</th>
<th>ETP</th>
<th>DN2</th>
<th>DN3</th>
<th>DN4</th>
<th>DP</th>
<th>CD4 SP</th>
<th>CD8 SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg\textsuperscript{2.9}</td>
<td>7/8\textsuperscript{a}</td>
<td>6/8\textsuperscript{a}</td>
<td>8/8\textsuperscript{a}</td>
<td>8/8\textsuperscript{a}</td>
<td>8/8\textsuperscript{a}</td>
<td>8/8\textsuperscript{a}</td>
<td>8/8\textsuperscript{a}</td>
</tr>
<tr>
<td>Tg\textsuperscript{4.2}</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Tg\textsuperscript{1.2}</td>
<td>4/7</td>
<td>2/7</td>
<td>3/7</td>
<td>4/7</td>
<td>4/7</td>
<td>4/7</td>
<td>4/7</td>
</tr>
<tr>
<td>Tg\textsuperscript{ΔTg\textsuperscript{1.2}}</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
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</table>

\textsuperscript{a}Eight of nine Tg\textsuperscript{2.9} mice whose EGFP expression in peripheral T cells was positive were analyzed.
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 Tc e 1 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 Tc e 1 contributes to GATA3 expression in Th2 cells, we analyzed EGFP expression of reporter Tg mice bearing Tc e 1 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 Tg2.9 and Tg1.2 Th2 cells, comparable to reporter expression in Tg4.2 and Tg1.7 mice (Figure 6 and Table 3). These data show that neither of the initially bisected larger Tc e 1 fragments is sufficient for the transcription of the EGFP reporter gene in Th2-polarized cells, while Tc e 1 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 Tc e 1 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 Tg4.2 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 Tc e 1 are involved in the regulation of Th2 transcription, we generated Tg mice bearing internal deletions of Tc e 1 (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 Δ2.7) or the 1.5-kb (Tg Δ1.5) segments of Tc e 1. As shown in Figure 7B and Table 3, naive CD4 T cells from Tg Δ2.7 and Tg Δ1.5 mice both expressed EGFP. After those cells were stimulated to undergo Th2 differentiation, both genotypes retained EGFP expression (Figure 7B and Table 3). These results indicate that the 2.7-kb and 1.5-kb segments of Tc e 1 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 Tc e 1 enhancer core fragment.

We also asked whether the 1.7-kb fragment of Tc e 1 contributes to transcription in Th2 cells by generating additional deletion mutants (Tg Δ1.7). Naive CD4 T cells from Tg Δ1.7 mice expressed EGFP (Figure 7B and Table 3), as expected. Th2-polarized stim-
clusters of constituent enhancers) rather than a classical mono-
super-enhancer (48) (i.e., an extended genomic domain harboring
t targets might regulate Gata3
proposed upstream regulators, we next examined which of these
transcription factor–binding sites, including sites for all 3 of these
T cells. These results imply that the transcriptional mecha-
nisms mediated by Tcet during T cell development are complex,
and at least partially redundant through activities residing wholly
within Tcet, and, therefore, that the amalgam of cis contributions
to T cell enhancement of Gata3 transcription contained within
Tcet may more closely resemble a locus control region (47) or
whether Tcet binds to Gata3
promoter (39), it is unknown
Gata3
expression
drive
through the 1b promoter, Tg Gata3
transgenic mice bearing only the
Gata3
transcription in DN cells (36), an observation recon-
firmed here (e.g., Figure 3B shows the failure to express EGFP in
mutant
mouse. Note that Tg Δ1.2 mice represent expres-
sion in T cells (39), was used as a positive control (Figure 8B).

We found that TCF-1 bound to site e (Figure 8A and Supple-
mental Table 3), located within the 1.2-kb enhancer core element,
in total thymocytes (Figure 8B). Since more than 80% of thymo-
cytes 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 TCF-1's occupancy at those sites by ChIP assays.

TCF-1, HEB, and RBP-J regulate Gata3 transcription by bind-
ing within Tcet. 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 fac-
tor 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 mul-
tiple stages (40, 50–52). Since Tcet contains dozens of putative
transcription factor–binding sites, including sites for all 3 of these
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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 Tc rb 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 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).

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### Table 3. EGFP expression in naive CD4+ T cells and Th2 cells of F0 Tg mice

<table>
<thead>
<tr>
<th>Tg</th>
<th>No. EGFP+ Tg mice/no. Tg mice (PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Naive CD4+ Th2</td>
</tr>
<tr>
<td>Tg2.9</td>
<td>8/8A 1/8A</td>
</tr>
<tr>
<td>Tg1.2</td>
<td>0/6 0/6</td>
</tr>
<tr>
<td>Tg1.7</td>
<td>4/7 1/7</td>
</tr>
<tr>
<td>Tg1.5</td>
<td>0/4 0/4</td>
</tr>
<tr>
<td>Tg1.2</td>
<td>0/7 0/7</td>
</tr>
<tr>
<td>Tg1.7</td>
<td>5/7 3/7</td>
</tr>
<tr>
<td>Tg1.5</td>
<td>9/9A 9/9A</td>
</tr>
<tr>
<td>Tg1.7</td>
<td>9/9B 6/9B</td>
</tr>
</tbody>
</table>

*Eight of nine Tg1.5 mice, nine of twenty Tg1.15 mice, and nine of ten Tg1.7 mice whose EGFP expression in peripheral T cells was positive were analyzed.*
expression through Tcε1 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 Tcε1 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 Tcε1.

Discussion

Here, we report that a complex enhancer, encoded by the distant Tcε1 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 Tcε1 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 transcription in T cells (36), while Tcε1 mediates this activity.

Tcε1 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
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

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–RBP-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.
maintain cells at the DP stage until a functional βTCDR 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 TceI, 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 TceI 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− cells (49). TCF-1 and LEF-1 may contribute to the regulation of Gata3 through TceI in order to maintain abundant Gata3 expression at the CD4 stage. Alternatively, once bound to TceI, 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 TceI 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 TceI 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 TceI 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 TceI, 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 TceI 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 TceI, 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 756 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), hinitis (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, TceI and other currently undefined cis elements may contain partially overlapping activity and interact with one another to coordinate regulation of Gata3 transcription during T cell development.

TceI, 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 TceI 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 define these 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 TceI was edited using CRISPR/Cas9, as described previously (42). gRNA sequences corresponding to sequences surrounding the 7.1 kb that define the boundaries of TceI (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 cojected 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 Tcell was confirmed by DNA sequencing of the PCR product (Supplemental Figure 2).

Tg^{Tc1} and Tg^{EGFP} mice were established as previously described (36), and Tg lines were used in this study. Founder Tg mice containing various truncated Tcell fragments were generated using standard methods in the University of Michigan Transgenic Animal Model Core. Plasmid DNAs were microinjected into (C57BL/6 × SJL)F1, 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 FACS Aria 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), CD8α (53-670; 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), TCRγδ (GL3; BioLegend and 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 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; BD Biosciences), 10 ng/ml recombinant mouse IL-4 (554432, BD PharMingen), CD16/32 ( clone H57-597; BD Biosciences), 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 (5 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 Tcell was performed using rVISTA 2.0 (http://rvisita.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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