TCF-1 and LEF-1 regulate T cell lineage choice and *Cd4* gene silencing by differential harnessing of Th-POK and Runx3

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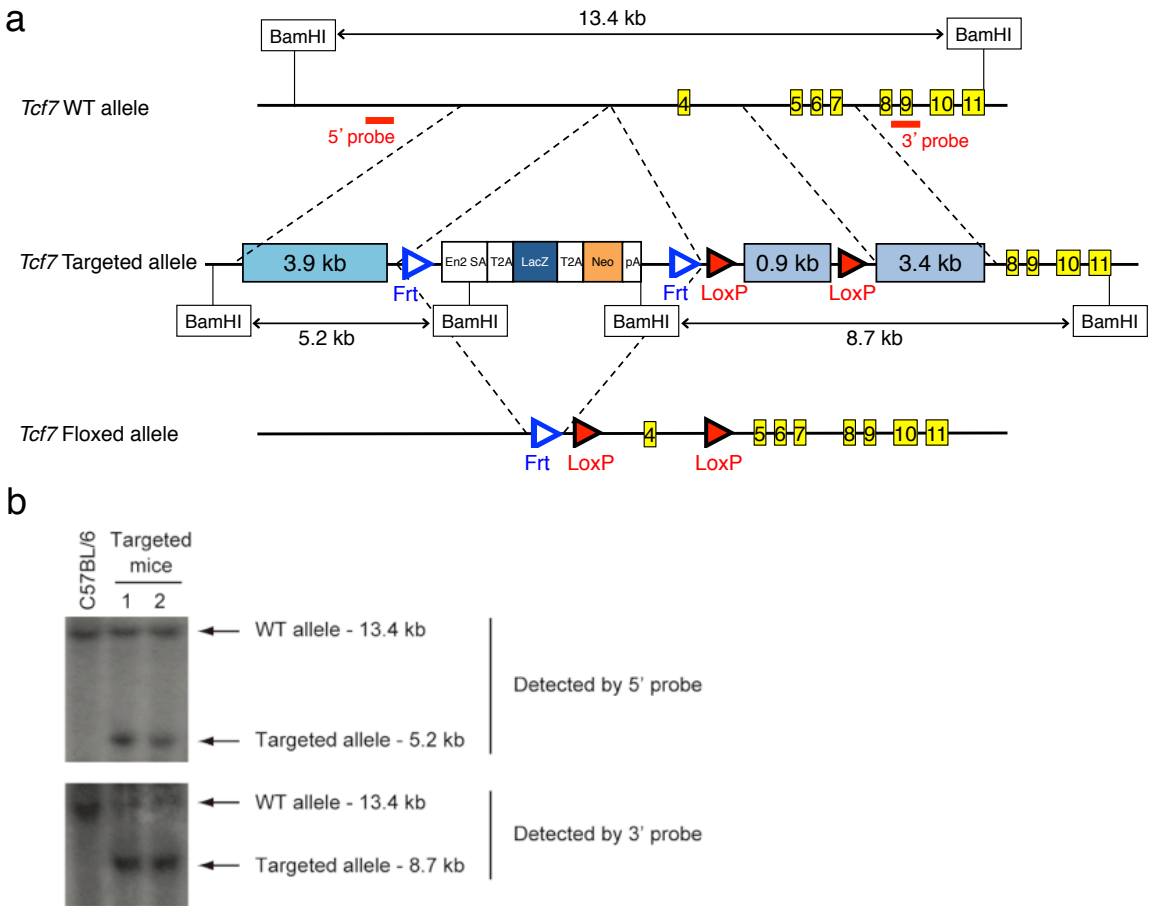
Steinke et al. Supplementary Figure 1.



Supplementary Figure 1. Conditional targeting of the *Tcf7* gene.

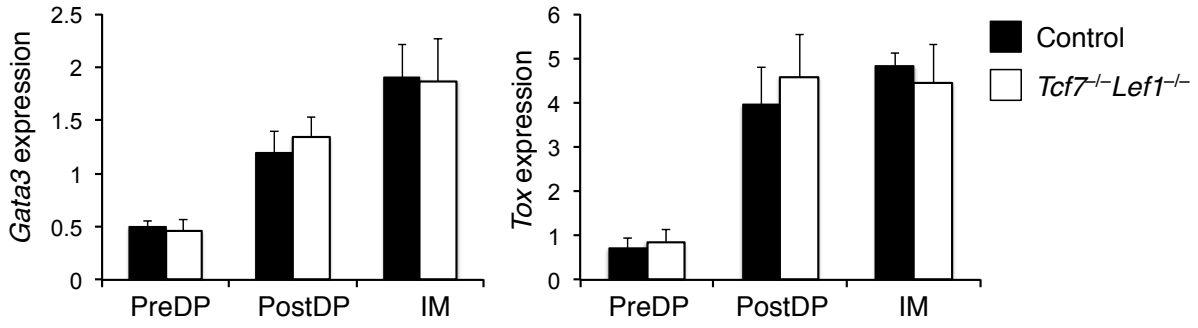
(**a**) Targeting strategy. The *Tcf7* gene was conditionally targeted by the International Knockout Mouse Consortium (IKMC, project 37596). Depicted on top is partial structure of the *Tcf7* gene with filled rectangles in yellow denoting exons (all numbered). The exon 4 of *Tcf7* was flanked by two LoxP sites, and deletion of this exon results in a nonsense frame-shift mutation. Also marked are key enzyme sites and relative locations of 5' and 3' probes used in Southern blotting.

Shown in the middle is the structure of *Tcf7*-targeted allele, highlighting the targeting arms, locations of inserted LoxP sites (filled triangles in red), Frt sites (open triangles in blue), β-galactosidase-neomycin resistant gene (LacZ-Neo) cassettes. Note that two extra BamHI sites were embedded in the LacZ-Neo cassette, and these two sites were used to facilitate detection of the targeted allele by Southern blotting.

By crossing with Rosa26-Flippase knock-in mice, the Frt site-flanked LacZ-Neo cassette was excised, giving rise to the *Tcf7*-floxed allele.

(**b**) Identification of targeted mice. Genomic DNA was extracted from tails of targeted mice, digested with BamHI, and Southern-blotted with the 5' or 3' probes. Both probes detect the WT allele at approximately 13.4 kb. The 5'-probe detects the targeted allele at ~ 5.2 kb (top panel), and the 3' probe detects the targeted allele at ~8.7 kb (bottom). The probes were amplified with the following primers: 5' probe: 5'-agggtgggcacagagatatg and 5'-gccagagctcagctgctaat; 3' probe: 5'-agccaaggtcattgctgagt and 5'-ccttcctgtgttgaggtggt.

Steinke et al. Supplementary Figure 2.

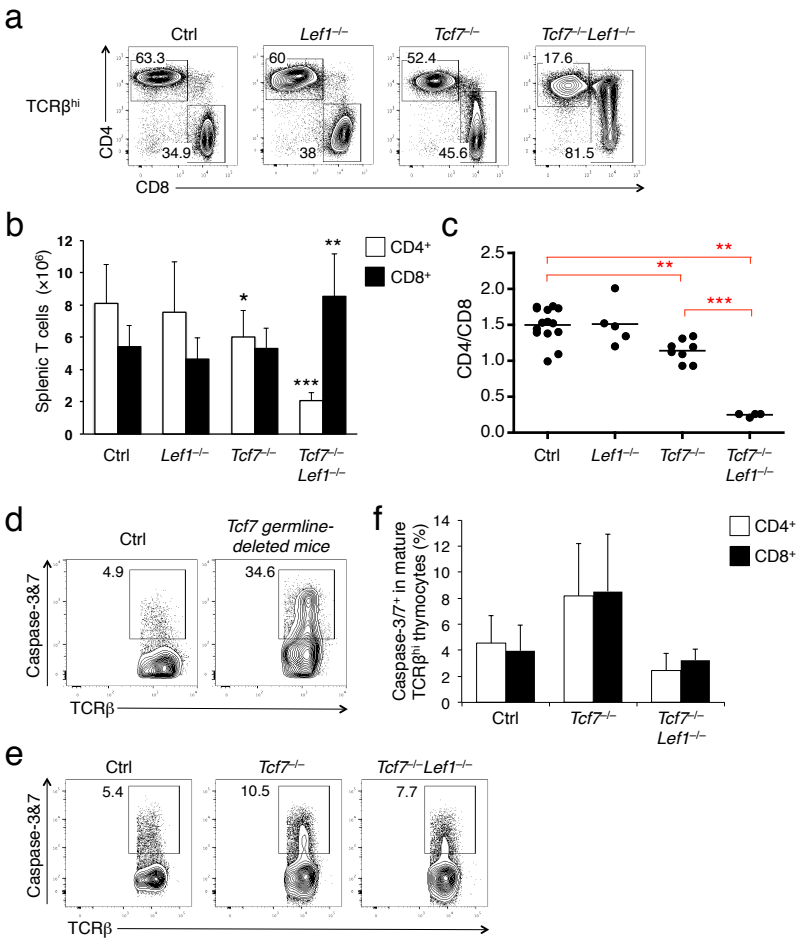


Supplementary Figure 2. Deficiency in both TCF-1 and LEF-1 does not affect TCR-dependent induction of GATA-3 and Tox.

Although CD69 expression was reduced in TCRβ^{hi} thymocytes from *Tcf7*$^{-/-}$*Lef1*$^{-/-}$ mice (**Fig. 1c**), the combination of CD69 and CD24 was sufficient to distinguish immature and mature subsets within the TCRβ^{hi} population. The surface-stained thymocytes from *Tcf7*$^{-/-}$*Lef1*$^{-/-}$ and littermate controls were sorted for 3 subsets, pre-select DP (PreDP), post-select DP (PostDP), and CD4^{+}8^{lo} intermediate (IM). *Gata3 and Tox expression was measured as an end outcome of TCR signaling in positively selected DP subsets*. The relative expression of each gene was normalized to *Hprt1*. Data are pooled results from 3 independent experiments and shown as means ± s.d. ($n \geq 3$). *Gata3* and *Tox* expression between control and *Tcf7*$^{-/-}$*Lef1*$^{-/-}$ within each subset was not statistically different ($p>0.4$).

Note that the post-select DP thymocytes from Tcf7$^{-/-}$*Lef1*$^{-/-}$ *mice may contain a fraction of CD8*$^{+}$ *T cells that had derepressed expression of CD4 (the CD8*4 cells). Because Gata3 and Tox were less abundantly expressed in CD8*$^{+}$ *T cells compared with DP thymocytes, CD8*4 cells unlikely contributed to elevating Gata3 and Tox expression in Tcf7*$^{-/-}$*Lef1*$^{-/-}$ *post-select DP thymocytes.*

Steinke et al. Supplementary Figure 3.



Supplementary Figure 3. Lack of TCF-1 and LEF-1 diminishes CD4$^+$ T cell output, independent of their role in thymocyte survival.

(**a**) and (**b**) Loss of TCF-1 or both TCF-1 and LEF-1 diminishes CD4$^+$ T cell output in the periphery. Splenocytes were surface-stained, and TCRβ$^+$ cells were analyzed for CD4$^+$ and CD8$^+$ lineage distribution. Representative and cumulative data are shown in **a** and **b**, respectively.

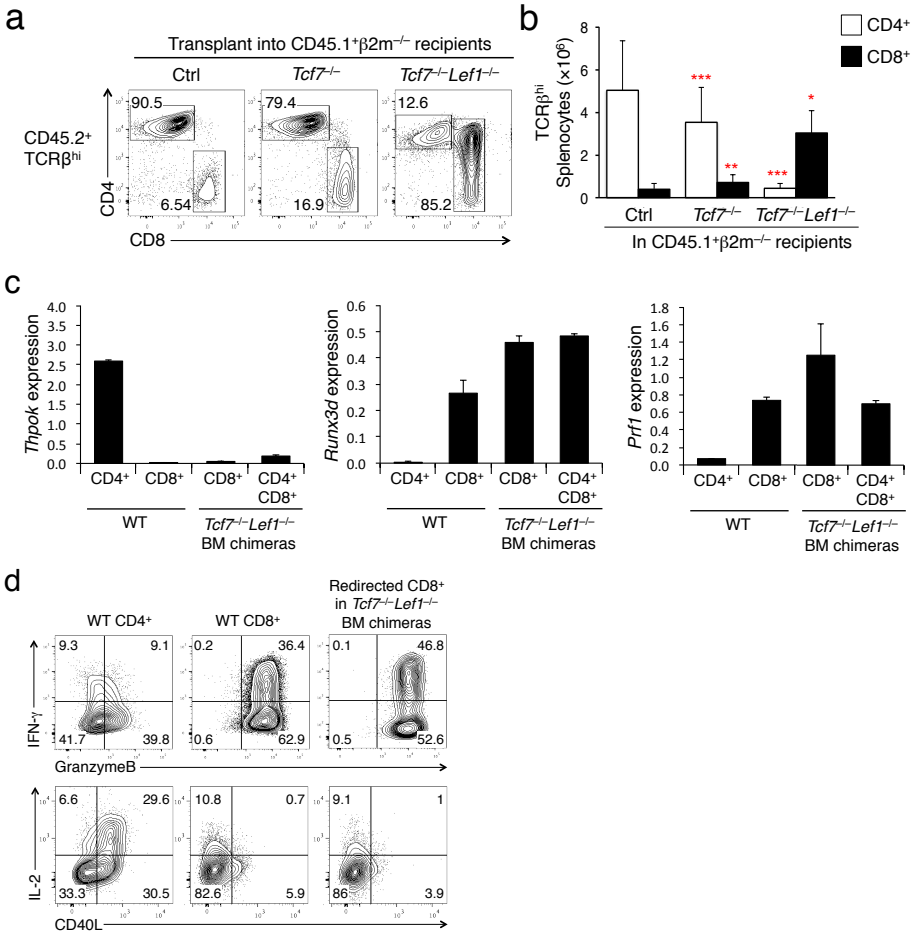
(**c**) Loss of TCF-1 and LEF-1 reverses the CD4/CD8 ratio in the periphery. The CD4$^+$ to CD8$^+$ ratio is calculated from **b**. Data are from ≥ 4 independent experiments. *, p<0.05; **, p<0.01; ***, p<0.001.

(**d**) Germline deletion of TCF-1 results massive cell death in TCRβ^{hi} thymocytes. Thymocytes from germline-targeted TCF-1 knockout mice and littermate controls were harvested, and Caspase-3&7 activation was measured in the TCRβ^{hi} subset.

(**e**) Late deletion of TCF-1 and LEF-1 alleviates death of thymocytes. TCRβ^{hi} thymocytes were analyzed as in **d**. The frequency of Caspase3&7-positive subset is shown. Data are representative from ≥ 3 experiments.

(**f**) Deficiency in TCF-1 and LEF-1 does not cause preferential death of CD4$^+$ SP T cells. CD4$^+$ or CD8$^+$ TCRβ^{hi} thymocytes were analyzed for Caspase activation. Cumulative data from 3 experiments are shown.

Steinke et al. Supplementary Figure 4.



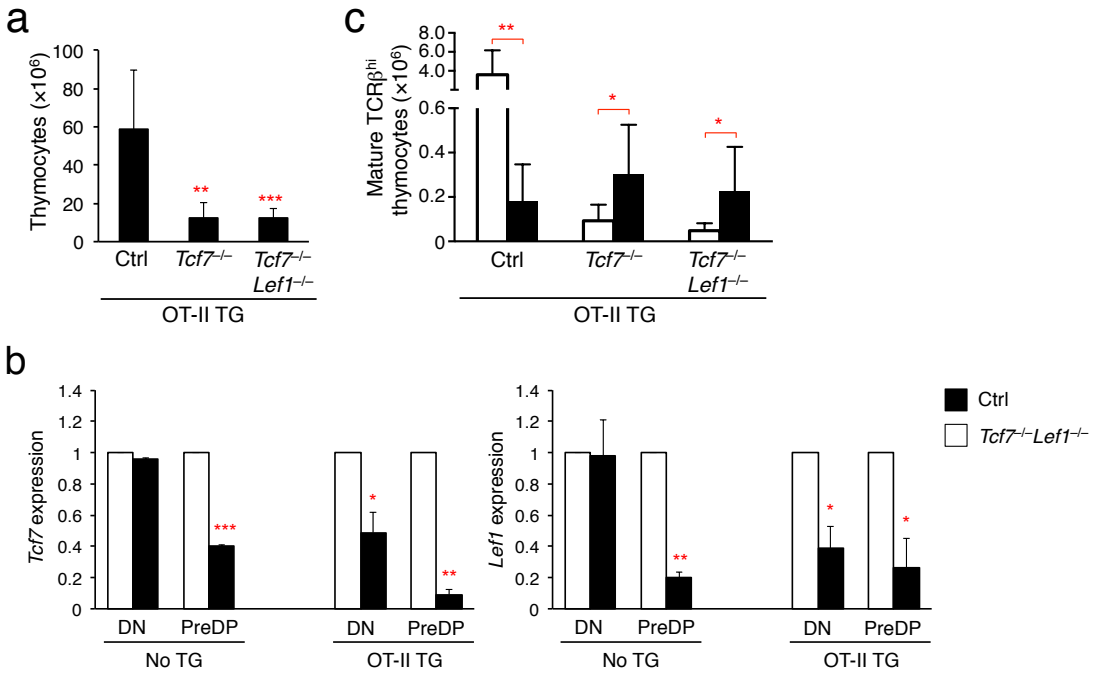
Supplementary Figure 4. The redirected CD8$^+$ T cells in *Tcf7$^{-/-}$Lef1$^{-/-}$*-reconstituted β2m$^{-/-}$ BM chimeras exhibit *bona fide* CD8$^+$ T cell characteristics. BM cells from *Tcf7$^{-/-}$*, *Tcf7$^{-/-}$Lef1$^{-/-}$*, or littermate controls were transplanted into lethally irradiated CD45.1$^+$ congenic β2m$^{-/-}$ mice. Six weeks later, splenocytes were isolated from the BM chimeras and used for downstream analysis.

(**a**) and (**b**) The redirected CD8$^+$ T cells in the absence of TCF-1 or both TCF-1 and LEF-1 persist in the periphery. Donor-derived CD45.2$^+$TCRβ$^+$ splenocytes were analyzed for CD4$^+$ and CD8$^+$ lineage distribution. Representative contour plots (**a**) are from 4 independent experiments with ≥ 4 recipients analyzed in each experiment. Numbers of mature CD4$^+$ and CD8$^+$ splenocytes in the BM chimeras are shown in **b** as means ± s.d. (n ≥ 14). *, p<0.05; **, p<0.01***; p<0.001.

(**c**) The redirected *Tcf7$^{-/-}$Lef1$^{-/-}$* CD8$^+$ T cells express CD8$^+$ T cell-characteristic genes. CD4$^+$ and CD8$^+$ splenic T cells were sorted from WT C57BL/6 mice, and CD8$^+$CD4$^-$ and CD8*4 CD45.2$^+$TCRβ$^+$ splenocytes were sorted from the *Tcf7$^{-/-}$Lef1$^{-/-}$*-reconstituted β2m$^{-/-}$ BM chimeras (*Tcf7$^{-/-}$Lef1$^{-/-}$* BM chimeras), followed by gene expression analysis.

(**d**) The redirected *Tcf7$^{-/-}$Lef1$^{-/-}$* CD8$^+$ T cells proficiently produce granzyme B and interferon-γ upon stimulation. Splenic T cells were isolated from WT B6 mice or *Tcf7$^{-/-}$Lef1$^{-/-}$* BM chimeras, and then activated by plate-bound anti-CD3 antibody and soluble anti-CD28 antibody in the presence of IL-2. Three days later, the cells were stimulated with PMA/Ionomycin in the presence of Golgi plug, and then surface-stained for CD40L, intracellularly stained for granzyme B, interferon-γ, and IL-2. For **c** and **d**, similar results were obtained for redirected *Tcf7$^{-/-}$* CD8$^+$ T cells (not shown).

Steinke et al. Supplementary Figure 5.



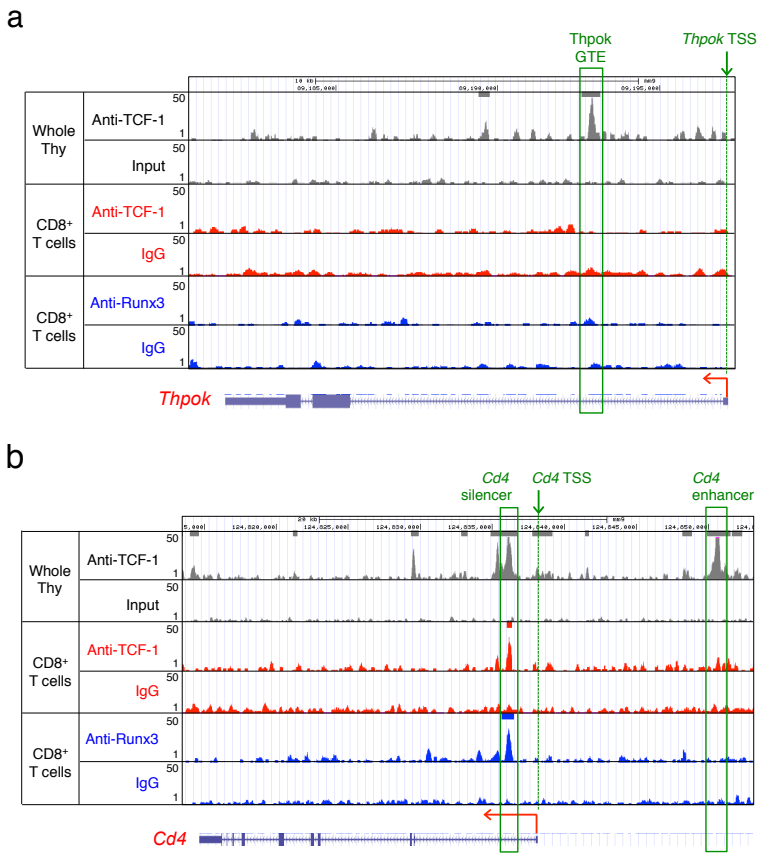
Supplementary Figure 5. Expression of a TCR transgene alters timing of CD4-Cre-mediated deletion of TCF-1 and LEF-1.

(**a**) Expression of the OT-II TG greatly diminished total thymocyte numbers in *Tcf7*$^{-/-}$ and *Tcf7*$^{-/-}$*Lef1*$^{-/-}$ mice compared with littermate controls. Also compare with **Fig. 1b**.

(**b**) CD4-Cre initiates deletion of *Tcf7* and *Lef1* at the DN stage in the presence of OT-II TG. Thymocytes from *Tcf7*$^{-/-}$*Lef1*$^{-/-}$ and littermate controls with or without the OT-II TG were isolated and surface-stained. Lineage-negative CD4^{-}CD8^{-} thymocytes were sorted as DN, and TCRβ^{+}CD69^{-} CD4^{+}CD8^{+} cells sorted as pre-select DP (PreDP) subsets. The expression of *Tcf7* and *Lef1* was measured by quantitative RT-PCR. Data are duplicate measurements of two samples. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Note that without the OT-II TG, CD4-Cre did not excise *Tcf7* and *Lef1* at the DN stage but initiated the deletion from the pre-select DP stage, consistent with our Western blot data in **Fig. 1a**. In contrast, in the presence of the OT-II TG, CD4-Cre initiated deletion of both *Tcf7* and *Lef1* from the DN stage. Because TCF-1 is critical for survival of early thymocytes (as seen in **Supplementary Fig. 3d**), early deletion of TCF-1 and LEF-1 in the presence of OT-II TG at least partly account for more severe reduction of total thymocytes as shown in (**a**).

(**c**) OT-II TG T cells adopt CD8^{+} T cell fate in the absence of TCF-1 or both TCF-1 and LEF-1. The numbers of CD4^{+} or CD8^{+} SP thymocytes were calculated from the mature Vα2^{+}TCRβ^{hi}CD24^{-} thymic subset. Data are means ± s.d. ($n \geq 5$-10). In spite of reduced total thymic cellularity upon deletion of TCF-1 or both TCF-1 and LEF-1, the mature OT-II^{+} thymocytes were predominantly CD8^{+}.

Steinke et al. Supplementary Figure 6.



Supplementary Figure 6. TCF-1 binding to the *Thpok* and *Cd4* gene loci based on ChIP-Seq.

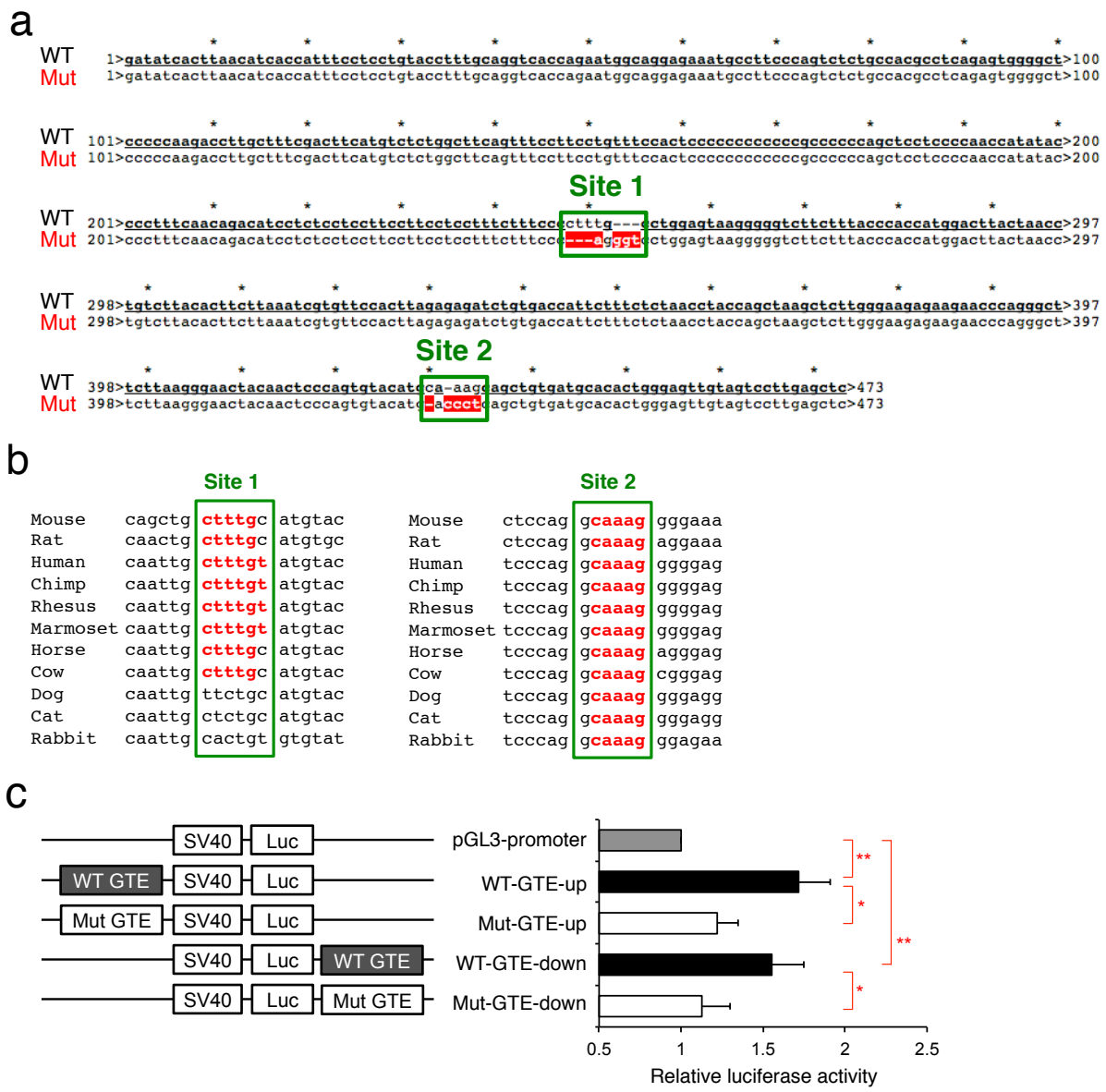
ChIP-Seq of TCF-1 in whole thymocytes was reported by Li L et al. (Blood 122, 902, 2013), and ChIP-Seq of Runx3 in CD8$^+$ T cells was reported by Lotem J et al (PLoS One, 8, e80467, 2013). The data were downloaded and processed for peak calling using MACS. Using the same stringent criteria (detailed in **Supplementary Fig. 8**), wherein 2,827 TCF-1 binding peaks were identified in CD8$^+$ T cells, we found 32,663 peaks in whole thymocytes. Possible reasons for the higher numbers of TCF-1 binding peaks in whole thymocytes include: 1) TCF-1 may regulate different target genes during thymocyte maturation stages. The binding events detected in whole thymocytes are a collection of all TCF-1 binding events at different stages; and 2) the ChIP-Seq control sample was from input DNA for peak calling, whereas ChIP-Seq of TCF-1 and Runx3 by us and Lotem J et al used IgG or non-immune serum-immunoprecipitated samples as control.

The ChIP-Seq track wiggle files were uploaded to the UCSC genome browser for visualization of enriched binding by the transcription factors. For the select gene locus, the transcription start site (TSS) and orientation are marked by arrows. The horizontal bars over TCF-1 or Runx3 tracks indicate the enriched binding peaks identified by MACS.

(**a**) shows enriched binding of TCF-1 at the *Thpok* GTE in whole thymocytes but not in CD8$^+$ T cells.

(**b**) shows co-occupancy of TCF-1 and Runx3 at the *Cd4* silencer in all cell types. TCF-1 is also associated with *Cd4* enhancer and weakly with *Cd4* promoter in whole thymocytes, consistent with reported TCF-1 binding to these regions by Huang Z et al (*J. Immunol*. 176, 4880, 2006). Note that no TCF-1 binding to *Cd4* enhancer and promoter in CD8$^+$ T cells.

Steinke et al. Supplementary Figure 7.



Supplementary Figure 7. The TCF-1 sites in the *Thpok* GTE are important for the GTE enhancer activity.

(**a**) The TCF-1 sites in the 473-bp *Thpok* GTE. The two TCF-1 sites are marked and mutant sequence aligned.

(**b**) Conservation of TCF-1 sites across different species, with core sequence highlighted.

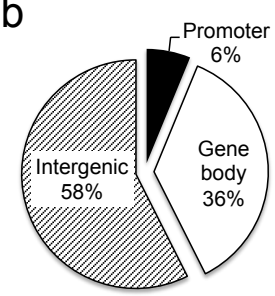
(**c**) Mutation of TCF-1 sites in the *Thpok* GTE abrogates its enhancer activity. 293T cells were transfected with the indicated luciferase reporter constructs along with an internal control pRL-TK. Forty-eight hours later, luciferase activity was measured as in **Fig. 6c**.

Steinke et al. Supplementary Figure 8.

a

	Setting for MACS peak calling			Numbers of TCF-1 binding peaks
	Fold enrichment	p value	FDR	
Stringent setting	≥ 4	$<10^{-5}$	<5%	2,827
Permissive setting	≥ 4	$<10^{-3}$	<5%	9,404

b



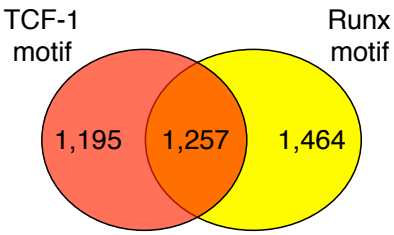
c

TCF-1 binding peak distribution	Peak numbers		
	Total	Overlap w/ H3K4me3 (%)	Overlap w/ H3K27me3 (%)
Promoter	585	86 (15%)	4 (0.7%)
Gene body	3,419	36 (1%)	9 (0.3%)
Intergenic		12 (0.2%)	15 (0.3%)

d

e

f



Supplementary Figure 8. Analysis of TCF-1 ChIP-Seq in CD8$^+$ T cells.

(**a**) Numbers of TCF-1 peaks identified using stringent and permissive settings of the MACS algorithm. Under the stringent setting, 2,827 peaks were defined as TCF-1 strong peaks, and under the permissive settings, 6,577 additional peaks were defined as TCF-1 weak peaks.

(**b**) Genomic distribution of all TCF-1 binding peaks. Promoter region is defined as "-5 kb to +1 kb" flanking the transcription start sites of known RefSeq genes.

(**c**) Overlap of TCF-1 binding peaks with H3K4me3 and H3K27me3 peaks in human naïve CD8$^+$ T cells. The homologous regions of the H3K4me3 and H3K27me3 peaks from human CD8$^+$ T cells were identified in mouse genome using the LiftOver tool. TCF-1 binding peaks in different genomic regions were then assessed for peak overlapping. The criterion for overlapping is that the closest boundary-to-boundary distance of two peaks is within 400 bp.

(**d**) TCF-LEF and (**e**) Runx motifs found in the weak TCF-1 binding peaks. The motif logos are shown. Note that the motifs found in TCF-1 weak peaks are consistent with those found in TCF-1 strong peaks (**Fig. 8c** and **8d**). (**f**) Venn diagram showing the motif distribution in the weak TCF-1 peaks.

Steinke et al. Supplementary Table 1. Primers used in quantitative PCR

For gene expression analysis		
Gene symbol	5’ primer	3’ primer
Hprt1	5′-GCGTCGTGATTAGCGATGATG	5′-CTCGAGCAAGTCTTTCAGTCC
Zbtb7b	5′-CCCTGCTCGAGTTTGCTTAC	5′-CTCGCTCACAGTCATCCTCA
Prf1	5′-GATGTGAACCCTAGGCCAGA	5′-GGTTTTTGTACCAGGCGAAA
Runx3	5′-AGGGAAGAGTTTCACGCTCA	5′-AGGCCTTGGTCTGGTCTTCT
Runx3d	5′-AGCACGTCCACCATCGAG	5′-TGCGACATGGCTTCCAACAG
Myb	5′-CTGAGCACCCAGCTGTTCTC	5′-GTCTTCCCACAGGATGCTGG
Gata3	5′-CTTATCAAGCCCAAGCGAAG	5′-CATTAGCGTTCCTCCTCCAG
Tox	5′-GAGGATGCCTCCAAGATCAA	5′-GCCTGGGTATCACGAAAGAA
Tcf7	5'-CAATCTGCTCATGCCCTACC	5'-CTTGCTTCTGGCTGATGTCC
Lef1	5'-TGAGTGCACGCTAAAGGAGA	5'-CTGACCAGCCTGGATAAAGC
For TCF-1 occupancy in genomic locations		
Genome locations	5’ primer	3’ primer
Gapdh	5′-TCTCCACACCTATGGTGCAA	5′-TTGCCGTGAGTGGAGTCATA
Hprt1	5′-TGAGCGCAAGTTGAATCTG	5′-GGACGCAGCAACTGACATT
Zbtb7b-A	5′-GGAGAAATAGCTGCGCTGAC	5′-CTAGTTTGGGACACCGGAAG
Zbtb7b-B	5′-ACACCAATGCCATTCAGGAT	5′-CTTGGGGAGAGAAGCTGAGA
Zbtb7b-C	5′-GCGGCCTTTGAACATAAAAA	5′-CCCCAGAAAAAGTGTCCTCA
Zbtb7b-D	5′-AGCCCTGGGTTCTTCTCTTC	5′-CCTCCTTTCTTTCCCCTTTG
Zbtb7b-E	5′-TTCCCCTTACAAATGCTTGG	5′-CAGGCTAGTTCCCTCCCTTT
Zbtb7b-F	5′-CCCCTCACTCCAATACCTCA	5′-AGAATTGGCCAGGGGTTAGA
Zbtb7b-G	5′-ACTGGACAGCCAATCCAAAG	5′-GTGCTTCTTTGACCCCGTAA
Axin2	5′-AAATCCACAGCGCAGTTTTT	5′-TTCAACCCAGGTCCTGTTTC
Lef1	5′-ATCCAGCTCCCCAAACTCTT	5′-GGCCCCTTTGTGTGACTAAA
Cd4 silencer	5′-GAACCACAAGGGTCGCTTAG	5′-AACAGAGGAAGGGTGTGTGG